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Self-association of sodium isoursodeoxycholate and sodium isohenodeoxycholate in water

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ABSTRACT

Bile salts (BS) form hydrophobic Small's primary micelles at concentrations above the critical micelle concentration (*CMC*), while at concentrations above 3*CMC* they form secondary micelles (by the association of primary micelles via H-bonds). In this paper the self-associations of the anions of isohenodeoxycholic acid (3-epimer of henodeoxycholic acid, ICD) and the anions of isoursodeoxycholic acid (3-epimer of ursodeoxycholic acid, IUD) are examined, since the thermodynamic parameters of their self-association have not yet been published. Forming of IUD aggregates with two or three building units is slightly more favorable via α sides of steroid skeletons, regarding hydrophobicity, while regarding steric repulsive interactions it is more favorable to associate via β sides. Due to this, IUD in the vicinity of the CMC can form primary micelles by association of IUD particles both from the convex side and from the concave side of the steroid ring system. Therefore, IUD is significantly more prone to initial micellization than bile salt derivatives whose steroidal skeletons contain equatorially oriented OH groups.

1. Introduction

Bile acid salts are well known in the physiology of digestion, as well as receptor modulators (Hofmann and Roda, 1984). In the pharmaceutical industry they are used in formulation of different types of drug carriers (micelles, mixed micelles, liposomes etc.) and for their promoting effects on some drug transport processes and pharmacodynamics (Thomas et al., 2008; Chen et al., 2009; Yang et al., 2012; Mrózek et al., 2013). Although they form relatively small micelles, the low value of their hemolytic potential justifies their biopharmaceutical application (Garidel et al., 2007; Poša and Kuhajda, 2010).

According to the Small's model, at the critical micelle concentration (*CMC*) bile acid salts (BS) form primary micelles whose building units are facing each other by the convex surfaces (β) of the steroid skeletons (*back-to-back association*) (Small et al., 1969). In Small's concept primary micelles formation follows the principle *all or nothing at all* (the pseudo phase separation model):

$$nBS \overset{Kn}{\leftrightarrow} M_n^p \quad (1)$$

where n is the aggregation number and M_n^p signifies primary micelle with the aggregation number n, i.e. where the size distribution of the aggregates is monodispersed and the properties of the system (water solution) suddenly change in the vicinity of the CMC. At higher total concentrations of surfactants and in the presence of salts like NaCl, KCl, etc., bile acid anions form secondary micelles - primary micelles bonded via hydrogen bonds (the equilibrium model)

Based on the BS aggregate's examination by the spin labeled probe molecule, Kawamura et al. (1989) suggested using the model of the disc shape micelle. In this model hydrophilic surfaces of the building units are oriented towards solution, while hydrophobic surfaces of the steroid skeletons are turned towards the interior of the aggregate. Kawamura's model allows the continual increase of the micelle size with the increase of the equilibrium concentration of the bile acid salts, i.e. it does not support Small's association of the primary micelles on the principle *all or nothing*.

Oakenfull and Fisher (1978, 1980), suggested completely opposite structure of BS micelles (primary micelles with H-bonds and secondary micelles with hydrophobic interactions), in relation to widely accepted Small's model.

Today, the Oakenfull and Fishers model of BS association is completely rejected, while the Small-Kawamura model of primary micelles with hydrophobic interactions is accepted. The principle *all or nothing* is completely abandoned and it is accepted that micelle formation is a polydispersed process, regarding aggregate size (Matsuoka and Yamamoto, 2017; Meier et al., 2018; Mustan et al., 2015; Natalini et al., 2014; Gomez-Mendoza et al., 2012; Madenci and Egelhaaf, 2010;

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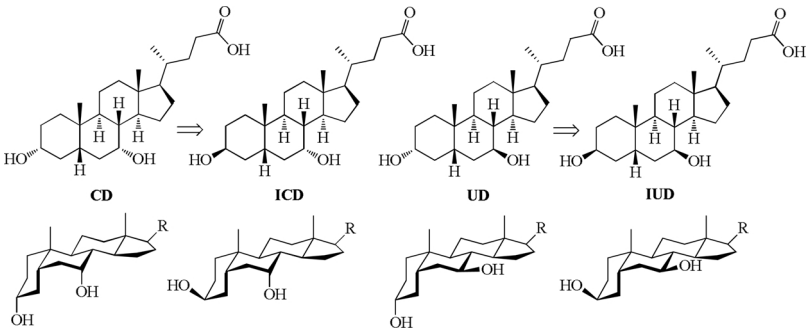

Fig. 1. Examined bile acids: isohenodeoxycholic acid (ICD) is the C3 epimer of henodeoxycholic acid and isoursodeoxycholic acid (IUD) is the C3 epimer of ursodeoxycholic acid (UD).

Calabresi et al., 2007; Garidel and Hildebrand, 2005; Garidel et al., 2000). Molecular dynamic methods also confirmed the existence of BS primary micelles with hydrophobic interactions and the existence of secondary micelles (Mustan et al., 2015; Haustein et al., 2014; Pártai et al., 2007).

So far, the thermodynamic functions of the micellization of isohenodeoxycholic acid (ICD) anions and isoursodeoxycholic acid (IUD) anions have not been reported (Fig. 1). Thus, the aim of this paper is to determinate the micellization parameters (critical micelle concentrations, aggregation numbers), the enthalpy of demicellization and the change in the heat capacity of demicellization, for ICD and IUD. Based on the thermodynamic functions of micellization and the pattern recognition analysis, the structures of micelles of ICD and IUD are assumed.

2. Materials and methods

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

Ursodeoxycholic acid (UD) and chenodeoxycholic (CD) acid (Sigma-Aldrich, Auckland, New Zealand) were used as starting compounds for the synthesis of their 3-epimer derivatives.

3β,7α-dihydroxy-5β-cholanic acid (ICD) and 3β,7β-dihydroxy-5β-cholanic acid (IUD) were obtained according to Lida and Chang (1982). All bile acids were transformed to their sodium salts by a known procedure (Roda et al., 1983).

2.2. Reverse phase HPLC method

The HPLC system Agilent 1100 Series (Agilent Technologies, Santa Clara, California, USA), equipped with degasser, binary pump, automatic injector and DAD detector with a software system for data processing Agilent ChemStation was used and the analyses were performed on a reversed-phase C-18 column: Zorbax Eclipse Plus C18 (250 mm x 3 mm, 5 um, 250 Å) column (Agilent Technologies, Santa Clara, California, USA). 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 the mobile phase were prepared in the concentration of 1 mg/ml. All separations were performed isocratically at a flow rate of 1 ml/min and a column temperature of 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).

