Scientific paper

# Hydrophobicity and Retention Coefficient of Selected Bile Acid Oxo Derivatives

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*Received: 02-03-2010*

## Abstract

Retention coefficients (k) of cholic acid and its keto derivatives are determined by means of Reversed Phase High Pressure Liquid Chromatography at different temperatures (303K, 309K, and 313K). At each studied temperature, retention factor decreases if the hydroxyl group in the cholic acid molecule replaces oxo group. In addition, the change of retention coefficient in a function of temperature ($\Delta k/\Delta T$) is dominant for the cholic acid while by increasing the number of oxo groups it decreases. Introduction of an oxo group in a bile acid molecule leads to the lower hydrophobicity of the β side of the steroid nucleus. Because of that, less interaction happens between β side of the steroid nucleus and stationary phase. For dehydrocholic acid (three- oxo derivative), the value for $\Delta k/\Delta T$ shows an exception of this explanation. This suggests that in this molecule the planar polarity is disturbed.

Partition coefficient K of nitrazepam (probe molecule) in micelles of bile acid salts at the examined temperatures shows a high linear correlation with retention factors of the selected bile acids. This indicates the importance of hydrophobic interactions in mixed micelles between the examined drug and bile acid salts.

Haemolytic potential (erythrocyte haemolysis, log (Lys50)) represents measure of membranotoxicity of bile acids. In addition, it is shown that haemolytic potential correlates highly with the retention coefficient.

All experiments that we conducted to obtain the values of K and log (Lys50) as well as their correlations with k, contribute to significance of retention coefficient as a measure of hydrophobicity in biopharmaceutical experiments.

**Keywords:** Hidrophobicity, retention coefficient, bile acids, cholic acid oxo derivatives

## 1. Introduction

Bile acid salts are bi- planar compounds with two functionally different molecular areas. Convex surface of steroid core (side β) is more hydrophobic, while the concave surface (α side) is less hydrophobic (hydrophilic) due to the side-chain hydroxyl and carboxyl groups.[1–4] The relationship between hydrophobic and hydrophilic surfaces of bile acid can vary by changing the number of OH groups on the α side of the steroid nucleus, introducing hydrocarbon groups or replacing OH groups with oxo groups.[1,5,6]

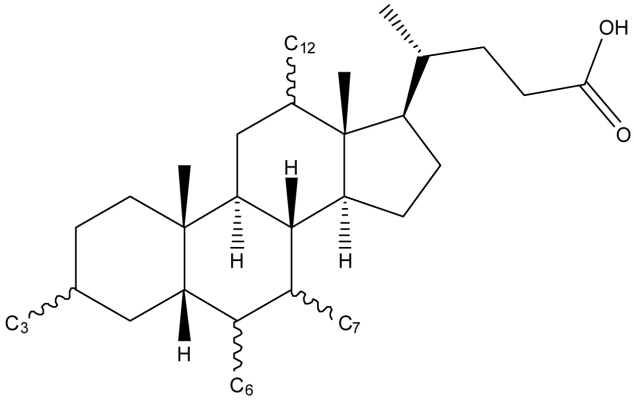
The relationship between hydrophobic and hydrophilic surfaces of bile acids is determined by their physicochemical properties such as self-association (micelle forming),[1,7,8] ability to solubilize cholesterol,[4] etc. If a bile acid is more hydrophobic, its critical micellar concentration is lower [1, 6] or it has a higher cholesterol solubilizing capacity.[4]

Hydrophilic-lipophilic balance (HLB) of a bile acid determines its binding for large conductance $Ca^{2+}$ -activated $K^+$ ($BK_{Ca}$) channels, which induces the endothelium dependent relaxation of smooth muscles.[11] Along with that, HLB value indicates that bile acids facilitate the movement of polar drugs through biological membranes.[5,12] In addition, membranotoxic effects of bile acids also depend on their HLB values.[13]

Hydrophobicity of a molecule is usually expressed as the logarithm of n- octanol -water partition coefficient. It can be expressed as well through chromatographic parameters that are more precise and reproductive than traditional "shake–flask" methods for obtaining partition coefficient.[14,15]

The aim of this work was to assess hydrophobicity of the cholic acid and its keto derivatives (Fig. 1) expressed by the retention coefficient (capacity factor)[16] and determined by Reversed Phase High Pressure Liquid Chromatography (RP HPLC) at temperatures 303K, 308K and

