CHOLESTEROL SOLUBILIZATION BY OXO DERIVATIVES OF SELECTED BILE ACIDS AND THEIR MEMBRANOTOXICITY

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This study is concerned with the effect of the structure of bile acids on the solubilization of cholesterol (cholesterol solubilizing capacity (C_{Chm}) and the equilibrium micellary solubilization of cholesterol (x_{Chm})). It was found that the replacement of the hydroxy group in the bile acid molecule with an oxo group results in a decrease of the solubilization power of cholesterol. The examined bile acids form two linear groups at the plane of critical micellar concentration (CMC) and solubilization power of cholesterol (C_{Chm} or x_{Chm}). The group I is formed by bile acids with lower CMCs and higher cholesterol solubilites (deoxycholic (**1**), chenodeoxycholic (**2**), hyodeoxycholic (**3**), cholic (**8**), 12-oxolithocholic (**4**), and 7-oxolithocholic (**5**) acids). On the other hand, the group II is formed by bile acids with higher CMCs and lower cholesterol solubilites (7-oxodeoxycholic (**9**), 12-oxochenodeoxycholic (**10**), 12α-hydroxy-3,7-dioxocholanic (**11**), 7,12-dioxolithocholic (**12**), 3,7-dioxocholanic (**6**), and 3,12-dioxocholanic (**7**) acids). The common conformational characteristics of the bile acid molecules was determined (orientation of OH or oxo grups considered to the mean plane of the steroid skeleton). They form joint groups in the plane of CMC – (C_{Chm} or x_{Chm}), and the linear function (C_{Chm} or x_{Chm}) = *f*(CMC). The osmotic resistance of erythrocytes determines the membranotoxicity of bile acids. 12-Oxolithocholic acid represents the best compromise with regard to the solubilization of cholesterol and membranotoxicity.

Keywords: Steroids; Structure-activity; Micelles; Bile acids; Cholesterol; Solubilization membranotoxicity.

The value of hydrophilic-lipophilic balance of bile acids exceeds 20, indicating that they are amphiphiles which form mixed micelles with hydrophobic molecules[1–4]. Hence some bile acids (chenodeoxycholic and ursodeoxycholic acid) are used as drugs for dissolving gallstones of cholesterol type[4,5], while some others exhibit a promotive effect on the transport of certain drugs through various biological membranes (nasal, epithelial, intestinal, etc.)[4–10]. It should be also noticed that bile acids damage cell membranes by withdrawing phospholipids from them[6]. In their experiment of monitoring erythrolysis, Bowe et al.[6] showed that bile acids with higher

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critical micellar concentrations (CMC) damage less erythrocytes. Poša et al.[11–14] and Roda et al.[15] showed that the replacement of the OH group in the molecule of a bile acid with the oxo group results in an increase of its CMC value, which is desirable from the aspect of preventing cell membrane damages. Besides, oxo derivatives of bile acids exhibit a promotive effect in the transport of some drugs[4,16–20].

In the literature, the membranotoxicity of the bile acids is usually determined through the hemolytic potential (erythrolysis)[6,9,12] but a less known parameter can be used, i.e., the osmotic resistance of the erythrocites[21]. The osmotic resistance of the erythrocytes is the ability of the erythrocyte membrane to lower the unsteady flux (netto flux) of the water molecules through the membrane, which is appearing when the cell is in the hypo- or hyperosmolar solution. The erythrocyte membrane is more resistant to the change of the solution osmolality in which it is in, if it has pores of smaller size and if the number of the pores is smaller, because the permeability of the membrane to the water is less[22].

The aim of this study was to determine the relationship between the structure of investigated bile acids, their cholesterol solubilizing capacity[23] (C_{Chm}) and the equilibrial micellary solubilization of cholesterol (x_{Chm}). The other goal was to find the structure of a bile acid, which dissolves cholesterol, but does not show a membranotoxicity.

This study of solubilization of cholesterol is primarily focused on the oxo derivatives of cholic (**8**), deoxycholic (**1**), and chenodeoxycholic (**2**) acids (Fig. 1), the subject that, as far as we know, has not been treated so far.

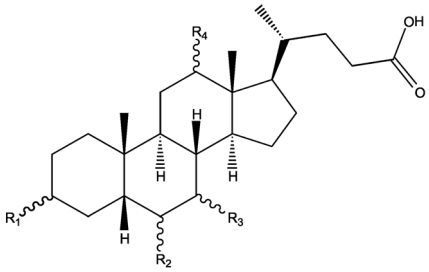
EXPERIMENTAL

Synthesis of Oxo Derivatives of Cholic, Deoxycholic and Chenodeoxycholic Acids

Cholic **8**, deoxycholic **1**, and chenodeoxycholic **2** acids (Sigma, 98%) were used as the starting compounds for the synthesis of their oxo derivatives.

3α-Hydroxy-12-oxo-5β-cholanoic (12-oxolithocholic) acid (**4**) and 3α,7α-dihydroxy-12-oxo-5β-cholanoic (12-oxochenodeoxycholic) acid (**10**) were prepared according to the procedure of Miljković et al.[24], while 3α,12α-dihydroxy-7-oxo-5β-cholanoic (7-oxodeoxycholic) acid (**9**) and 3α-hydroxy-7-oxo-5β-cholanoic (7-oxolithocholic) acid (**5**) were obtained according to Tullar[25]. 3α-Hydroxy-7,12-dioxo-5β-cholanoic (7,12-dioxolithocholic) acid (**12**) was synthesized by a selective oxidation of the 7α-hydroxy group of 3α,7α-dihydroxy-12-oxo-5β-cholanoate following the procedure of Tullar[25]. The starting compound for obtaining 12-hydroxy-3,7-dioxo-5-cholanoic (12-hydroxy-3,7-dioxocholanic) acid (**11**) was methyl cholate, selectively oxidized in a one-pot reaction according to Kuwada et al.[26]. 3,7,12-Trioxo-5β-cholanoic (3,7,12-trioxocholanic) acid (**13**), 3,12-dioxo-5β-cholanoic (3,12-dioxocholanic) acid (**7**), and 3,7-dioxo-5β-cholanoic (3,7-dioxocholanic) acid (**6**) were obtained according to Fieser and Rajagopalan[27]. Hyodeoxycholic acid (**3**; 98%) was bought