2.3. Isothermal titration calorimetry, ITC

Thermometric titration experiments (Poša et al., 2016) were performed at T (10, 15, 20, 30, 40, and 50 °C) with a thermal activity monitor (TAM) isothermal heat-flow microcalorimeter (ThermoMetric LKB 2277, Lund, Sweden) and twin cells, supplied with a sample cell and a reference cell. A sample cell, equipped with a stirring facility and a Lund microtitrator, was loaded with 2 ml of water. A stirring rate of 60 rpm was applied and titrant (0.5 ml of BS solution in water at ≈ 10CMC) was injected into a cell at 90-min intervals, using injection volume of 10 µL. The experiment was computer-controlled via DigiTam 4.1 software. The noise level of the calorimeter baseline during measurements was within ± 0.05 µJsec^{-1}.

2.4. Determination of average aggregation numbers by the Moroi-Matsuoka-Sugioka method

The solid bile acid was suspended in distilled water by stirring by 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 -h equilibration, the pH of the clear solution was measured with the Boeco BT-675 pH/MV/ION meter (Boeckel + Co, Hamburg, Germany) without separation of the solid phase, and taking care not to disturb it (Sugioka and Moroi, 1998; Matsuoka and Moroi, 2002). This gave one point on the titration curve and the procedure was repeated to obtain about 20 points, of which at least 5 were in the micellar region. Measurements were performed at room temperature.

2.5. Determination of fractions of bound counter ions

The pNa values were determined using a Radiometer TitraLab 845 titrator (Hach Lange GmbH, Düsseldorf, Germany) with the ion selective electrode ISE21Na and the reference electrode RedRod201 (Ag/AgCl) at room temperature. The calibration was carried out with NaCl solutions (Poša and Popović, 2013).

3. Results and discussion

3.1. Hydrophobicity and the steroid skeleton

In the examined group of bile acids (Fig. 1), according to the reversed phase (HPLC) capacity factor (k), the most hydrophobic molecule is CD, since in this derivative both OH groups of steroid skeleton are α orientated and mutually sin-axial (the C7 OH group is in axial orientation in B ring of the steroid skeleton, while the C3 OH group is in equatorial orientation in A ring).

However, due to the *cis* binding of A–B rings of the steroid skeleton, the main axis of rotation of cyclohexane A ring is rotated 60° relative to the same axis of B cyclohexane ring, which results in a mutually parallel orientation of the C3 and the C7 OH groups, i.e. the C3 OH group has apparent axial orientation. These OH groups can form hydrogen bonds only with water molecules of the CD hydration shell from the concave surface (α side) of the steroid skeleton - stabilized water molecules (SWM) (Fig. 2. Newman's projection formulas of the vicinal diatomic

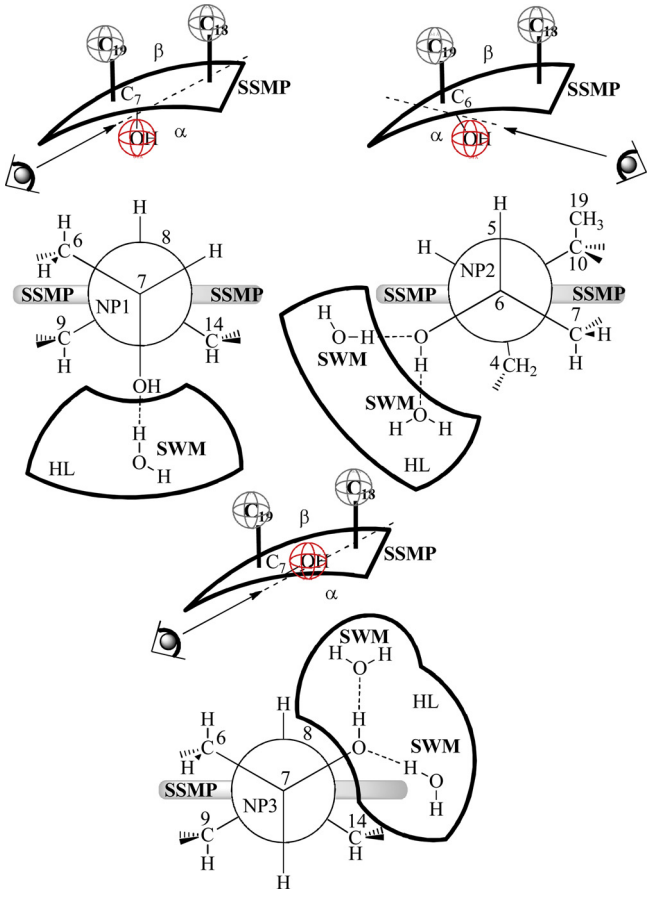

Fig. 2. The influence of the stereochemistry of the steroid OH group on the stabilization of water molecules in the hydration shell of bile acid (SSMP = steroid skeleton mean plane, SWM = stabilized water molecules, HL = hydration layer).

systems (**NP1**: C7(α-*a*-OH)-C8); **NP2**: C6(α-*e*-OH)-C5 i **NP3**: C7(β-*e*-OH)-C8)) from the steroid skeletons of bile salts, taking into account the spatial orientation of OH groups (*a* = axial i *e* = equatorial orientation)).

The water molecules of the hydration layer above the convex surface of the CD steroid skeleton are not thermodynamically stabilized with hydrogen bonds, so they have lower entropy (due to the decrease of mobility), in comparison to the water molecules of the interior (bulk) of water solution - non-stabilized water molecules (NSWM). The presence of NSWM makes a convex surface of the steroid skeleton (β side) become the hydrophobic surface of CD molecule, together with the C12 lateral side, which enters the whole hydrophobic surface. Binding of CD at the hydrophobic stationary phase over the β side of steroid skeleton is driven by the thermodynamic force resulted from transferring of NSWM to bulk water, which increases system entropy – the hydrophobic effect. The C7 epimer of CD, henodeoxycholic acid (UD), has a significantly lower value of *k* (Table 1).

Namely, the C7 OH group of UD is in a β equatorial orientation and spatial position of this OH group (compared to the position of α axial OH group in CD) is switched 120° towards the angular methyl groups. Accordingly, the C7 OH group of UD can form hydrogen bonds with the water molecules of the hydration layer above the convex surface of the steroid skeleton and above the C7 lateral side of the steroid skeleton (Fig. 2. **NP3**). Thus, the number of NSWM at β side of the UD steroid skeleton decreases, i.e. the hydrophobicity of the UD steroid skeleton's β side in comparison to the CD β side is decreased. As the 3 OH group of UD is in the pseudo axial orientation, i.e. it is placed directly above α side of steroid skeleton's A ring, it stabilizes a greater number of water

Table 1
Hydrophobicity (HPLC capacity factor: *k*), average aggregation number (*n*) and fractions of bound counter ions (*β*) at *CMC*. Measurements were made at $T = 25\ ^{\circ}C$ and $p_a = 1.00 \times 10^5$ Pa.