313K. Also, this work deals with determination of partition coefficient of nitrazepam (probe molecule),[17] as well as, haemolytic potential (membranotoxicity) of bile acids.[5] The evaluation of the nitrazepam partition coefficient in bile acid micelles is important for its biopharmaceutical use. Probe molecules partition coefficient indicates the level of its intake into the micelle, and hence modification of its bioavailability. In addition, the goal of this work was to determine correlations between the retention factor and partition coefficient and between the retention coefficient and haemolytic potential at different temperatures (303K, 308K and 313K). If high linear correlation is proved then the retention factor can be used to conduct predictive analytics. Bile acids oxo derivatives were included in the examination because their retention coefficients are not yet determined and their pharmacological use increases.[18–23]




| Trivial name (abbreviation) | Substituents $C_3$ | $C_6$ | $C_7$ | $C_{12}$ |
|---|---|---|---|---|
| cholic acid **(C), (1)** | α-OH | | α-OH | α-OH |
| hyocholic acid **(HC), (2)** | α-OH | α-OH | α-OH | |
| 12-oxochenodeoxycholic acid **(12-oxoCD), (3)** | α-OH | | α-OH | =O |
| 7-oxodeoxycholic acid **(7-oxoD), (4)** | α-OH | | =O | α-OH |
| 7,12-dioxolithocholic acid **(7,12-dioxoD), (5)** | α-OH | | =O | =O |
| 3, 7, 12-trioxocholanoic acid **(3,7,12-trioxoC), (6)** | =O | | =O | =O |

**Figure 1.** Investigated oxo- derivatives of bile acids.

# 2. Experimental

## 2. 1. Chemicals

Cholic acid 98% purity obtained from Sigma, New Zealand was used as the starting compound for the synthesis of its keto derivatives. The syntheses of cholic acid keto derivatives and their transformation to sodium salts were carried out according to the previously described procedures.[6,18,24] Hyocholic acid 98% purity was purchased from Sigma, New Zealand, methanol of HPLC grade from Carlo Erba reagenti, Italy, $KH_2PO_4$ and $Na_2PO_4$ were obtained from Lachner, Czech Republic. Nitrazepam 99, 98% purity was purchased from Sigma, Aldrich and NaCl pro analysis from Merck, Germany. Defibrinated rabbit blood was purchased fresh from Remel Biological Products Lexena, KS.

## 2. 2. Instruments

The HPLC system Agilent 1100 Series, equipped with degasser, binary pump, automatic injector and DAD detector with software system for data processing AgilentChemStation was used and the analyses were performed on a reversed-phase C-18 column: Eclipse Plus C18 (250 mm × 3mm, 5 μm, 250 A°) column (Zorbax SD).

Spectrophotometric determination of partition coefficient of nitrazepam in bile salts micelles was carried out according to De Castro *et al.*,[17] using Agilent 8453 spectrophotometer equipped with the Peltier thermostated cell holder

## 2. 3. HPLC Analysis

The mobile phase was 0.01 M phosphate buffer (0.2 M $KH_2PO_4$ and 0.2 M $Na_2PO_4$): methanol = 70:130 v/v maintained at pH 7 and the injection volume was 10 μL. Solutions of bile acids and their derivatives in mobile phase were prepared in concentration of 1 mg/ml. All separations were performed isocratically at a flow rate of 1 ml/min and a column temperature changing from 303–313K (303K, 309K, and 313K). The detection was performed at 210 nm.[25]

## 2. 4. Data Treatment

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

$$k = \frac{t_x - t_0}{t_0} \quad (1)$$

where $t_x$ and $t_0$ are the retention times of the bile acids and the unretained solvent front respectively.

Critical micellar concentrations of bile salts (cholic acid and its keto derivatives) used for calculation of partition coefficient of nitrazepam were taken from Poša *et al.*[24] while the critical micellar concentration of hyocholic acid was taken from Roda *et al.*[1]

Erythrolysis was determined from defibrinated rabbit blood according to Bowe *et al.*[5] Bile acids solutions of the following concentrations were prepared: (C, HC: 2–20 mM; 12-oxoCD, 7-oxoD: 2–80 mM; 7, 12-dioxoD,

2,7,12-trioxoC: 2–160 mM.). Critical micellar concentration was expected to be achieved in these concentration ranges.