FIG. 1
Structures of tested bile acids



Bile acid (abbreviation)	Substituent			
	R_1	R_2	R_3	R_4
3α,12α-dihydroxy-5β-cholanoic acid; deoxycholic acid (D (**1**))	α-OH			α-OH
3α,7α-dihydroxy-5β-cholanoic acid; chenodeoxycholic acid (CD (**2**))	α-OH		α-OH	
3α,6α-dihydroxy-5β-cholanoic acid; hyodeoxycholic acid (HD (**3**))	α-OH	α-OH		
3α-hydroxy-12-oxo-5β-cholanoic acid; 12-oxolithocholic acid (12-oxoLC (**4**))	α-OH			=O
3α-hydroxy-7-oxo-5β-cholanoic acid; 7-oxolithocholic acid (7-oxoLC (**5**))	α-OH		=O	
3,7-dioxo-5β-cholanoic acid; 3,7-dioxocholanic acid (3,7-dioxoC (**6**))	=O		=O	
3,12-dioxo-5β-cholanoic acid; 3,12-dioxocholanic acid (3,12-dioxoC (**7**))	=O			=O
3α,7α,12α-trihydroxy-5β-cholanoic acid; cholic acid (C (**8**))	α-OH		α-OH	α-OH
3α,12α-dihydroxy-7-oxo-5β-cholanoic acid; 7-oxodeoxycholic acid (7-oxoD (**9**))	α-OH		=O	α-OH
3α,7α-dihydroxy-12-oxo-5β-cholanoic acid; 12-oxochenodeoxycholic acid (12-oxoCD (**10**))	α-OH		α-OH	=O
12α-hydroxy-3,7-dioxo-5β-cholanoic acid; 12-hydroxy-3,7-dioxocholanic acid (12-OH-3,7-dioxoC (**11**))	=O		=O	α-OH
3α-hydroxy-7,12-dioxo-5β-cholanoic acid; 7,12-dioxolithocholic acid (7,12-dioxoLC (**12**))	α-OH		=O	=O

from Sigma (New Zealand). All bile acids were transformed to their sodium salts by a known procedure[15].

Cholesterol (95%; Aldrich) was recrystallized three times from hot 95% ethanol, followed by preparation of crystalline monohydrate[23].

Determination of Bile Salt Cholesterol Solubilizing Capacity

The bile salt solubilizing capacity was determined according to the procedure of Armstrong and Carey[23]. Finely powdered cholesterol monohydrate (100 mg, 0.2580 mmol) was placed in an Ehrlenmeyer flask and mixed with 10 ml of a buffered aqueous suspension of sodium salt of the bile acid (150 mM, 0.15 M NaCl, pH 7.4, $H_2PO_4^-/HPO_4^-$). In the case of sodium salts of 12-hydroxy-3,7-dioxocholanic (**11**), 3-hydroxy-7,12-dioxocholanic (**12**), and 3,7,12-trioxocholanic (**13**) acids, because of their detection limits, the suspension volume was 20 ml. The flask content was incubated on a water bath (37 °C) for 7 days while stirring on a magnetic stirrer (300 rpm). After that, the suspension was filtered on a preheated (37 °C) filter (0.22 μm). In the case of deoxycholic (**1**), 12-oxolithocholic acid (**4**), chenodeoxycholic (**2**), and cholic (**8**) acids, samples (100 μl) were taken directly from the filtrate, whereas in the case of the other bile acids, the filtrate was evaporated to dryness. If the salts tested were of hyodeoxycholic (**3**) or 7-oxolithocholic (**5**) acid, the residue after the evaporation was mixed with distilled water (5 ml), whereas in the case of 12-oxochenodeoxycholic (**10**), 7-oxodeoxycholic (**9**), 3,12-dioxocholanic (**7**), 3,7-dioxocholanic (**6**), 7,12-dioxolithocholic (**12**), 12-hydroxy-3,7-dioxocholanic (**11**), and 3,7,12-trioxocholanic (**13**) acids, the dry residues were dissolved in distilled water (1.00 ml) and then an aliquot (100 μl) was taken for cholesterol analysis. Cholesterol was determined by enzymatic method with cholesterol oxidase (Reanal, Budapest), measuring absorbance at 500 nm on an Agilent 8453 spectrophotometer, using distilled water as the blank.

In this work, the bile salt solubilizing capacity C_{Chm} is expressed as the number of moles of dissolved cholesterol monohydrate per mole of bile acid (mole fraction of dissolved cholesterol)[23].

Determination of Equilibrium Micellar Solubilization of Cholesterol Based on the Curve of Solubilization

The equilibrium micellar solubilization of cholesterol by bile acid salts was determined according to the modified version of the experiment for the determination of lecithin solubilization[28]. Suspensions of cholesterol monohydrate (10 mg, 0.0258 mmol) and Na salt of bile acid (20 ml, 0.15 mM NaCl, pH 7.4, $H_2PO_4^-/HPO_4^-$) were prepared so that bile acid concentrations were in the range from 2 to 10 mM. If no cholesterol solubilization was achieved, new series of solutions was prepared ranging from 10 to 20 mM, etc. The suspension of cholesterol monohydrate and bile acid was thermostated at 37 °C in a water bath for 48 h while stirring on a magnetic stirrer (300 rpm). After that, the suspension was filtered on a preheated (37 °C) filter (0.22 μm). The concentration of cholesterol was determined directly from the filtrate by enzymatic method (Reanal, Budapest). If the solubilization of cholesterol was not achieved by one of the bile acids up to 250 mM (the concentration of the bile acid), then the experiment for that bile acid was repeated with the bile salt and with the concentration of cholesterol monohydrate in the suspension lowered to 1 mg (0.0025 mmol). The equilibrium concentration x_{Chm} (mole fraction of dissolved cholesterol)

is determined by the curve of dependence between cholesterol solubilization and bile salt concentration.