Compound	*k*	*n*				*β*
		40 mM	50 mM	60 mM	70 mM	
CD	8.48	8.0	8.0	8.2	8.4	0.25
ICD	2.70	3.0	3.0	3.0	3.0	0.21
UD	2.97	4.8	5.0	5.2	5.2	0.14
IUD	3.29	6.0	6.0	6.0	6.2	0.10

Standard uncertainties *u*, are: $u(T) = 0.1\ ^{\circ}C$, $u(p) = 0.002$ MPa, $u(n) \leq 0.3$, $u(\beta) \leq 0.2$, $u(E_b) \leq 0.5$.

molecules from the hydration layer of the concave surface than from the convex surface. On the other hand, the C7 β equatorial OH group stabilizes water molecules of the hydration layer from the convex surface (β side) of UD. Equatorial orientation of the C7 OH group of UD is not positioned directly above the steroid skeleton and therefore α side of the UD steroid skeleton is less hydrophobic (more hydrophilic) than β side. The C3 epimer of CD, isohenodeoxicholic acid (ICD), according to its *k* value is less hydrophobic than UD. In ICD, the C3 OH group has β axial orientation, which means that it stabilizes (with hydrogen bonds) the water molecules of the hydration layer above β side of steroid skeleton's A ring. Nevertheless, B, C and D rings on β side of ICD remain hydrophobic as it is in CD, due to the convexity of the steroid skeleton. On the concave surfaces of ICD, there is the C7 α axial OH group, which gives hydrophilic character to this surface of the ICD molecule. The C3 β OH group of ICD is in axial orientation, as it is placed directly above the A ring of the steroid skeleton. Conversely, the C7 β OH group of UD is in an equatorial orientation and it is not placed directly above the B ring. Accordingly, the C3 β axial OH group of ICD can stabilize more water molecules of the hydration layer above A ring (on β side) than the C7 β equatorial OH group above the B ring of UD can do. Therefore, the β side of the steroid skeleton of UD is more hydrophobic that β side of ICD steroid skeleton. Certainly, for both derivatives (UD and ICD), β side of the steroid skeleton is more hydrophobic than α side. For the C3 epimer of UD, isoursodeoxycholic acid (IUD), it could be expected that it has a *k* value lower than ICD and UD derivatives, owing to the β orientation of the C3 and the C7 OH groups. However, according to the experimentally determined value of *k,* IUD is more hydrophobic that ICD and UD (Table 1). A molecule of IUD has the same orientation of the C3 OH group as the molecule of ICD, implying that the stabilization of water molecules of the hydration shell is possible only from β side, and not from α side of the A ring of an IUD steroid skeleton. The C7 OH group of IUD has identical spatial orientation as the C7 OH group in UD, implying that stabilization of water molecules in the hydration layer of B ring is absent from α side of the ring. Thus, the hydrophobicity of IUD is inversed in relation to CD, ICD and UD. In IUD, the β side of a steroid skeleton becomes the less hydrophobic part of the molecule (i.e. more hydrophilic), while α side of a steroid ring system becomes the more hydrophobic (i.e. less hydrophilic). Consequently, it could be assumed that IUD binds to the hydrophobic stationary phase by its convex surface (α-side). Hydrophobicity of IUD is, according to the obtained *k* value, similar to the hydrophobicity of hiodeoxycholic acid (HD, $k = 3.49$) (Poša and Pilipović, 2017). Namely, HD has the C6 α equatorial OH group which forms an angle of 30° degrees with the steroid skeleton mean plane (SSMP), with regard to the α side of steroid skeleton (Fig. 2. **NP2**). Similarly, the C7 β equatorial OH group of IUD forms an angle of 30° degree with the SSMP, with regard to the β side of steroid skeleton. Hence, in respect to the hydrophobic surfaces (β side of HD and α side of IUD), the C6 OH group of HD and the C7 OH group of IUD have identical spatial orientations. Molecules of HD and IUD have similar orientations of the C3 OH group, with respect to their hydrophobic surfaces. However, a bit lower value of hydrophobicity of IUD related to

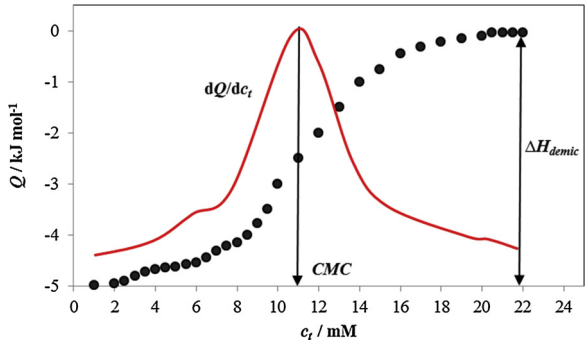

Fig. 3. Titration of 100 mM IUD in water at 10 °C: reaction enthalpy (-Q) vs. the total detergent concentration in the reaction cell (the red full line is the first derivative of the titration curve).

HD is the result of a larger distance between the C3 and the C7 OH groups in the molecule of IUD, compared to the distance between the C3 and the C6 OH groups in the molecule of HD. A larger distance between the OH groups in molecules causes a greater disturbance in the continuity of their hydrophobic surface.

3.2. Micellisation: thermodynamic functions of micellisation

Surfactants in water solutions above a certain concentration of the monomer – the critical micelle concentration (*CMC*) – form micellar aggregates (self-associations), in which the hydrophobic surface of the surfactant is protected from the solvent. For deriving thermodynamic functions of micellization and for simultaneous determination of *CMC* values, it is most suitable to follow the process of self-association by isothermal calorimetric titration (ITC) (Fig. 3) (Garidel et al., 2000; Garidel and Hildebrand, 2005; Anderson et al., 2016; Kroflič et al., 2012). In an ITC experiment, actually, the process of demicellisation is observed, i.e. successive dilution of the bile acid salt in micellar solution (BS):

$$M_n^p \overset{demicelisation}{\leftrightarrow} nBS \tag{2}$$

In the pseudo phase separation approximation of demicellisation, when BS concentration achieves the critical micelle concentration during dilution, the chemical potential of the micellar pseudo phase ($\mu_m^{\cdot}$) is equal to the chemical potential of BS from water solution (μ_{aq}):

$$\mu_m^{\cdot} = \mu_{aq} = \mu_{aq}^0 + RTlnCMC \tag{3}$$

$$\Delta G_{demic}^0 = \mu_{aq}^0 - \mu_m^{\cdot} = -RTlnCMC \tag{4}$$

Differentiating the equation for the Gibbs energy of demicellisation (ΔG_{demic}^0) by temperature and taking into account the Gibbs-Helmholcz relation, the next equation is derived:

$$-\left(\frac{\partial(G_{demic}^0/T)}{\partial T}\right)_p = \frac{\Delta H_{demic}^0}{RT^2} = \left(\frac{\partial lnCMC}{\partial T}\right)_p \tag{5}$$

where ΔH_{demic}^0 is the enthalpy of demicellisation (derived from the ITC results, Fig. 3).