## 3. Results and Discussion

Replacement of hydroxyl groups in a cholic acid molecule (1) with oxo groups yields the keto derivatives (3), (4), (5), (6), whose capacity factor (k) decreases with the increase of the number of oxo groups introduced (Table 1.).

**Table 1.** Calculated k values of bile acids and their derivatives at different temperatures

| Bile acids | | Temperature [K] | | | $\Delta k_{313-303}/\Delta T$ | $\Delta k_{303-313}/k_{303}$ |
|---|---|---|---|---|---|---|
| | | 303 | 308 | 313 | | |
| **(1)** | **C** | 4.340±0.042 | 3.840±0.035 | 3.191±0.040 | –0.115 | 0.265 |
| **(2)** | **HC** | 2.957±0.020 | 2.828±0.023 | 2.514±0.029 | –0.044 | 0.150 |
| **(3)** | **12-oxoCD** | 1.827±0.014 | 1.775±0.025 | 1.549±0.021 | –0.027 | 0.152 |
| **(4)** | **7-oxoD** | 1.667±0.018 | 1.627±0.020 | 1.429±0.017 | –0.024 | 0.142 |
| **(5)** | **7,12-dioxoL** | 0.465±0.005 | 0.458±0.005 | 0.410±0.007 | –0.005 | 0.118 |
| **(6)** | **3,7,12-trioxoC** | 0.329±0.003 | 0.321±0.002 | 0.286±0.003 | –0.004 | **0.131** |

$n = 5$

Oxidation of α axial (*a*) OH groups of cholic acid (OH groups at C7 and C12 methylene group in the steroid nucleus) gives oxo groups whose oxygen atoms have α equatorial (*e*) position. Thus, oxygen atoms at C7 and C12 oxo group shift 60° with respect to α (*a*) orientation of the OH group (Newman's projection formula) and form the angle of –30° with the mean plane of the steroid skeleton mean plane (SSMP). Oxidation of α equatorial (*e*) OH group (C3 OH group) gives oxo groups whose oxygen atoms have β (*e*) orientation i.e. form an angle of 30° with the SSMP (Fig. 2). It means that substitution of OH groups in cholic acid molecule with oxo groups yields derivatives whose oxygen atom is shifted to the β side of the steroid nucleus and that is why the polar surface of the bile acid is changed. Namely, solvent molecules (i. e. water molecules) in the solvatation shell of cholic acid are strictly stabilized by hydrogen bonds from the α side of the steroid skeleton while for oxo derivatives this stabilization is possible from both sides of steroid nucleus.[6,24] Hence, their polar surface increases, while their capacity factor decreases (Table 1).

After the adsorbtion of bile acids on the hydrophobic stationary phase, solvent molecules that are not stabilized by hydrogen bonds move to the hydrophilic mobile