Determination of Osmotic Resistance

Osmotic resistance was determined from rat (Wistar) blood[21]. A number of 14 groups of rats was used in the experiment (one group for every investigated bile acid and one control group). Every group was formed from 7 experimental animals. In the determination of the osmotic resistance of the erythrocytes, the drugged animal (narcosis by ether) was injected in the tail vein by a solution (3 mM, pH 7.4, $H_2PO_4^-/HPO_4^-$) of the Na-salt of the investigated bile acid in the dose of 0.01 mmol/kg (in the controle group by a saline solution). After the distribution phase of the bile acids (after 45 min), the blood was drained from the left chamber of the heart muscle of the experimental animal in a test tube with anticoagulans (citrate blood). The sample of the citrate blood (100 µl) was inserted in a series of hypotonic solutions of NaCl (0.00, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, and 0.75%) and in isotonic solution of NaCl (0.90%). The solutions were centrifuged (3 min, 1500 rpm). The concentration of hemoglobine was determined spectrophotometrically[29] from the supernatant. The hemolysis was confirmed in the hypotonic solution if the relative increase (δ_{HB}) of the concentration of hemoglobin ($c_{HB\text{-}h}$) in the hypotonic solution relative to the concentration of the hemoglobin in the isotonic solution ($c_{HB\text{-}i}$) (0.90% NaCl) was more than 0.1 (10%): $\delta_{HB} = (c_{HB\text{-}h} - c_{HB\text{-}i})/c_{HB\text{-}i} > 0.1$.

Data Treatment

Hierarchical classification, correlations and principial component analysis (PCA) were obtained using the program package of Statistica 8.0. The 3D models (energetically most favorable) of bile acids were generated according to the MOPAC protocol (ChemBio3D Ultra 11.0).

RESULTS AND DISCUSSION

Micellar Solubilization of Cholesterol

The capacity of cholesterol solubilization (C_{Chm}) was determined by the Armstrong and Carey method[23] (Table I) by which the investigated bile acid salts were in equimolar amount in the solutions where their concentrations are above CMC. The equilibrial micellar solubilization of cholesterol (x_{Chm}) was determined, too, based on the curve of solubilization (Fig. 2). The values of the cholesterol solubilization capacity and equilibrial micellary cholesterol solubilization were statistically significantly different in these bile acids: deoxycholic, chenodeoxycholic, cholic, hyoholic, and 7-oxolytocholic (Table I). These different values of cholesterol solubilization in the above mentioned bile acids can be explained by the micellar forms they form. Based on the partai molecular dynamics simulation[30], it is known that cholic acid below 30 mM forms dimers with hydrogen bonds as well as

Small's micelles with the hydrophobic nucleus. This decreases the capacity of cholesterol solubilization by the decreased number of micelles with hydrophobic cages. Other explanation offer the values of the equilibrium constants (K) in forming mixed micelles of bile acid salts and cholesterol. The K values are very low with the bile acids with two or three oxo groups. That is why the change in concentration (above CMC) is affecting very little the cholesterol solubilizing capacity.

Experimental data for the cholesterol solubilizing capacity does not differ statistically from the literature data[23] (Table I).

With the increase of the number of the OH groups as well as of the substitutions of the OH groups with the oxo groups in the studied bile acids, the solubilization of cholesterol is decreasing (the fall of the value of C_{Chm} and x_{Chm}) as well as their tendency to self aggregation[11,12] (the value of the CMC rises, Table I). The effect of the oxo groups in the bile acids on their solubilization of cholesterol can be explained by the orientation of the oxo

TABLE I
Experimental values and literature data

Bile acid	CMC (lit.[11,12]) mM	Exp. mean±SD (n = 7)		mean±SD (lit.[23])
		c_{chm} mol/mol BA	x_{chm} mol/mol BA	c_{chm} mol/mol BA
Deoxycholic **1**	5.50	0.072±0.003	0.057±0.004	0.069±0.01
Chenodeoxycholic **2**	5.75	0.061±0.002	0.048±0.003	0.056±0.003
Hyodeoxycholic **3**	17.00	0.0065±0.005	0.0051±0.0005	
12-Oxolithocholic **4**	18.50	0.016±0.005	0.013±0.005	
7-Oxolithocholic **5**	22.50	0.0058±0.003	0.0047±0.0004	
3,7-Dioxocholanic **6**	75.00	0.0012±0.004	0.0010±0.0005	
3,12 Dioxocholanic **7**	72.00	0.0010±0.004	0.0010±0.0005	
Cholic **8**	8.00	0.032±0.002	0.026±0.002	0.032±0.002
7-Oxodeoxycholic **9**	60.00	0.0017±0.0004	0.0016±0.0003	
12-Oxochenodeoxycholic **10**	65.00	0.0022±0.0005	0.0024±0.0003	
12α-Hydroxy-3,7-dioxocholanic **11**	102.00	0.00053±0.0020	0.00053±0.00020	
7,12-Dioxolithocholic **12**	100.00	0.00075±0.00025	0.00070±0.0003	
3,7,12-Trioxocholanoic **13**	140.00	0.00±0.00015	0.00±0.00010	

group to the mean plane of the steroidal skeleton. These orientations impact the stability of mixed micelles formed by bile acids and cholesterol.

By the oxidation of the α axial (α(α)) OH groups of cholic acid (**8**) (OH groups with C7 and C12 methylene groups of the steroidal skeleton), and α(α) OH group of deoxycholic (**1**) and chenodeoxycholic (**2**) acids, oxo groups are formed with oxygen atoms with α equatorial (*e*) position (Fig. 3). Based on this the oxygen atoms of C7 and C12 oxo groups are shifted by 60° related to the positions of α(α) OH groups (Newman's projection formula). With the mean plane of the steroid skeleton (SSMP), the oxygen atoms form the angle of –30°. By the oxidation of the α equatorial (*e*) OH group (C3 OH group of cholic (**8**), deoxycholic (**1**), and chenodeoxycholic (**2**) acids), an oxo group is formed with the oxygen atom of β(*e*) orientation and it forms a 30° with the SSMP (Fig. 3). This means that with the substitution of OH groups with oxo groups, derivates are formed in which the oxygen atom is shifted to the β side of the steroid skeleton.

