Effect of sodium salts of 3α,12α-dihydroxy-7-oxo-5β-cholanoic and 3,7,12-trioxo-5β-cholanoic acids on verapamil hydrochloride in biophysical–chemical model experiments

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Abstract: It is known that certain bile acids have a promotive effect on the action of some drugs. Special attention is paid to bile acids having oxo groups instead of OH groups in the steroid skeleton of their molecule, since these derivatives have a lower hemolytic potential (membrane toxicity). This study examined the effects of sodium salts of 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (7-oC) and 3,7,12-trioxo-5β-cholanoic acid (3,7,12-toC) on the adsorption of verapamil hydrochloride on activated carbon (model of the cell membrane). The interaction was followed by measuring the effect of verapamil on the functional dependence between the spin-lattice relaxation time T_1 (protons of the C18 angular group of the bile acid molecule) and the bile acid concentration in deuterated chloroform (model of the cell membrane lipid phase). Whether a depot effect of verapamil exists when 7-oC and 3,7,12-toC (in the form of methyl esters) are present in chloroform was also investigated. It was found that 7-oC exhibited a significant effect in the experiments with verapamil, whereas 3,7,12-toC showed no difference of the measured parameters with respect to the control. This indicates that bile acid molecules should have OH groups bound to the steroid nucleus, in order to exhibit an effect on the monitored physico–chemical parameters of verapamil.

Key words: Bile acid oxo derivatives; verapamil; spin-lattice relaxation time.

INTRODUCTION

Bile acids are amphiphilic molecules[1,2] which, apart from their well-known physiological roles, such as micellar solubilization of lipids during digestion and regulation of biosynthesis and cholesterol homeostasis,[3] also participate in a

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189

number of metabolic pathways as regulators, *i.e.*, modulators of nuclear receptors (farnesoid X (FXR)) of enzymes, ionic channels (large conductance Ca^{2+}-activated K^+ channel (BK_{Ca})), *etc*.[4,5] More recently, bile acid analogues have been increasingly used to treat metabolic disturbances (obesity, hypertriglyceridemia, type 2 diabetes, atherosclerosis and hypertension). Pharmacological applications of certain bile acids are based on their ability to increase drug transport through the cell membrane or through the complex blood-brain barrier. In these interactions, the mechanism of bile acid action is observed as a change of the integrity of the membranes of complex biological structures (epithelial, buccal, dermal, *etc*.) by weakening or breaking *tight junctions* between cells, thus enhancing paracellular transport. In addition, when bile acids are present at levels close to their critical micellar concentrations (*CMC*) or exceeding them, they modify the structure of the cell membrane by withdrawing phospholipids and forming mixed micelles, which results in increased disorder, *i.e.*, increased probability of the formation of water pool in the cell membrane.[4] Besides, on the membrane surface or in its interior, bile acids may form complexes with some drugs which mediate the rate of drug passage through the cell membrane.[3,6–9]

Oxo derivatives of bile acids are suitable for pharmacological investigations in view of the high values of their *CMC*s, which diminish their membrane toxicity, *i.e.*, hemolytic potential.[10]