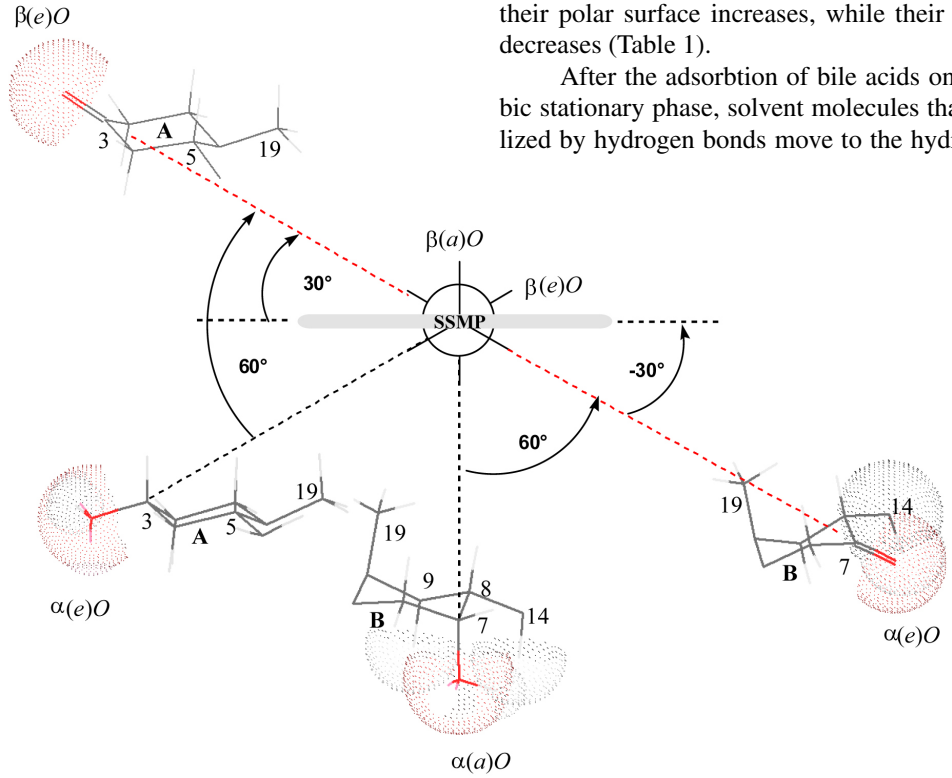



**Figure 2.** Position of the oxygen atom in Newman projection formula after substitution of OH groups in A and B rings of steroid skeleton in the cholic acid molecule (SSMP = steroid skeleton mean plane).

phase giving thus a positive entropic contribution to the adsorbtion (total entropy of adsorbtion is negative).[24] Desolvated hydrophobic surface of a bile acid steroid nucleus links with the surface of absorbents via hydrophobic interaction (London dispersion forces, dipole-induced dipole interactions) giving thus a negative enthalpic contribution to the adsorbtion process.[26] Within oxo derivatives of cholic acid, the number of stabilized water molecules in solvatation shell increases with decrease in the number of none stabilised water molecule which leave the solvatation shell. This lowers the positive entropic contribution of adsorbtion. Along with that, the hydrophobic surface is reduced so the hydrophobic interactions between stationary phase and oxo derivatives are decreased. That lowers the negative enthalpic contribution. In Table 1 it is noticeable that the relative effect of the decrease in the capacity factor ($\Delta k_{303-313}/k_{303}$) at higher temperatures is the highest for the cholic acid while for its keto derivatives (3), (4), (5), (6) the capacity factor decreases with the introduction of oxo groups except for the dehydrocholic acid (6). Additionally, parameter $\Delta k_{313-303}/\Delta T$ has the highest value for the cholic acid while values for this parameter in the case of its keto derivatives decrease. These parameters for dehydrocholic acid (6) and dioxo derivative (5) do not differ statistically. This can be explained by observing the balance between a bile acid in mobile phase $BA_{mf}$ and a bile acid adsorbed on the hydrophilic stationary phase $BA_{sf}$:[4]

$$BA_{mf} \rightleftharpoons BA_{sf} \qquad (2)$$

The equilibrium mentioned above is characterised by a constant $K_{ad} = [BA_{sf}]/[BA_{mf}]$ which is, according to Jones *et al*., connected to the retention factor via next equation: [27]

$$k = \frac{K_{ad} V_{hc}}{V_{pf}} \qquad (3)$$

where $V_{hc}$ and $V_{pf}$ represent the volumes of the hydrocarbon and polar phases respectively. When the temperature is changing, we can say that $V_{hc}/V_{pf} \approx const$. This leads to next equation:

$$\frac{dk}{dT} \approx \frac{dK_{ad}}{dT} \qquad (4)$$

According to Van't Hoff reaction isochor for solutions, this can be written as: [26]