The presence of the oxo groups decreases the stability of the mixed micelles of the bile acids and cholesterol, because water molecules enter the gap – fiord[12,13,19,31], which is situated on the surface of the micelle where the two monomers collide (Small's type of primary micelles). The water molecules are binding with hydrogen bonds to the oxo groups and form

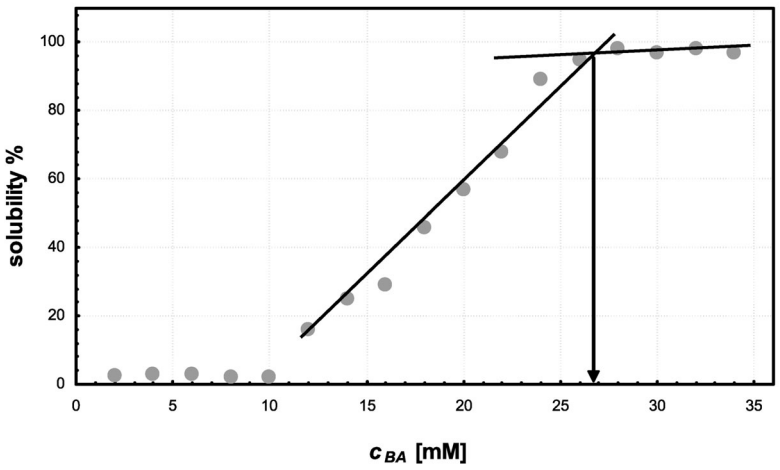


FIG. 2
The example of the curve of solubilization of cholesterol monohydrate (1 mg) with 7-oxolithocholic acid (**5**), the arrow in the figure shows the concentration of the bile acid in which the solubilization is 100%, and based on this concentration, the x_{Chm} is calculated

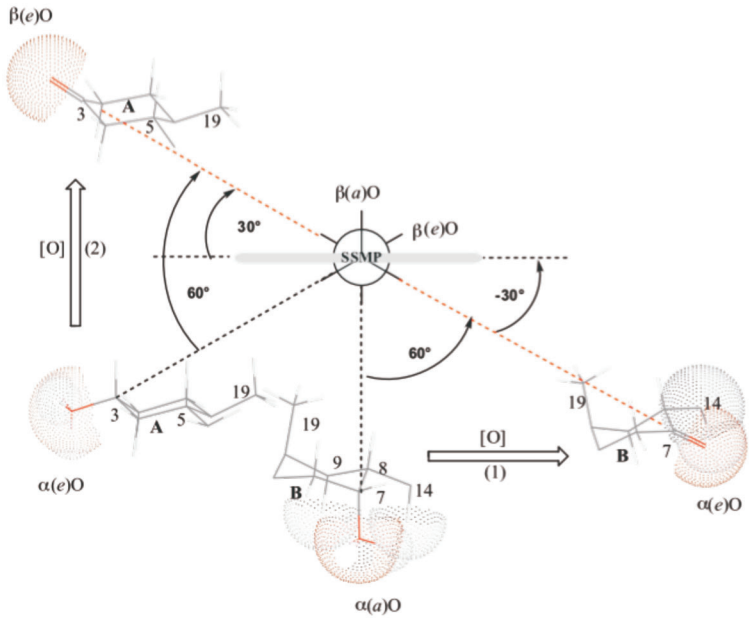


FIG. 3

The shift of the oxygen atom to the steroid skeleton main plain (SSMP) by the oxidation of the α axial (1) and α equatorial (2) OH groups to the oxo groups. A and B ring cut of the steroid system rings

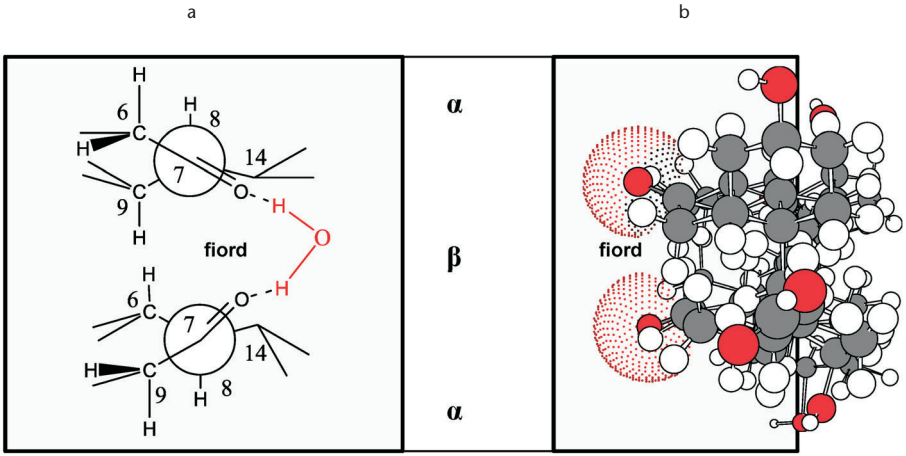


FIG. 4

The fiord effect: cut from the structure of the micelle of the 7-oxodeoxycholic acid (9) in the Newman projection (a), the cross section of the micelle (b)

hydrogen bond bridges – water molecules between the two monomers aggregate, which is stabilizing the micelle without cholesterol. This kind of stabilization is not possible if the bile acid monomer has α axial OH groups[12,13,19]. This is lowering the hydrophobicity of the inner cage of the micelle. By this reducing, the possibility of accepting the hydrophobic molecule guest (cholesterol), and this is decreasing the solubilization of cholesterol with the oxo derivates of bile acids (Fig. 4). Kawamura et al.[32] explained on the similar way the decreased power of solubilization of cholesterol with tauroursodeoxycholic acid. This bile acid has a C7 β equatorial OH group which is shifted to the angular methyl group, and therefore in the Small micelle C7 OH groups of the neighbor bile acids form hydrogen bonds. Hence the micelle is more compact and it is harder for the cholesterol to incorporate into the hydrophobic cage.