From the Eq. (5) it is apparent that if $\Delta H_{demic}^0 < 0$ the function $\ln CMC = f(T)$ decreases ($(\partial lnCMC/\partial T)_p < 0$), while on the contrary, if $\Delta H_{demic}^0 > 0$ the function $\ln CMC = f(T)$ increases ($(\partial lnCMC/\partial T)_p > 0$)). From the Eq. (5), it is also apparent that in the case $\Delta H_{demic}^0 = 0$ the function $\ln CMC = f(T)$ has the extreme minimum point (Fig. 4i Table 2).

Similarly to the Eq. (5), during the solubilization of the hydrophobic molecules from pure monocomponent liquid phase to water phase (as molecules of surfactants from the micellar pseudo phase during demicellization move to bulk water), the water solubility of the hydrophobic molecules from the pure monocomponent phase (expressed in molar fraction of the solute in a water solution, x) is described by equation: (Privalov and Gill, 1989).

$$\left(\frac{\partial lnx}{\partial T}\right)_p = \frac{\Delta H_{sol}^0}{RT^2} \tag{6}$$

where ΔH_{sol}^0 is the change of the enthalpy of solubilization of one mole of hydrophobic molecule. The function $\ln x = f(T)$ also has the U shape

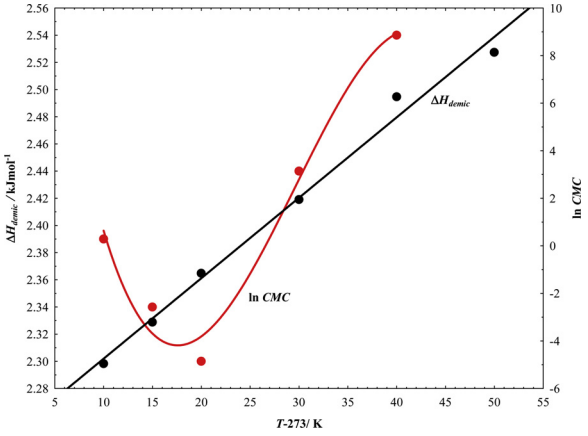

Fig. 4. Dependence of ln*CMC* from temperature (red points) that fits with third order polynomial function (red curve) and dependence of enthalpy of demicellization from temperature (black points) that fit to line (black colour); example for IUD is $T_H = 23$ °C.

Table 2
The critical micelle concentration (*CMC*), the change of enthalpy of demicellization (ΔH_{demic}), change of the Gibbs energy of demicellization (ΔG_{demic}), the change of the entropy of demicellization (ΔS_{demic}) and the change of the heat capacity for demicellization process (ΔC_p) for BS derivatives measured with ITC method.

Compound	T /°C	*CMC* /mM	ΔH_{demic} kJmol^{-1}	$T\Delta S_{demic}$ kJmol^{-1}	ΔG_{demic} kJmol^{-1}	ΔC_p /Jmol^{-1} K^{-1}
CD	10	6.0	−6.75	−18.79	12.04	369
	15	5..5	−3.88	−16.33	12.45	
	20	5.0	−2.25	−15.16	12.91	
	30	5.7	1.90	−11.11	13.00	
	40	6.1	5.30	−7.97	13.27	
	50	6.5	8.21	−5.32	13.52	
ICD	10	28.0	−3.79	−12.20	8.41	200
	15	27.5	−2.34	−10.94	8.60	
	20	26.3	−1.09	−9.95	8.86	
	30	27.2	1.58	−7.5	9.08	
	40	28.4	3.02	−6.24	9.26	
	50	31.0	4.14	−5.18	9.32	
UD	10	18.0	−4.11	−13.56	9.45	245
	15	17.6	−2.91	−12.57	9.66	
	20	17.2	−1.51	−11.40	9.89	
	30	18.0	0.79	−9.33	10.12	
	40	19.5	3.36	−6.88	10.24	
	50	21.0	5.67	−4.70	10.37	
IUD	10	11.0	−4.95	−15.56	10.61	333
	15	10.4	−3.21	−14.14	10.93	
	20	10.0	−1.15	−12.37	11.22	
	30	11.5	1.95	−9.30	11.25	
	40	12.7	6.27	−5.09	11.36	
	50	13.0	8.14	−3.52	11.66	

Standard uncertainties u, are: $u(T) = 0.1$ °C, $u(p) = 0.002$ MPa, $u(\Delta H_{demic}) \leq 0.2$ kJ mol^{-1}T (10, 15) °C, $u(\Delta H_{demic}) \leq \pm 0.3$ kJ mol^{-1}T (20), $u(\Delta H_{demic}) \leq 0.1$ kJ mol^{-1}T (10, 40, 50) °C, $u(\Delta G_{demic}) \leq 0.3$ kJ mol^{-1}T (10, 15) °C, $u(\Delta G_{demic}) \leq 0.6$ kJ mol^{-1}T (20) °C, $u(\Delta G_{demic}) \leq 0.3$ kJ mol^{-1}T (30, 40, 50) °C, $u(T\Delta S_{demic}) \leq 0.4$ kJ mol^{-1}T (10, 15) °C, $u(T\Delta S_{demic}) \leq 0.7$ kJ mol^{-1}T (20) °C, $u(T\Delta S_{demic}) \leq 0.4$ kJ mol^{-1}T (30, 40, 50) °C, $u(CMC) \leq 0.2$ mMT (10, 15) °C, $u(CMC) \leq 0.5$ mMT (20) °C, $u(CMC) \leq 0.2$ mMT (30, 40, 50) °C, $u(\Delta C_p) \leq 8$Jmol^{-1} K^{-1}.

(with the slope (6)), as the function $\ln CMC = f(T)$.This similarity suggests the importance of the hydrophobic effect in the process of BS micellization (demicellization). For each examined BS, for the dependence of $\ln CMC$ from T, the U curve is derived, as shown for IUD (Fig. 4), i.e. at a certain temperature the *CMC* has minimum value (Table 2), which confirms that the hydrophobic effect (interaction) has the crucial role in the process of micellization of the examined BS. The *CMC* value for IUD at 20 °C is 10.0 mM. Roda et al. (1983) gained the value of 10.5 mM (at 25 °C) for the critical micelle concentration using tensiometer, which confirms the *CMC* value for IUD derived with the ITC method.