$$\frac{d \ln K_{ad}}{dT} = \frac{\Delta U}{RT^2} \qquad (5)$$

Based on that, if a process is exothermic, as it is during the bile acid adsorbtion ($\Delta U < 0$), the $K_{ad}$ value and $k$ value decrease with the temperature rise according to expressions (Eq. 3) i (Eq. 4). It can be seen from the equation (Eq. 5) that a more negative $\Delta U$ (exothermic reaction) diminishes the equilibrium constant. Since the cholic acid (1) has the largest hydrophobic surface among all examined bile acids, its $\Delta U$ value is the most negative. In other words, the decrease of the capacity factor with elevation of temperature is the largest for the cholic acid (Table 1). Hydrophobic surface of its keto derivatives decreases, and at the same time, the value of $\Delta U$ becomes less negative. According to equations (Eq. 3), (Eq. 4) and (Eq. 5), the absolute values of parameters $\Delta k_{313-303}/\Delta T$ and $\Delta k_{303-313}/k_{303}$ decrease. However, for the hydrocholic acid (6) with three oxo groups in the steroid skeleton one would expect the minor decrease in relative capacity factor. Nevertheless, the decrease of the capacity factor $\Delta k303_{-313}/k_{303}$ for hydrocholic acid is larger than for its examined derivatives with two oxo groups (Table 1). This can be explained by planar polarity.[4] The cholic acid molecule has the largest planar polarity since its β side of the molecule is separated from the hydrophilic α side. For its mono- and diketo- derivatives planar polarity decreases because the β side of the molecule becomes more hydrophilic (less hydrophobic) due to shift of the oxo group toward SSMP. Moreover, partial inversion of polarity occurs for dehydrocholic acid because β side of the molecule becomes more polar because of displacement of the oxygen atoms at C3, C7 and C12 oxo group (Fig. 3), and α side becomes less polar (more hydrophobic) due to appearance of the hydrophobic island on the α side of the steroid skeleton.

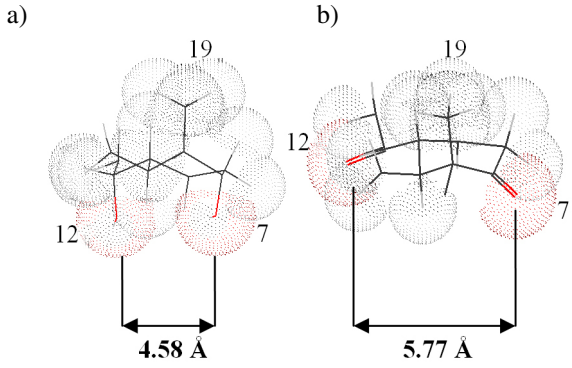



**Figure 3.** Distance between oxygen atoms at C7 and C12 for cholic acid (a) and for dehydrocholic acid (b)

We can assume that, some additional adsorbtion is possible on the α side (on hydrophobic island) (Fig. 4) on already adsorbed dehydrocholic acid (6) (over its β side). This additional hydrophobic interaction makes the value of $\Delta U$ little more negative than for diketo- derivatives of the cholic acid (5) so its value of parameter $\Delta k_{303-313}/k_{303}$ is a bit higher than for a 7, 12- dioxolithocholic acid (5). Values of parameters $\Delta k_{313-303}/\Delta T$ do not statistically differ.

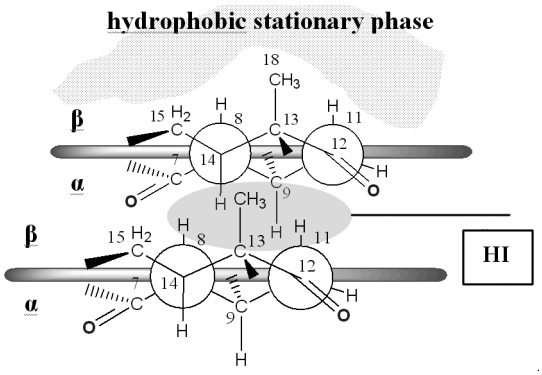



**Figure 4.** Assumed dehydrocholic acid (6) adsorbtion on the hydrophobic stationary phase, HI = hydrophobic island

Hyocholic acid (2), also involved in the examination, is less hydrophobic than the cholic acid (1) although both of them possess three OH groups. The OH group at C6 in the hyocholic acid molecule has the α (*e*) orientation the same as oxo groups at C7 and C12 of keto derivatives of the cholic acid. However, according to the retention coefficient (Table1), hyocholic acid (2) is more hydrophobic than C7 and C12 mono keto derivatives of cholic acid (3), (4). It is the consequence of higher steric protection of the C6 hydroxyl group which can be seen if it is positioned in the appropriate partial conformations of butane (Fig. 5) with two groups in synclinal conformation (methylene group at C4 and C7 OH group). Each oxo group (at C7 and C12) is covered with one anticlinal group (in appropriate Newman's projection formula C7 oxo and C14 methylene groups are in the interactive anticlinal position; the same holds for C12 oxo group and C17 methylene group). According to that, equatorial C6 OH group have a smaller contribution to solvatation shell stabilisation of water molecules.