The aim of this study was to use a biophysical-chemical model to examine experimentally the interaction of 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (7-oC) and 3,7,12-trioxo-5β-cholanoic acid (3,7,12-toC) with verapamil hydrochloride (Fig. 1). Namely, it is known that in *in vitro* experiments certain bile acid salts increase the adsorption of verapamil hydrochloride in the intestinal epithelium in rats, which assumes the formation of a complex between the drug (non-ionized form) and bile acid molecules (non-ionized form) in the lipophilic membrane phase[9]. The study was also concerned with the influence of Na salts of 7-oC and 3,7,12-toC on the adsorption of verapamil hydrochloride on activated carbon (model of the cell membrane surface and small intestine surface). Finally, the study dealt with the interaction between these bile acids (non-ionized form) and verapamil (molecular form, *i.e.*, base) in $CDCl_3$ (hydrophobic interior of the cell membrane), studied by the ^{1}H-NMR relaxation method. When experiment was concerned with the modeling of the processes in the small intestine or in the intercellular space, the bile acid was assumed to be in the form of its sodium salt and verapamil in the form of hydrochloride (adsorption on activated carbon), whereas when the experiment was concerned with modeling in the membrane lipid phase (NMR relaxation experiment), the bile acid and verapamil were assumed to be in their non-ionized forms.
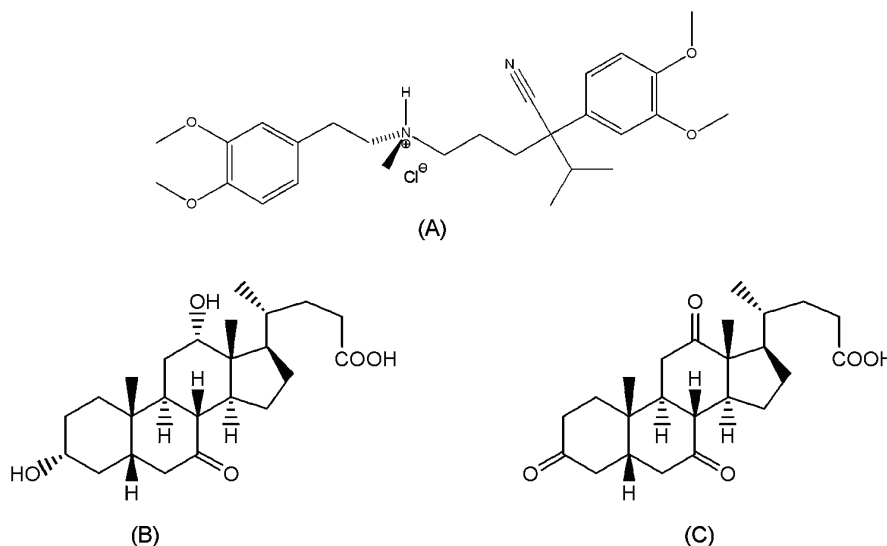
Fig. 1. A) Verapamil hydrochloride (5-[(3,4-dimethoxyphenyl)methylamino]-2-(3,4-dime-thoxyphenyl)-2-isopropylvaleronitrile, hydrochloride), B) 3α,12α-dihydroxy-7-oxo-5β-cho-lanoic acid (7-oxodeoxycholic acid, 7-oC) and C) 3,7,12-trioxo-5β-cholanoic acid (3,7,12-trioxocholanoic acid; 3,7,12-toC).

EXPERIMENTAL

Materials

Cholic acid (Sigma, New Zealand, 98 %) was used for the synthesis of 7-oC according to the procedure by Tullar,[11] whereas 3,7,12-toC was prepared by the procedure of Fieser and Rajagoplan.[12] The other employed chemicals were obtained commercially, *i.e.*, Verapamil hydrochloride, verapamil (Sigma, New Zealand, 99.9 %), $CDCl_3$ (Aldrich, 99.99 %), $CHCl_3$ (Sigma, HPLC grade), KH_2PO_4 (Lachner, analytical reagent grade), Na_3PO_4 (Lachner, analytical reagent grade), $FeCl_3$ (Lachner, 98 %), $CrCl_3$ (Lachner, 98 %) and activated carbon (Darco G-60 powder). Double distilled water was used throughout.

Adsorption on activated carbon

For this purpose, a series of solutions of verapamil hydrochloride of the concentration of 0.125, 0.25, 0.5, 0.75, 1.00, 1.25, and 1.50 mg ml^{-1}, pH 7.4, were made to contain also sodium salts of 7-oC or 3,7,12-toC at concentrations of 0.5 *CMC* and *CMC*. The control was the solution of the drug without a bile acid. To each solution, including the control, 20 mg of activated carbon was added and the suspension was stirred for 30 min. After centrifugation (3000 rpm, 10 min), the verapamil hydrochloride concentration was measured in the supernatant by spectrophotometry (Agilent 8453) at 280 nm. The results are presented as the equilibrium amount of the adsorbed drug per unit mass of the adsorbent as a function of drug concentration in the supernatant.

Adsorption of verapamil hydrochloride was also measured in the presence of 10 mM solution of iron(III) chloride and chromium(III) chloride at 0.5 *CMC* of the sodium salt of 7-oC at pH 7.4. In doing this, the adsorbent was separated and the tested drug was extracted with chloroform (2×5 ml) from the supernatant pH 3.5, then the extract was passed through a solid-

-phase extraction column (Oesis), to bind the metal ions potentially present in the chloroform. The concentration of verapamil (molecular, *i.e.*, basic, form) in the chloroform solution was determined spectrophotometrically (285 nm).

^{1}H-NMR relaxation experiment

The starting solutions of 7-oC and 3,7,12-toC acids (non-ionized form, 60 mM in CDCl$_3$) were diluted with deuterated chloroform to obtain solutions of the concentrations ranging from 2 to 60 mM, each solution being also 4 mM in verapamil (molecular, *i.e.*, basic form). Control measurements were performed in pure CDCl$_3$. The experiments were performed at 23 °C on a Bruker AC-250 instrument. The ^{1}H-NMR spectra were recorded in a spectral window of 3200 Hz. The spin-lattice relaxation time was determined by fast inversion–recovery experiments (180°-τ-90°-AQC).[13-15] The area of the bile acid signal was measured at nine different times τ.