In the plane which define CMC and the capacity of cholesterol solubilization C_{Chm}, two linear groups can be identified (Fig. 5). The first group is formed by deoxycholic (D (**1**)), chenodeoxycholic (CD (**2**)), cholic (C (**8**)), 12-oxolithocholic (12-oxoLC (**4**)), hyodeoxycholic (HD (**3**)) and 7-oxolithocholic (7-oxoLC (**5**)) acids, and the second one is formed by the mono and dioxo derivates of cholic acid (7-oxoD (**9**), 12-oxoCD (**10**), 12-OH-3,7-dioxoC (**11**), 7,12-dioxoLC (**12**)), 3,7,12-trioxocholanic (3,7,12-trioxoC (**13**)), 3,12-dioxocholanic (3,12-dioxoC (**7**)) and 3,7-dioxocholanic (3,7-dioxoC (**6**)) acids.

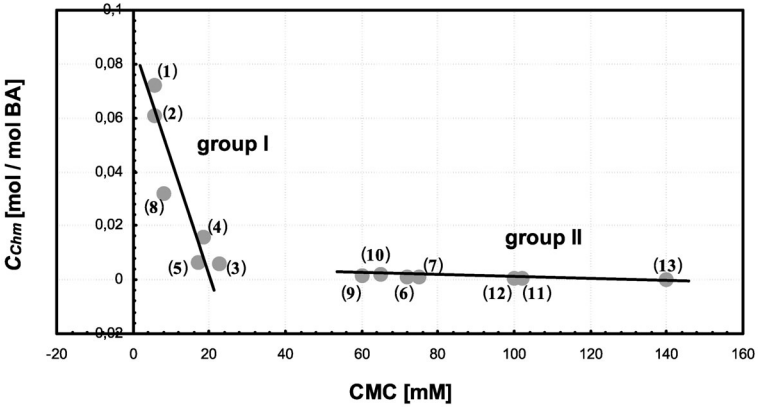


FIG. 5

The correlation between the cholesterol solubilization capacity C_{Chm} and the critical micellar concentrations CMC of bile acids. (**1**): D; (**2**): CD; (**3**): HD; (**4**): 12-oxoLC; (**5**): 7-oxoLC; (**6**): 3,7-dioxoC; (**7**): 3,12-dioxoC; (**8**): C; (**9**): 7-oxoD; (**10**): 12-oxoCD; (**11**): 12-OH-3,7-dioxoC; (**12**): 7,12-dioxoLC; (**13**): 3,7,12-trioxoC

The small value of the correlation coefficient between the CMC of bile acids and C_{Chm} (r = –0.677) is the result of forming of the linear groups I and II. It is known in the literature that bile acids form linear congener groups, e.g., the group of cholic acid and its oxo derivates, dihydroxy derivates of bile acids and its oxo derivates, etc.[33]. In this case, the linear groups are not "pure" homologue groups because the group II is formed by the oxo derivates of the cholic acid, and the oxo derivates of deoxycholic acid and chenodeoxycholic acid, too. It is determined by stereochemical (conformational) analysis that the bile acids from the group I satisfy the next equation:

$$\max 2\alpha(e)\mathrm{O} \wedge 0\alpha(a)\mathrm{O} \vee \max 2\alpha(a)\mathrm{O} \wedge \max 1\alpha(e)\mathrm{O} \qquad (1)$$

where O marks the oxygen atom (OH or oxo group), α(*e*) presents α equatorial orientation, while α(*a*) corresponds to the axial orientation. According to the mentioned equation, the group I consists of the bile acids with maximum two atoms of oxygen (OH or oxo groups) in the α equatorial position without the α axial OH group in the steroidal skeleton (like hyodeoxycholic acid (**3**): C3-α(*e*)OH, C6-α(*e*)OH; 7-oxolithocholic acid (**5**): C3-α(*e*)OH, C7-α(*e*)oxo group; 12-oxolithocholic acid (**4**): C3-α(*e*)OH, C12-α(*e*)oxo group), as well as of that with maximum two OH groups in the α axial orientation and one O atom (OH or oxo group) in the α equatorial orientation (cholic acid (**8**): C3-α(*e*)OH, C7-α(*a*)OH, C12-α(*a*)OH). Moreover, in the first group, there are also those bile acids which have a lesser number of O atoms from the maximal number in the appropriate orientations according to Eq. (*1*), like deoxycholic (**1**) (C3-α(*e*)OH, C12-α(*a*)OH) and chenodeoxycholic (**2**) (C3-α(*e*)OH, C7-α(*a*)OH) acids.

If the stereochemical (conformational) condition of Eq. (*1*) is satisfied, the oxygen atoms can be on different C atoms as well as the OH and oxo groups, in the plane of CMC – C_{Chm} bile acids form a group which is determined by the linear function:

$$C_{Chm} = 0.0774 - 0.0035\ \mathrm{CMC} \qquad (2)$$

$$r = -0.9090$$

Bile acids that do not refer to the limit of Eq. (*1*) form group II. The linear regression equation for the group II is:

$$C_{Chm} = 0.0031 - 0.0002\ CMC \quad (3)$$

$$r = -0.8950$$

The tested bile acids in the plain of CMC – x_{Chm} also form two groups which are identical with the groups from the plain CMC – C_{Chm}. The linear regression equation for the group I is:

$$x_{Chm} = 0.0613 - 0.0027\ CMC \quad (3)$$

$$r = -0.91149$$

The linear regression equation for the group II is:

$$x_{Chm} = 0.0030 - 2.35 \times 10^{-5}\ CMC \quad (2)$$

$$r = -0.84413$$

Between the parameters of solubilization of cholesterol C_{Chm} and $x_{Chm,}$ there is a high correlation (Pearons correlation: 0.99989), which implies that both parameters contain the same information about the bile acid structure and its cholesterol solubilization.