Taking into account the equation that defines the change of the Gibbs energy of demicellization $\Delta G^0_{demic} = \Delta H^0_{demic} - T\Delta S^0_{demic}$ ($\Delta G^0_{demic} = -\Delta G^0_{mic}$), at the temperature T_H, $\Delta H^0_{demic} = 0$ (T_H = 23 °C for IUD, Fig. 4), it follows: $\Delta G^0_{demic} = -T\Delta S^0_{demic}$. According to this, the process of demicellization (micellization) at T_H is determined solely by the change of entropy (ΔS^0_{demic}). The extreme value of the function $\ln CMC = f(T)$ corresponds to the point of minimum at the temperature T_H. Furthermore, bile salts at the temperature T_H have the lowest value of the critical micelle concentration (the hydrophobic effect manifests itself to the greatest extent), i.e. at the temperature T_H bile salts have the greatest tendency to self-associate – process of micellization is entropically driven (Garidel et al., 2000; Garidel and Hildebrand, 2005). The $T\Delta S^0_{demic}$ function grows with the increase of temperature (Fig. 5), since the increase of temperature decreases the difference in the content of entropy between NSWM and water molecules from bulk solution (hydrophobic surface of the steroid skeleton is protected from the solvent in micelle, but during disintegration of micelle the hydrophobic surfaces of the micelle building units are being exposed).

If (Fig. 5) $T\Delta S^0_{demic}$ is extrapolated to the value $T\Delta S^0_{demic} = 0$ (at the temperatureT_S), the value of 75 °C is gained for T_S (usually the surfactants with a conformationally flexible hydrophobic segment and polar head have T_S value around 100 °C). The change of the Gibbs energy of demicellization at T_S is determined exclusively by the change of the enthalpy of demicellization ($\Delta G^0_{demic} = \Delta H^0_{demic}$), i.e. by heat energy that should be brought to the system in order to disintegrate micelle into monomers (by breaking up the hydrophobic interactions between monomers in the micelle). Therefore, the opposite process, i.e. micelle formation, above T_S is enthalpy driven (Paula et al., 1995). The function ΔG^0_{demic} compared to the functions ΔH^0_{demic} and $T\Delta S^0_{demic}$ changes slightly with temperature (Figs. 4 and 5), which is characteristic for the occurrence of the effect of entropic-enthalpic compensation in some processes.

Values of ΔH^0_{demic} increase linearly with the temperature (Fig. 4 and Table 2) for CD, ICD, UD and IUD bile acid anions. According to that, in an analyzed range of temperatures, the change in heat capacity of demicelization:

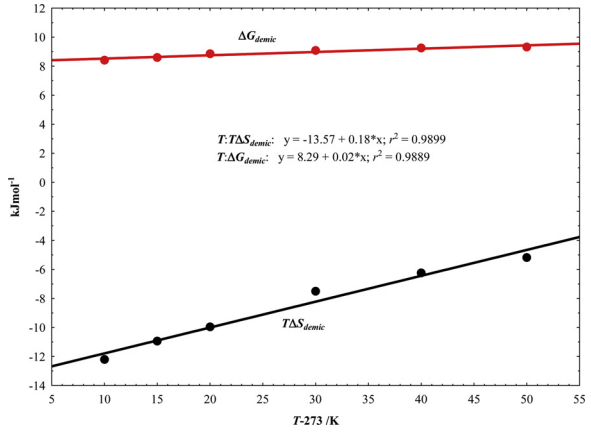

Fig. 5. Dependence of the Gibbs energy and $T\Delta S_{demic}$ of demicellization from temperature, example for ICD.

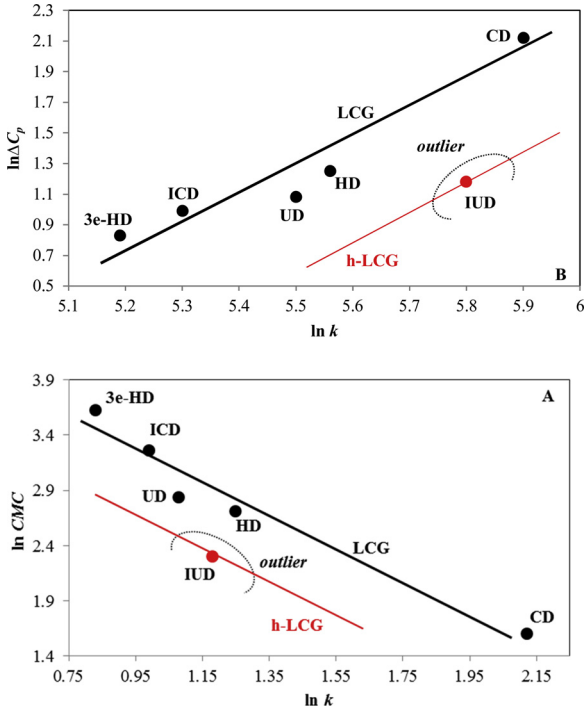

Fig. 6. A: Linear dependence between $\ln k$ and $\ln CMC$, B: Linear dependence between $\ln k$ and $\ln \Delta C_p$ (LCG = linear congeneric group, h-LCG = hypothetical linear congeneric group).

$$\Delta C_p^{demic} = \left(\frac{\partial \Delta H^0_{demic}}{\partial T}\right)_p \tag{7}$$

is constant, and $\Delta C_p^{demic} > 0$, meaning that during the demicellization, the hydration of the hydrophobic surface of steroid skeletons of monomers occurs. Namely, binding of the counter ion at the examined BS micelles is poorly expressed (Table 1) and can be neglected due to a small value of the counter ion fraction (Garidel et al., 2000; Garidel et al., 2005). Therefore counter ion hydration in demicellization can be neglected, i.e. ΔC_p^{demic} and originates from hydration of the hydrophobic surfaces of the steroid skeletons which are in the micellar pseudo phase protected from water molecules (Paula et al., 1995). Usually with the increase of the hydrophobic surface of steroid skeleton, the value of ΔC_p^{demic} grows. Among the examined BS, values of ΔC_p^{demic} decrease in the following order: CD > IUD > UD > ICD which is in agreement with declination of their hydrophobicity (k values) (Tables 1 and 2).