Depot experiment

The experiment was performed on an apparatus specially made for this purpose.[8] It consisted of two vertical glass tubes **A** and **B** mounted on a glass cylinder **C** (Fig. 2). Tube **A** contains an aqueous solution of verapamil hydrochloride (5 ml, 30 mM, pH 7.4) and it represents the verapamil hydrochloride solution in contact with the intestine epithelium. In the control experiment, the horizontal cylinder **C** contained chloroform, whereas in the experiments involving bile acids it contained solutions of the bile acid methyl ester in chloroform, 18 mM in one and 36 mM in the other experiment. The horizontal cylinder **C** corresponds to a complex membrane structure in the biological system (*e.g.*, the intestine epithelium membrane), whereas tube **B** contains buffer (5 ml, pH 7.4). The head of one peristaltic pump supplying fresh buffer pH 7.4 and to the head of a second peristaltic pump conducting the buffer from the tube **B**. As the pumping flow rate of both pumps was 2 ml min^{-1}, there was no change in the solution volume in tube **B** (Master flex 7523-60, pump head: L/S-Easy-Load II 77200-60, L/S-Tubing 13). The pumping flow rate is 2 ml min^{-1}; hence there is no change in the solution volume in tube **B**. In this way, the blood flow in a capillary around a tissue was modeled. In the horizontal cylinder **C**, three magnets are placed in the middle and two at the sites of its connection to the vertical tubes (300 rpm). After 3 h, the chloroform solution from the horizontal tube **C** was withdrawn (through tube **A**) and the verapamil concentration determined by spectrophotometry (Agilent 8453).
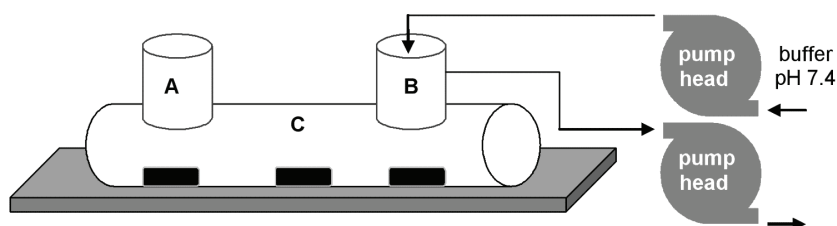

Fig. 2. Sketch of the setup for testing the depot effect.

RESULTS AND DISCUSSION

Adsorption on activated carbon

This part of the investigation represents the model of the surface action of bile acid salts at the water–cell membrane interface, where the bile acid salts are

adsorbed, modifying thus the boundary surface of the cell membrane.[7] Hjelm *et al.*[16] and Nicol *et al.*[17] in their studies of mixed micelles between lecithin (phospholipid) and bile acid salts proposed the model of caped-rod micelle in which the lecithin molecules are arranged radially, with the polar groups oriented towards the surface and separated by bile acid molecules, whereby the *β*-side of the steroid skeleton is oriented towards the hydrocarbon residues of the fatty acids from the lecithin molecules. This model for the orientation of bile acid molecules can also be acceptable for the case of their incorporation in the phospholipid cell membrane, where the hydrophilic *α*-side of the bile acid molecules is oriented towards the extracellular space. A similar situation arises also in the adsorption of bile acid salts on activated carbon, where the *α*-side of the steroid skeleton is directed to the interior of the solution.

The adsorption isotherms of verapamil hydrochloride in the presence and absence of Na-7-oC in the aqueous medium at the concentration of 0.5 *CMC* are shown in Fig. 3. The curve of verapamil hydrochloride adsorption shows an abrupt rise with increasing equilibrium concentration of the drug, and saturation is attained already at a relatively low concentration. The effect of Na-7-oC on verapamil hydrochloride was evidenced by a change of its saturation mass per unit mass of adsorbent. Namely, the Na-7-oC steroid skeleton has two proton donor--acceptor OH groups that form hydrogen bonds with the methoxy groups and nitrile group of verapamil hydrochloride, and the formation of this complex increased the amount of verapamil hydrochloride adsorbed. In the presence of Na--3,7,12-toC (0.5 *CMC*), however, the saturation mass of verapamil hydrochloride decreased abruptly (0.075±0.012) compared to the control value (0.138±0.006), which is probably the result of the formation of the complex with verapamil hyd-
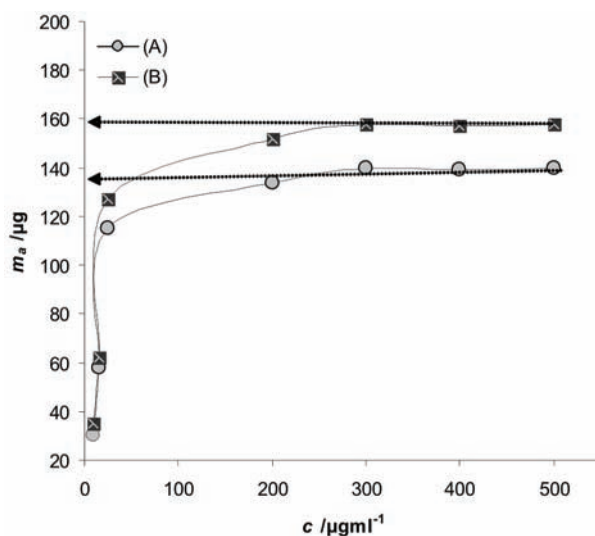