Table 2 shows partition coefficients of nitrazepam in bile acid micelles at different temperatures. At each examined temperature, the partition coefficient of nitrazepam is the highest for the cholic acid (1) while it decreases with the increase of the number of oxo groups. This is a consequence of decreasing the hydrophobicity of micelle core due to the increase of polar surface and a presence of the "fiord" effect, which is also a characteristic of keto derivatives of a bile acid.[24] With temperature rise for each examined bile salt, the partition coefficient of nitrazepam in micelles falls down. This is a result of escalation in molecule vibration frequency of the bile salts and the probe molecule (nitrazepam) which destroys the structure of the mixed micelle i.e. micelle hydrophobic core is more exposed to water.[28]

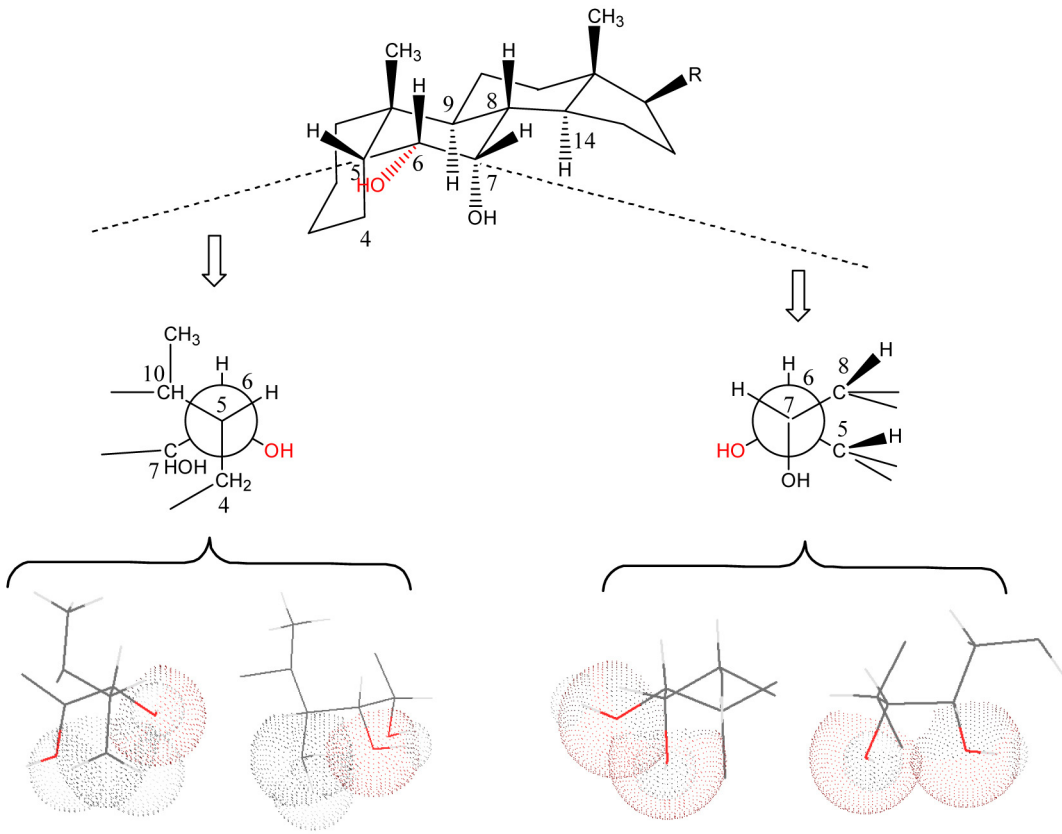



**Figure 5.** Steric protection of C6 equatorial OH group for the hyocholic acid.

**Table 2.** Partition coefficients $K$ [$M^{-1}$] of nitrazepam in bile salts micelles

| Bile acids | Temperature [K] | | |
|---|---|---|---|
| | 303 | 308 | 313 |
| **(1) C** | 2260±13 | 2242±17 | 2007±35 |
| **(2) HC** | 1998±20 | 1974±16 | 1848±27 |
| **(3) 12-oxoCD** | 1705±18 | 1689±21 | 1603±38 |
| **(4) 7-oxoD** | 1695±27 | 1680±22 | 1610±29 |
| **(5) 7,12-dioxoL** | 1452±14 | 1431±17 | 1402±16 |
| **(6) 3,7,12-trioxoC** | 1437±24 | 1430±29 | 1406±25 |

$n = 5$

Monovariate linear regressions with high values of the coefficient of determination ($R^2$) are obtained (Table 3) for the partition coefficient $K$ of nitrazepam in micelle of the selected bile acids (dependent variable) and their capacity factors (independent variable) at each examined temperature. This indicates the importance of hydrophobic interactions between bile acid salts and probe molecule in mixed micelle, and the importance of relationship between hydrophilic and hydrophobic surfaces steroid skeleton of bile acids.