Values of the aggregation numbers (n) for the examined BS decrease as their hydrophobicity declines. However, a very low value of the ICD aggregation number (Table 1) is probably due to the fact that the C3 β axial OH groups in ICD dimmer micelles during further association sterically prevents access to the spatial region of micelle in the vicinity of the steroid skeleton's A ring. Namely, the formation of aggregates, consisted of four monomers, began with the formation of dimmers (Gouin and Zhu, 1998) in a parallel association (carboxylate functions of both monomers are placed on the same side of the aggregate), over the convex surfaces of steroid skeletons. Dimmer aggregates generated in this way mutually associate over the steroid skeleton's A rings (Small, 1971). In the case of ICD dimmer micelles, the axial β C3 OH groups spatially prevent the association of dimmers. Formation of ICD micelle with $n = 3$ is possible only if one ICD monomer is associated to the dimmer ICD micelle over its hydrophobic C12 lateral side, wherein the self-association ends (BS = ICD):

$$BS \overset{BS}{\leftrightarrow} M_2^p \overset{BS}{\leftrightarrow} M_3^p \neq M_4^p \tag{8}$$

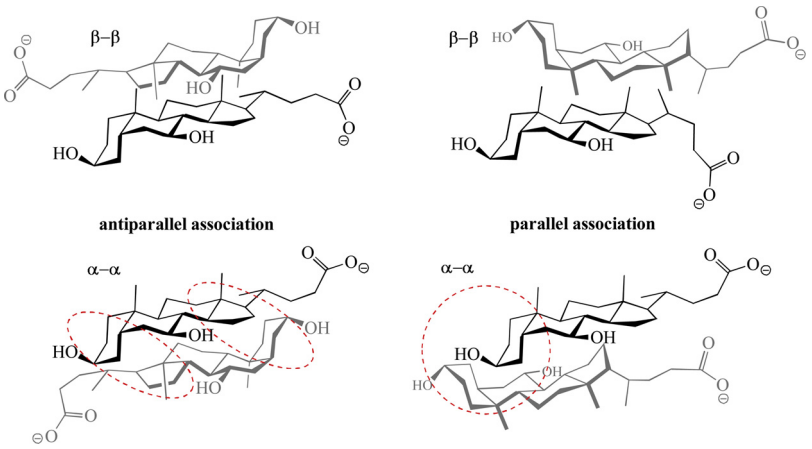

Fig. 7. In IUD dimmer IUD, during the association over α-side of the steroid skeletons, due to the *cis* binding of A/B rings, steric repulsive interactions are generated between A–D rings and A-A rings of two IUD anions.

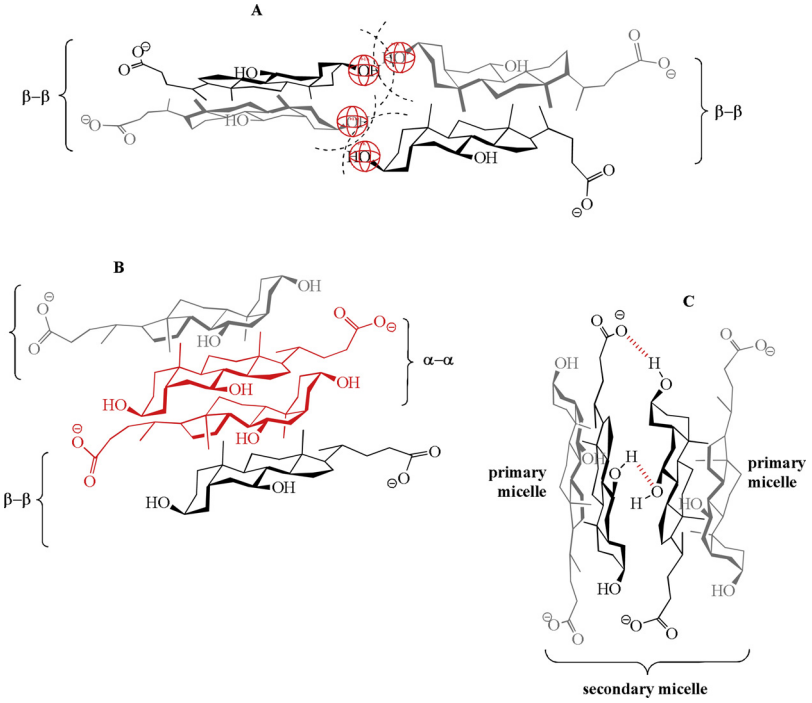

Fig. 8. Possible IUD micelles with aggregation number 4, A: steric inhibition of micellization by the β axial C3 OH group, B: micelles in which the building units are linked by hydrophobic interactions along α side and β side of the steroid skeleton and C: secondary micelles.

3.3. Pattern recognition and hypothetical structure of IUD micelle

For the pattern recognition analysis (recognition of patterns and regularities in data, i.e. classification of data into different classes), the group of examined molecules (Fig. 1) is expanded with the molecules of hiodeoxycholic acid (HD) and 3-epimer of hiodeoxycholic acid (3e-HD), whose association parameters were determined earlier (Poša and Pilipović, 2017). In the planes $\ln k - \ln CMC$ and $\ln k - \ln \Delta C_p^{demic}$ the derivatives CD, UD, ICD, HD and 3e-HD form linear congeneric group (LCG), which reflects the similarity in the set of molecules and is expressed by a linear function of a given molecule parameter (in this case, hydrophobicity) (Fig. 6):

$$\ln k = const. - \tan\alpha_{LCG} \ln CMC \quad (9)$$

$$\ln k = const. + \tan\alpha_{LCG} \ln \Delta C_p^{demic} \quad (10)$$

while IUD is an outlier (Otto, 2007; Adikaram et al., 2014). If linear congeneric group (9) and (10) exists (Fig. 6), the next equation is valid (Poša et al., 2015; Poša and Popović, 2017):

$\Delta G_m^0(i) = b_i \Delta G_a^0(r),\ A_{hf}(i) = c_i A_{hf}(r)\}\mathrm{b_i} = \xi_{\mathrm{LCG}}\mathrm{c_i} + \sigma_{\mathrm{LCG}}\ \mathrm{I}\colon \forall\ \mathrm{i} \bigwedge\ \mathrm{r} \in$ LCG (11).In equation (11) $\Delta G_a^0(r)$ represents the change of the standard Gibbs free energy of adsorption (at the hydrophobic stationary phase in the RPHPLC experiment) of the referent *r* molecule (any molecule from LCG); $\Delta G_m^0(i)$ represents the change of the standard Gibbs free energy of micellization of molecule *i*; $A_{hf}(i)$ corresponds to the hydrophobic surface of the steroid skeleton *i*; $A_{hf}(r)$ corresponds to the hydrophobic surface of the referent molecule from the LCG. Parameters b_i and c_i (11) are the coefficients of the decomposition of vectors $\Delta G_m^0(i)$, $A_{hf}(i)$ into one-dimensional vectors spaces of the ΔG_a^0, and A_{hf} using base vectors $\Delta G_a^0(r)$, $A_{hf}(r)$. However, these vector decompositions are not random and there is a linear relation between the coefficients of decomposition: $b_{i=\xi_{LCG}} c_i + \sigma_{LCG}$ which is specific to a given hydrophobic linear congeneric group, i.e. the set of molecules that form the given LCG group. If micelle formation is entropically driven, it can be shown that $\Delta G_m^0(i) = b_i \Delta G_a^0(r)$ is proportional to $\ln k$ and $\ln CMC$ (and thus