The grouping of the bile acids in the plane CMC and C_{Chm} (CMC – x_{Chm}) is confirmed by the hierarchical method of grouping based on the values of CMC and C_{Chm} (centroid rule of interrelationship). In the formed dendrogram, there are two groups, too (Fig. 6). In the group A, there can be identified two subgroups (a and b), for the subgroup Aa its characteristic that the molecules of the bile acids have C3-α(*e*) OH group and maximally two α(α) OH groups. For the subgroup Ab the characteristic is the presence of α(*e*) oriented oxygen atom without α(*a*)O. In the B group, there are two subgroups, too. For the bile acids of subgroup Ba, the presence of β(*e*)O (C3 oxo group) is characteristic, while the bile acids from the Bb group do not have β(*e*)O. The dendrogram shows significant similarity between hyodeoxycholic (**3**) and 7-oxolithocholic (**5**) acids, their values of x_{Chm} are statistically not significantly different ($p < 0.05$) (Table I). This similarity in cholesterol solubilization is present in both hyodeoxycholic (**3**) and 7-oxolithocholic (**5**) acids, because the oxygen atoms bound to the steroidal skeleton have the same orientation towards the mean plain of the steroid skeleton (Fig. 7). Even if the above mentioned conformational explanation

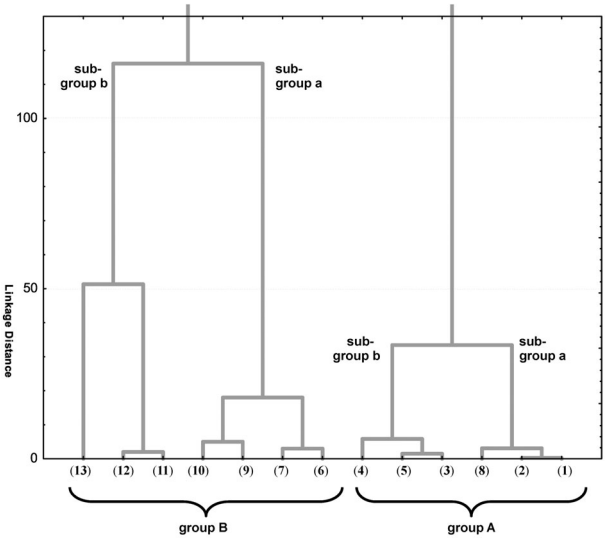


FIG. 6

The hierarchical grouping of the investigated bile acids in relation to CMC and C_{Chm}. (**1**): D; (**2**): CD; (**3**): HD; (**4**): 12-oxoLC; (**5**): 7-oxoLC; (**6**): 3,7-dioxoC; (**7**): 3,12-dioxoC; (**8**): C; (**9**): 7-oxoD; (**10**): 12-oxoCD; (**11**): 12-OH-3,7-dioxoC; (**12**): 7,12-dioxoLC; (**13**): 3,7,12-trioxoC

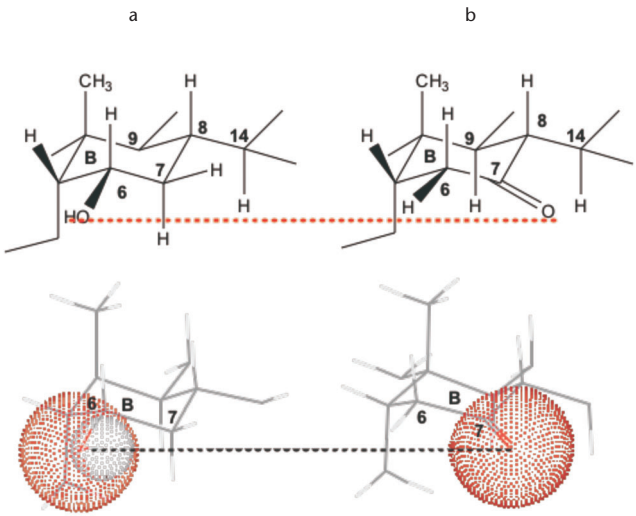


FIG. 7

The position of oxygen atoms: the B ring of the hyodeoxycholic acid (a), the B ring of the 7-oxolithocholic acid (b)

is valid for the 12-oxolithocholic acid (**4**), this bile acid has much higher value of x_{Chm} than the two bile acids mentioned before.

This difference is the consequence of the shielding of C12 oxo group with the side chain[13] (formation of a hydrogen bond between C12 oxo and side-chain COOH hydroxy groups, or alternatively, among C12 oxo group, water and side-chain COO^- group if the COOH group is deprotonated). Bertolesi et al.[34] found the hydrogen bond between the side chain and the C12 oxo group of the dehydrocholic acid (**13**). Due to this shielding, 12-oxolithocholic acid (**4**) is more hydrophobic than 7-oxolithocholic (**5**) or hyodeoxycholic (**3**) acid (R_{M0} retention index in the reverse thin layer chromatography, molecular lipophilicity, for the 12-oxoLC is 4.68 while for the 7-oxoLC 4.49)[28].

Osmotic Resistance

In this experiment where the osmotic resistance is determined, the erythrocytes are in a hypotonic solution, there is a netto flux of water molecules J_{water} from the solution in to the intracellular space of the erythrocytes if the permeability of water molecules are sufficiently large. The J_{water} is described by the next equation[22]:

$$J_{water} = p_{water}\,\Delta\pi \approx p_{water}\,RT\Delta c_{NaCl}\,.$$

In the upper equation, $\Delta\pi$ is the difference between the osmotic pressure on the surface of the membrane (inside the cell the osmotic pressure is large, while in the hypotonic solution the osmotic pressure is very low, so the water molecules are moving in the cell), Δc_{NaCl} describes the difference of the concentration of the salt between the intracellular liquid of the erythrocytes and the hypotonic solution. As the NaCl concentration of the solution in which the erythrocytes are lower, $\Delta\pi$ is getting higher (also the Δc_{NaCl} is increasing), so according to the upper equation, the water flux should be rising. But, if the permeability of the membrane to water is not changing, besides the high $\Delta\pi$ there is no transport of the water molecules to the cell and hemolysis is not encountered. If the xenobiotic does not damage the membrane (membranes small permeability is not changed), it is considered that a 0.45% NaCl solution does not have enough $\Delta\pi$ to start the netto flux of the water molecules through the membrane, and to create hemolysis[21,22]. If the hemolysis is reached in the hypotonic solution with the concentration above 0.45% NaCl, then it is considered that the molecule lowers the osmotic resistance of the erythrocytes.