**Table 3.** Linear regression parameters between partition coefficients of nitrazepam K and capacity factors k of bile acids

| Statistical parameter | Temperature [K] | | |
|---|---|---|---|
| | 303 | 308 | 313 |
| $a_0$ **(intercept)** | 1353.41±15.13 | 1320.24±23.86 | 1317.47±19.57 |
| $a$ **(slope)** | 209.45±6.05 | 232±10.88 | 210.00±10.42 |
| $R^2$ | 0.99633 | 0.99133 | 0.99027 |
| *sd* | 22.69 | 33.07 | 26.61 |
| *F* | 1085.86 | 457.00 | 407.26 |
| *p* | 0.0000 | 0.0000 | 0.0000 |

Membranotoxicity of bile acids can be easily determined by following the haemolysis of erythrocytes in the function of their concentrations. Table 4 represents logarithm of concentrations of bile acids in which 50 % of erythrocyte is lysed (log (*Lys* 50)) at three different temperatures (Fig. 6).

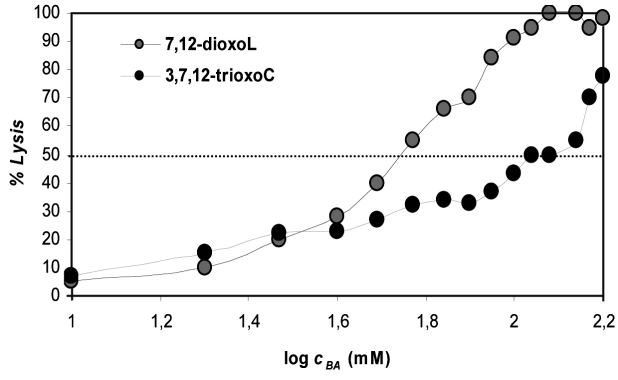



**Figure 6.** Percentage of erythrocytes lysed as a function of bile acid concentration (303 K; 0.15 mM NaCl; pH 7.4).

Considering this, we can conclude that at each examined temperature the cholic acid has the highest haemolytic potential (the smallest value of log (*Lys* 50)) while it decreases with the increase of number of oxo groups in the steroid skeleton. With temperature elevation haemolythic potential of nitrazepam decreases, while the influence of temperature on retention coefficient and partition coefficient lowers. Values of log (*Lys* 50) at 308K and 311 K are not statistically different ($p < 0.05$). During lyses of erythrocyte, membrane phospholipids form mixed micelles with bile salts. According to Hjelm, bile salts and phospholipids probably form micelles in the shape of rods.[9] Micelle core is composed of the residuals of fatty acids from phospholipids. The polar head of phospholipids is situated on the external surface. The β side of bile acid salts is oriented towards residuals of fatty acids while the α side is oriented to the hydrophilic solution. The effect of covering hydrophobic micelle interior is more effective if the bile acid is more hydrophobic. This explains the decrease of haemolytic potential (value of log (*Lys* 50) with the introduction of oxo groups instead of hydroxyl groups. In two mentioned experiments dehydrocholic acid (6) and 7,12- dioxolithocholic acid (5) behaved similarly. However, in haemolysis experiment they were significantly different for both values of log (*Lys* 50) and shape of the erythrocyte lyses curve (Fig. 6). The experiment of haemolysis, unlike two other experiments suggests that dehydrocholic acid is less hydrophobic than dioxo- derivatives of cholic acid (5). This can be explained analysing the structure of mixed micelles. Probably the repulsion between negative dipoles of oxo groups appears on the external surface of the molecule, while for bile acids which beside oxo group have a hydroxyl group, there is a possibility of forming the hydrogen bonds among two neighbour molecules which additionally stabilises micelle.