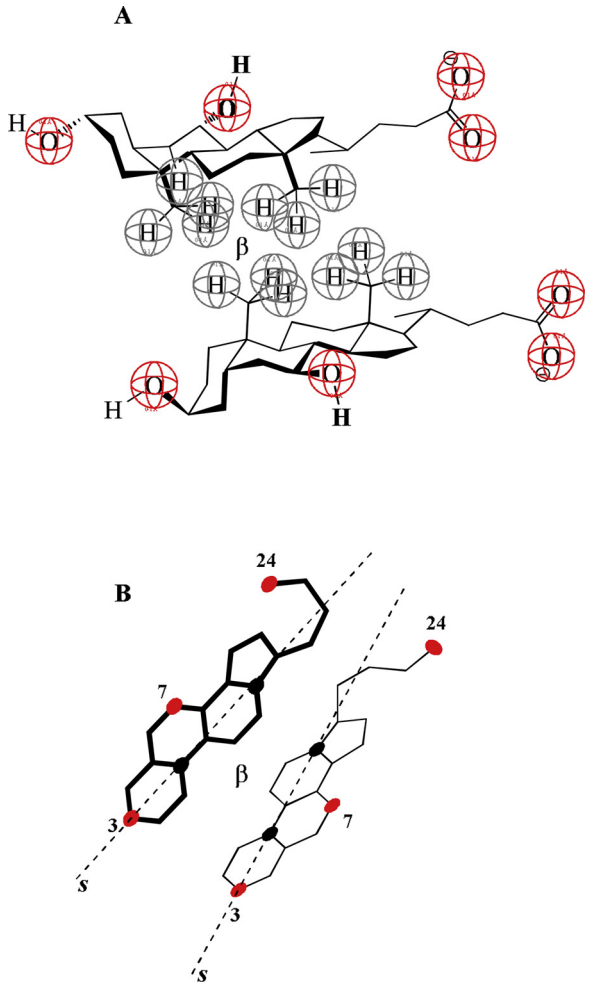

Fig. 9. β-β parallel association of IUE anions (A: conformational formulas, B: representation with molecular graph).

$\ln k - \ln CMC$ are mutually proportional). Namely, if the process is entropically driven, the number of NSWM determines the equilibrium of the binding of BS molecules at the hydrophobic stationary phase and the equilibrium of their self-association over the hydrophobic surfaces of steroid skeletons.

If the following equation is written for ΔC_p^{demic}:

$$\Delta C_p^{demic} = (\frac{\partial \Delta H^0_{demic}}{\partial T})_p = (\frac{\partial H^0_{aq}}{\partial T})_p - (\frac{\partial H^0_m}{\partial T})_p = C_p^{aq} - C_p^m \quad (12)$$

it can be seen that ΔC_p^{demic} is the difference between the heat capacity of the monomer from the water solution (C_p^{aq}) and the heat capacity of the monomer from the micellar pseudo phase (C_p^m). In the process of demicellization the weak hydrophobic interactions between hydrophobic surfaces of the micellar binding units in micelles are replaced with the hydrogen bonds between the first layer of water molecules that hydrate steroid skeleton hydrophobic surface of bile salts and the second layer of water molecules from the interior of the solution ($C_p^{aq} > C_p^m$). The larger the hydrophobic surface of the micellar binding unit is, the higher C_p^{aq} becomes (and consequently ΔC_p^{demic} becomes higher, as well). From this it follows that $\Delta G_m^0(i) = b_i \Delta G_a^0(r)$ is proportional to $\ln k$ and $\ln \Delta C_p^{demic}$ (and thus $\ln k - \ln \Delta C_p^{demic}$ are mutually proportional). Therefore, it can be said that the linear congeneric group from the Fig. 6 is the "hydrophobic "linear congeneric group.

For IUD, which is an outlier related to the LCG (Fig. 6), it is valid:

$$\Delta G_m^0(IUD) = b_{IUD} \Delta G_a^0(r),\ A_{hf}(IUD) = c_{IUD} A_{hf}(r)\} b_{IUD} \neq \xi_{LCG} c_{IUD} + \sigma_{LCG} \quad (13)$$

If $b_{IUD} \Delta G_a^0(r)$ is presented as the sum of the excess Gibbs energy G^E which describes an excess of Gibbs energy in relation to what follows from equation (11) and if $\hat{b}_{IUD} \Delta G_a^0(r)$ is the predicted Gibbs energy of the hydrophobic effect within the standard Gibbs energy of micelle formation, according to $A_{hf}(IUD) = c_{IUD} A_{hf}(r)$ and the equation $\hat{b}_{IUD} = \xi_{LCG} c_{IUD} + \sigma_{LCG}$ derived from the LCG (11) it follows:

$$\Delta G_m^0(IUD) = \hat{b}_{IUD} \Delta G_a^0(r) + G^E,\ A_{hf}(IUD) = c_{IUD} A_{hf}(r)\} \hat{b}_{IUD} = \xi_{LCG} c_{IUD} + \sigma_{LCG} \quad (14)$$

The excess Gibbs energy G^E (14) may originate from the formation of hydrogen bonds between the micelle building units of the IUD micelle - in this case IUD is a true outlier relative to LCG (11). On the other hand, IUD can in fact be a member of a new hydrophobic linear congeneric group (Fig. 6) whose members have different functional dependence between the hydrophobic surface of a steroid skeleton and the standard Gibbs energy of micellization, which is not included in the equation (11), i.e. the hydrophobic congeneric group which includes IUD is not described by the Eqs. (9) and (10). Namely, in the bile acids CD, UD, ICD, HD and 3e-HD (LCG), β side of a steroid skeleton is the most hydrophobic surface of the molecules. During the initial micellization (formation of dimmer aggregates) of these bile acids, in the vicinity of the critical micelle concentration (since the linear congeneric group is determined by micellization parameters around the CMC values), the Small-Kawamura primary micelles are formed over hydrophobic β side of the steroid system of rings. In IUD, although α side of the steroid skeleton is a bit more hydrophobic than β side, it should be noticed that this hydrophobic difference occurs in the hydrophobic binding at the octadecyl stationary phase (RPHPLC). As octadecyl residue has a flexible conformation, it is easily adapted to both the convex and the concave surface of the steroid skeleton, i.e. it does not resemble geometric surfaces. However, in the IUD dimmer micelles, in which the building units are mutually oriented over α sides of steroid skeletons, there are repulsive interactions between A and D rings of the IUD steroid skeletons. This is not the case in the formation of dimmer micelles where the steroid skeletons are mutually hydrophobically bonded over β sides of steroid skeletons (Fig. 7).