Table II shows the values of δ_{HB} for the examined bile acids. In hypotonic solutions of NaCl with the concentration above 0.45%, hemolysis is present in deoxycholic (**1**) and chenodeoxycholic (**2**) acids, while by cholic acid (**8**), the hemolysis is present already at 0.45%. Therefore, these bile acids lower the osmotic resistance of the erythrocytes. With the use of principal component analysis PCA on the Table II, the calculated principal components (PC1, PC2 and PC3) explain 98.99% of the complete variance of Table II. In the space of the score of principal components (Fig. 8), the examined bile acids form two clusters. The cluster I consists of the following bile acids: deoxycholic (**1**), henodeoxycholic (**2**), and cholic (**8**) acids, while the cluster II of the oxo derivates of the bile acids and hyodeoxycholic acid (**3**). The grouping of the bile acids in the space of principal components is confirming the conclusion based on the criteria $\delta_{HB} > 0.1$ that bile acids **1**, **2** and **8** decrease the osmotic resistance of the erythrocytes. These bile acids form a divided cluster (Fig. 8). The presence of hyodeoxycholic acid (**3**) in the group of the bile acids with the oxo group in the steroidal skeleton confirms the importance of the equivalent stereochemical orientation of α C6 OH group in hyodeoxycholic acid (**3**) and C7 oxo group in 7-oxolithocholic acid (**5**) (Fig. 7). To relate the degree of cholesterol solubilization of the investigated bile acids and their osmotic resistance, the Pearsons correlations are calculated between the C_{Chm} and the scores of principal component (PC1, PC2 and PC3). Significant correlation ($r = 0.981$; $p = 0.001$) exists between C_{Chm} and PC1 (PC1 explains 81.54% total variance in Table II). This means that from the values of the data δ_{HB} of the bile acid salts (Table II), the principal component PC1 has "extracted" the information about the hydrophobicity because it defines the solubilization of the studied molecule as the membranolytical activity of the bile acids[23,28]. The tested bile acids in the plane of C_{Chm} – PC1 form two groups, too, as in the plane of C_{Chm} – CMC with identical elements (Fig. 9). The hydrophobic bile acids which decrease the osmotic resistance of the erythrocytes (deoxycholic (**1**), chenodeoxycholic (**2**), and cholic (**8**) acids) are in the right positive corner of the plane C_{Chm} – PC1. The presence of oxo groups or α(*e*) OH groups instead of α(*a*) OH groups is decreasing the hydrophobicity of the β side of the steroidal skeleton, and this leads to the decrease in the membranotoxic activity. The bile acids froming the group II (Fig. 9) do not display membranolytic activity, but they practicaly do not dissolve cholesterol. The following bile acids from the group I, 12-oxolithocholic acid (**4**) > 7-oxolithocholic acid (**5**) ≈ hyodeoxycholic acid (**3**), are solubilizing cholesterol to some degree and in vivo do not decrease the osmotic resistance of the erythrocytes (Fig. 9).

TABLE II

The osmotic resistance, gray color marks hemolysis $\delta_{HB} \geq 0.1$, with dark gray color representing the concentration of NaCl after which the hemolysis occurs, i.e., the studied molecule lowers the osmotic resistance of erythrocytes

BA	Concentration of NaCl, %															
	0.75	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35	0.30	0.25	0.20	0.15	0.10	0.05	0.00
c.g.[a]	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.02±0.01	0.01±0.01	0.06±0.02	0.08±0.02	0.11±0.03	0.10±0.03	0.12±0.03
1	0.06±0.01	0.09±0.01	0.12±0.01	0.14±0.02	0.13±0.02	0.17±0.03	0.24±0.04	0.24±0.04	0.27±0.05	0.25±0.04	0.29±0.04	0.26±0.04	0.28±0.05	0.31±0.05	0.30±0.04	0.39±0.05
2	0.07±0.01	0.09±0.01	0.09±0.02	0.12±0.02	0.16±0.04	0.19±0.03	0.25±0.05	0.26±0.05	0.26±0.04	0.27±0.05	0.31±0.05	0.29±0.04	0.30±0.05	0.34±0.06	0.36±0.05	0.42±0.07
3	0.06±0.01	0.05±0.00	0.04±0.00	0.06±0.01	0.05±0.01	0.05±0.01	0.08±0.01	0.07±0.01	0.09±0.02	0.11±0.01	0.12±0.02	0.15±0.02	0.18±0.02	0.20±0.03	0.23±0.02	0.25±0.05
4	0.04±0.00	0.05±0.00	0.05±0.01	0.07±0.01	0.05±0.01	0.06±0.01	0.09±0.02	0.08±0.01	0.12±0.02	0.13±0.02	0.14±0.02	0.17±0.02	0.20±0.02	0.24±0.03	0.28±0.04	0.29±0.06
5	0.06±0.01	0.07±0.01	0.04±0.00	0.06±0.01	0.06±0.02	0.05±0.01	0.06±0.01	0.07±0.02	0.11±0.01	0.12±0.02	0.13±0.02	0.15±0.02	0.19±0.02	0.22±0.02	0.24±0.05	0.24±0.05
6	0.06±0.00	0.06±0.01	0.04±0.00	0.05±0.01	0.05±0.01	0.07±0.01	0.09±0.01	0.08±0.02	0.08±0.02	0.11±0.01	0.11±0.01	0.14±0.02	0.17±0.02	0.21±0.04	0.23±0.05	0.26±0.04
7	0.05±0.00	0.06±0.01	0.04±0.00	0.06±0.01	0.06±0.01	0.08±0.02	0.06±0.02	0.09±0.02	0.10±0.01	0.11±0.02	0.13±0.02	0.15±0.02	0.18±0.02	0.22±0.03	0.25±0.05	0.24±0.04
8	0.05±0.00	0.06±0.01	0.06±0.01	0.08±0.01	0.09±0.02	0.06±0.02	0.10±0.01	0.13±0.02	0.16±0.02	0.20±0.03	0.23±0.04	0.25±0.04	0.28±0.05	0.28±0.05	0.30±0.06	0.36±0.05
9	0.05±0.00	0.05±0.00	0.05±0.00	0.06±0.01	0.07±0.02	0.06±0.02	0.08±0.02	0.08±0.01	0.10±0.02	0.11±0.02	0.12±0.02	0.14±0.01	0.18±0.02	0.22±0.03	0.23±0.04	0.27±0.05
10	0.05±0.00	0.04±0.00	0.04±0.00	0.06±0.01	0.06±0.01	0.05±0.01	0.07±0.02	0.07±0.02	0.09±0.01	0.12±0.02	0.14±0.02	0.18±0.03	0.19±0.02	0.22±0.03	0.24±0.04	0.25±0.03
11	0.06±0.01	0.05±0.00	0.06±0.00	0.06±0.01	0.05±0.01	0.05±0.01	0.07±0.01	0.08±0.02	0.08±0.02	0.10±0.02	0.12±0.01	0.15±0.02	0.17±0.03	0.20±0.03	0.22±0.03	0.23±0.03
12	0.04±0.00	0.05±0.00	0.05±0.01	0.06±0.01	0.07±0.02	0.06±0.01	0.07±0.01	0.07±0.02	0.08±0.01	0.11±0.01	0.11±0.01	0.14±0.02	0.18±0.02	0.20±0.02	0.23±0.04	0.23±0.04
13	0.05±0.00	0.04±0.00	0.04±0.00	0.05±0.01	0.05±0.01	0.05±0.01	0.06±0.01	0.07±0.01	0.08±0.02	0.08±0.01	0.08±0.01	0.10±0.01	0.12±0.01	0.15±0.02	0.18±0.02	0.20±0.03