**Table 4.** Logarithm of haemolytic potential log (*Lys* 50)

| Bile acids | Temperature [K] | | |
|---|---|---|---|
| | 303 | 308 | 313 |
| **(1) C** | 0.52±0.05 | 0.65±0.05 | 0.63±0.04 |
| **(2) HC** | 1.04±0.06 | 1.10±0.05 | 1.09±0.07 |
| **(3) 12-oxoCD** | 1.44±0.05 | 1.51±0.07 | 1.54±0.08 |
| **(4) 7-oxoD** | 1.35±0.06 | 1.53±0.07 | 1.54±0.07 |
| **(5) 7,12-dioxoL** | 1.73±0.07 | 1.77±0.06 | 1.81±0.08 |
| **(6) 3,7,12-trioxoC** | 2.04±0.08 | 2.11±0.09 | 2.13±0.10 |

$n = 5$

Table 5 represents statistical parameters of linear regression between log (*Lys*50) and the capacity factor k of bile acids. Statistical parameters indicate the significance of hydrophobicity of bile acids and for erythrocyte hae-

molysis. According to the coefficient of determination, the capacity factor *k* is less important in describing partition coefficient than in describing the log *(Lys*50).

**Table 5.** Linear regression parameters between logarithm of haemolytic potential log (*Lys*50) and capacity factor of bile acids

| Statistical parameter | Temperature [K] 303 | 308 | 313 |
|---|---|---|---|
| $a_0$ **(intercept)** | 2.01±0.07 | 2.11±0.08 | 2.16±0.08 |
| ***a* (slope)** | 0.34±0.03 | 0.37±0.03 | 0.45±0.04 |
| $R^2$ | 0.96626 | 0.96211 | 0.96026 |
| ***sd*** | 0.10 | 0.11 | 0.11 |
| ***F*** | 114.56 | 101.00 | 96.70 |
| ***p*** | 0.0004 | 0.0005 | 0.0006 |

# 4. Conclusion

Retention coefficient *k* decreases if the OH groups of cholic acid are replaced by oxo groups (*k* decreases in the following order: **C>12-oxoCD>7-oxoD>7, 12-dioxoL>3, 7, 12-trioxoC**). The influence of the temperature rise is more significant for retention coefficient, if the examined bile acid is more hydrophobic. Retention coefficients of bile acids (obtained by RP HPLC) are suitable for describing their behaviour in experiments where the alterations of relationship between hydrophilic and hydrophobic surface of the steroid skeleton is important (partition coefficient of nitrazepam in bile acid salts micelles and haemolytic potential). Conducted experiments suggest that retention coefficient and its dependence on temperature properly describes structural changes due to the substitution of hydroxyl groups with an oxo group in a cholic acid molecule. This allows the usage of this parameter in Quantitative Structure Activity Relationship (QSAR) models. In addition, retention coefficient can be a good substitution for many molecular descriptors that have identical values for each bile acid congeneric group (for example Wiener index for the cholic acid and it's keto derivatives is the same for all of them and its value is 2045).

# 5. Acknowledgement

The Ministry of Science and Technological Development of the Republic of Serbia (Project No. 142005B) supported this study.

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## Povzetek

Z metodo reverzne visokotlačne tekočinske kromatografije smo pri 303 K, 309 K in 313 K določili retencijski koeficient ($k$) holne kisline in njenih ketonskih derivatov. Ugotovili smo, da se pri vseh temperaturah $k$ zmanjša, če hidroksilno skupino v holni kislini nadomesti okso skupina, kar kaže na to, da vpeljava okso skupine vodi do manjše hidrofobnosti β strani steroidnega jedra, kar povzroči šibkejšo interakcijo s stacionarno fazo. Za porazdelitveni koeficient $K$ nitrazepama, ki smo ga uporabili kot sondo v micelah žolčne kisline, smo ugotovili linearno zvezo z retencijskim koeficientom, kar kaže na pomembnost hidrofobnih interakcij v mešanih micelah, ki jih tvorijo preiskovane učinkovine in soli žolčne kisline. Tudi za hemolitični potencial, ki predstavlja merilo membranotoksičnosti žolčne kisline, smo ugotovili zvezo z retencijskim koeficientom. Zaključimo lahko, da retencijski koeficient lahko predstavlja merilo za hidrofobnost v biofarmacevtskih eksperimentih.