Therefore, in self-association of IUD in the vicinity of the CMC (dimmer micelles), α side of the steroid skeleton is more favorable regarding hydrophobicity and β side is more favorable regarding steric repulsive interactions. This means that IUD anions can form Small's primary micelles by mutual association both over the convex (β costs) and over the concave (α side) surface of steroid skeletons. For IUD, the hydrophobic self-association (in the vicinity of the CMC) is a more probable process, with respect to the bile salts in which β side of the steroid skeleton is the most hydrophobic molecular surface (at the same time β side does not contribute to steric repulsive interactions during micellization). Namely, in CD, UD, ICD, HD and 3e-HD, during the formation of the primary micelles, steroidal skeletons of the two building units must have a specific spatial orientation (steroid skeletons mutually oriented over β sides), while in IUD, for the successful formation of primary micelles there are twofold more spatial orientations that can lead to aggregation (a higher degree of freedom). The hypothetical linear congeneric group h-LCG from Fig. 6 would be composed of anions of bile acids in which dimmer micelles would be formed by the association along α sides, as well as by the association along β sides of the steroid skeletons. Above the critical micelle concentration, where the micelles with a larger aggregation number are formed (Table 1), IUD anions in the micelles can be hydrophobically linked exclusively over β side of steroid skeletons, up to aggregation number 4 (similarly to ICD, steric inhibition of the aggregate formation with n = 4 by the β axial C3 OH groups, Fig. 8A).

The micelles of IUD anions with aggregation number 4 (as well as larger micelles up to n = 6. Table 1) are formed by simultaneous hydrophobic aggregation of the building units along α side, β side and lateral C12 side of the steroid skeleton (Fig. 8B), or by formation of the

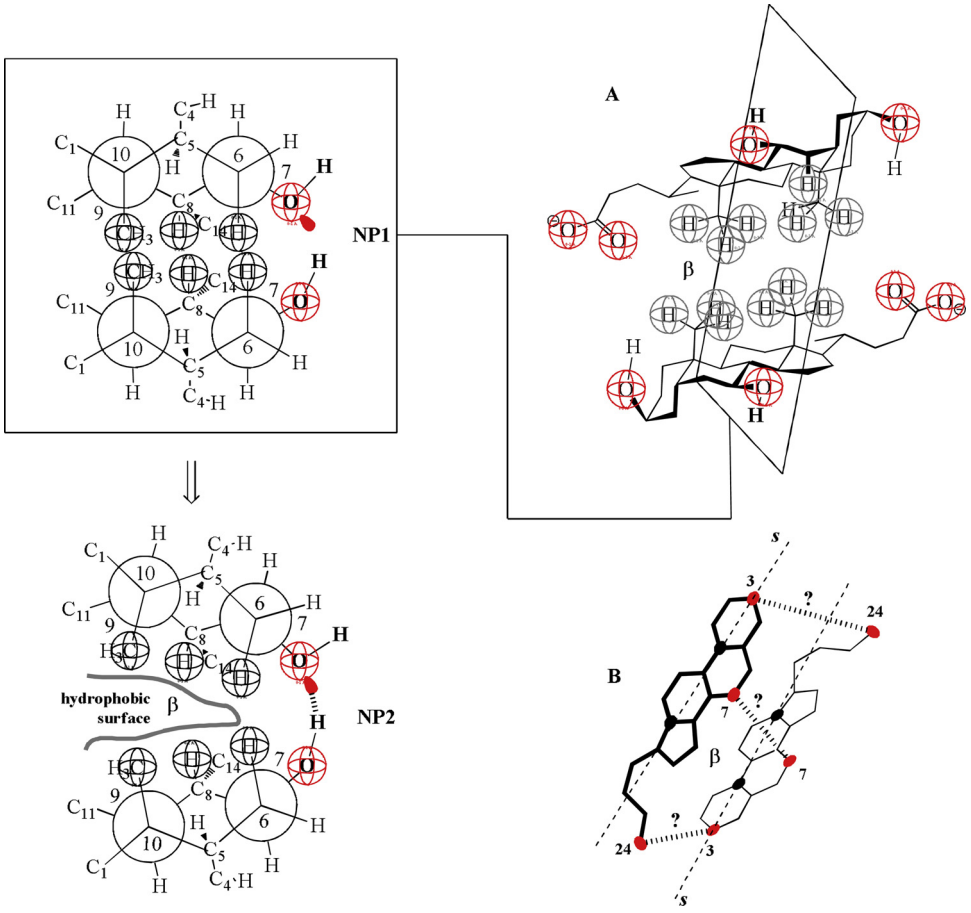

Fig. 10. β-β antiparallel association of IUD anion (A: conformational formulas, B: representation with molecular graph).

secondary micelles (by connecting primary dimmer or triple IUD micelles with H-bonds, Fig. 8C). Due to the equatorially-oriented OH groups, there are steric reasons why the H-bonds cannot be formed in the IUD primary micelles. In β-β parallel association (carboxyl groups are on the same side of the aggregate), the C7 OH groups of IUD are on the opposite sides of such a dimmer aggregate (i.e. related to the *s* symmetric axis of the main molecular subgraph that are mutually *trans* oriented), thus, stereochemically they do not form hydrogen bonds, while the C3 OH groups are too distant (Fig. 9).

In β-β antiparallel association, related to the *s* axis, the C7 OH groups are *cis* oriented (Fig. 10), thus fulfilling the conditions for the formation of hydrogen bonds. Nevertheless, for the formation of hydrogen bonds the steroid skeletons of IUD should move from the state of complete overlapping of convex surfaces (β sides) (Fig. 10 **NP1**) to the state of partial overlapping of convex surfaces over the C7 lateral sides of steroid skeletons (Fig. 10 NP2).

Thermodynamic stabilization of the micelle is greater if larger part of the hydrophobic surface area of the micellar building unit is protected from the solvent. However, the NP2 state is not a thermodynamically stable state, as hydrophobic hydration increases with respect to the NP1 state, which is not entropically favorable (transfer of water molecules from bulk water to hydrophobic surface of the micellar building units). In α-α parallel and α-α antiparallel association, for similar reasons as in β-β associations, the hydrogen bonds could exist only in secondary IUD micelles (via mutual association of the primary micelles).

4. Conclusions

According to the values of *k*, *CMC* i ΔC_p the hydrophobicity of bile salts decreases in the following sequence: CD > IUD > UD > ICD. However, as IUD contains two β equatorial OH groups in its steroid skeleton, it could have been expected that IUD is the least hydrophobic BS in the abovementioned sequence. The mutual distance between the C7 and the C3 OH groups, as well as the conformation of the steroidal skeleton of IUD, result in the smallest difference in the hydrophobicity between α and β side of its steroid skeleton, among the examined BS. In planes *lnk* – ln*CMC* and ln*k* – $\ln\Delta C_p^{demic}$ derivatives CD, UD, ICD form linear congeneric groups, while IUD is the outlier. The IUD is probably not a true outlier in relation to the hydrophobic linear congenital group (11), but a member of a new hydrophobic congeneric group. Namely, in IUD there are twice as many favorable spatial orientations of steroid skeletons that can result in the formation of primary micelles near the critical micellar concentration. In the vicinity of the CMC, IUD can form primary micelles by association of IUD particles both from the convex side and from the concave side of the steroid ring system.

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