[a] c.g., control group; $n = 7$.

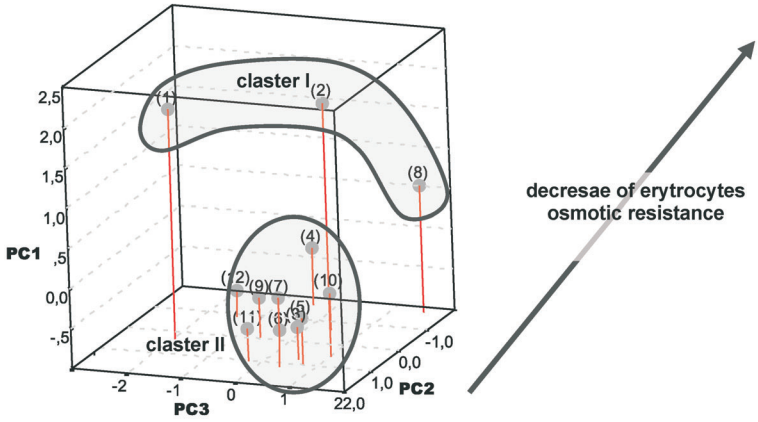


FIG. 8

The forming of the clusters in the examined bile acids in the field of the principal component score (PC1, PC2 and PC3 are explaining the 98.99% of the complete variance in Table II), based on the δ_{HB} data (Table II). (**1**): D; (**2**): CD; (**3**): HD; (**4**): 12-oxoLC; (**5**): 7-oxoLC; (**6**): 3,7-dioxoC; (**7**): 3,12-dioxoC; (**8**): C; (**9**): 7-oxoD; (**10**): 12-oxoCD; (**11**): 12-OH-3,7-dioxoC; (**12**): 7,12-dioxoLC; (**13**): 3,7,12-trioxoC

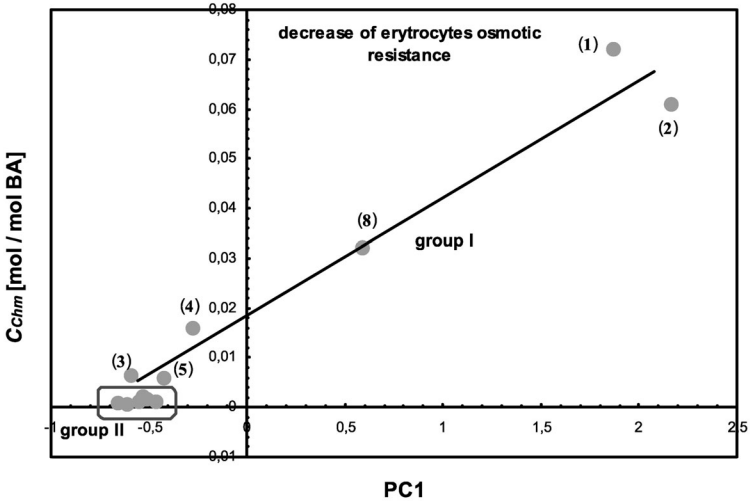


FIG. 9

The grouping of the bile acids in the plain C_{Chm} – PC1 (the PC1 explains 81.54% of the total variance from Table II). (**1**): D; (**2**): CD; (**3**): HD; (**4**): 12-oxoLC; (**5**): 7-oxoLC; (**6**): 3,7-dioxoC; (**7**): 3,12-dioxoC; (**8**): C; (**9**): 7-oxoD; (**10**): 12-oxoCD; (**11**): 12-OH-3,7-dioxoC; (**12**): 7,12-dioxoLC; (**13**): 3,7,12-trioxoC

The lowering of the osmotic resistance of the erythrocytes by deoxycholic (**1**), chenodeoxycholic (**2**), and cholic (**8**) acids is the consequence of their higher power of lecithin (phospholipid) solubilization[28], and these bile acids change the integrity of the membranes and the permeability by forming mixed micelles with the phospholipids of the cell membrane.

CONCLUSION

The cholesterol solubilizing capacity (C_{Chm}) and the equilibrial micellary solubilization of cholesterol (x_{Chm}) show significant interrelated correlation. With the substitution of the α axial OH group with oxo group or with the α equatorial OH group, the oxygen atom is shifted towards the mean plane of the steroidal skeleton of the bile acids. This leads to the lowering of the cholesterol solubilizing power and the membranolytical activity. Based on the ability to solubilize cholesterol monohydrate (C_{Chm} and x_{Chm}) and the osmotic resistance, the optimal bile acid is 12-oxolithocholic acid (**4**), which has 26% capacity of solubilization of the chenodeoxycholic acid (**2**), but does not show membranolytical activity.

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