Fig. 3. Adsorption isotherms (adsorbed mass of verapamil hydrochloride per unit mass of activated carbon m_a) as a function of the equilibrium concentration of verapamil hydrochloride c: A) without bile acid salts – control, and B) with Na-7-oC, pH 7.4.

POŠA *et al.*

rochloride (Table I). Namely, Na-3,7,12-toC competes in the process of verapamil hydrochloride adsorption, occupying part of the activated carbon surface, increasing thus the coverage of the adsorbent, which lowers the adsorption of the drug. In fact, in the process of adsorption on activated carbon, each of the investigated bile acid salts acts as a competitor to verapamil. However, the adsorbed Na-7-oC forms a hydrogen-bonded complex with verapamil hydrochloride, which then shifts the equilibrium towards its adsorption.

TABLE I. Saturation masses of verapamil hydrochloride, pH 7.4

Na salt of bile acid	Concentration of Na salts of bile acids	Saturation mass of verapamil hydrochloride, mg g^{-1}		
		Buffered solution	Buffered solution + Fe(III)	Buffered solution + Cr(III)
Control	–	0.138±0.006	0.130±0.008	0.135±0.007
7-oC	0.5 *CMC*	0.156±0.004	0.072±0.010	0.071±0.010
	CMC	0.105±0.006	0.069 ±0.008	0.058 ±0.04
3,7,12-toC	0.5 *CMC*	0.078±0.010	0.084±0.007	0.080±0.010
	CMC	0.073±0.012	0.075±0.008	0.070±0.005

If the sodium salts of the tested bile acids were present at levels exceeding their CMC values, then the saturation mass of verapamil hydrochloride decreased compared to the control value (Table I). In the presence of Na-7-oC (0.105± ±0.006), the decrease of the verapamil hydrochloride saturation mass was significantly larger than in the presence of Na-3,7,12-toC (0.073±0.012). Namely, the Na-7-oC has a larger hydrophobic area[18] and hence is more efficient in forming mixed micelles with verapamil hydrochloride in solution, which results in a shift of the equilibrium towards desorption. In the presence of Na-3,7,12-toC, the verapamil hydrochloride saturation mass is not statistically different from that observed at 0.5 *CMC*. Since the three oxo groups in the Na-3,7,12-toC were shifted to the *β*-side of the steroid skeleton, which caused a decrease in its hydrophobic area, this bile acid salt has a lower tendency to form mixed micelles with verapamil hydrochloride.

Ions of chromium(III) and iron(III) hindered the formation of the hydrogen--bonded complex between verapamil hydrochloride and Na-7-oC. Namely, the presence of these ions at the 0.5 *CMC* of Na-7-oC caused no increase in the saturation mass of verapamil hydrochloride, but its decrease was observed compared to the control value (Table I). This is probably a consequence of the formation of complexes between Na-7-oC and the metal ions, which hinders the formation of hydrogen bonds with verapamil hydrochloride.

^{1}H-NMR relaxation experiment

Formation of the aggregates between 7-oC (non-ionized form) and verapamil (molecular form, 4 mM, to avoid self-association) in deuterated chloroform was

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investigated by the ^{1}H-NMR relaxation technique. Fig. 4A shows the ^{1}H-NMR spectra of a mixture of 7-oC (60 mM) and verapamil (4 mM) recorded by the $180°$-τ-$90°$-AQC method are shown in Fig. 4A. On the other hand, Fig. 4B presents the time τ dependence of the area of the signal I of the angular C18 methyl group of 7-oC after inversion by $180°$ (which are the corresponding spectra from Fig. 4A). The parameter of this functional dependence is the spin-lattice relaxation time T_1, which was also determined for the other investigated concentrations c_{BA}, both for 7-oC itself and for the mixture 7-oC + verapamil (4 mM). The functional dependence between the concentration c_{BA} and the spin-lattice relaxation time T_1 is shown in Fig. 4C, from which it can be seen that at the bile acid concentration exceeding 8 mM (curve I: 7-oC in CDCl$_3$, without verapamil) an abrupt jump appears, which indicates a change in the size (mass) of the molecule under observation, $i.e.$, the formation of aggregates. Namely, the observed increase in the mass of the particles retards their thermal motion, which also re-
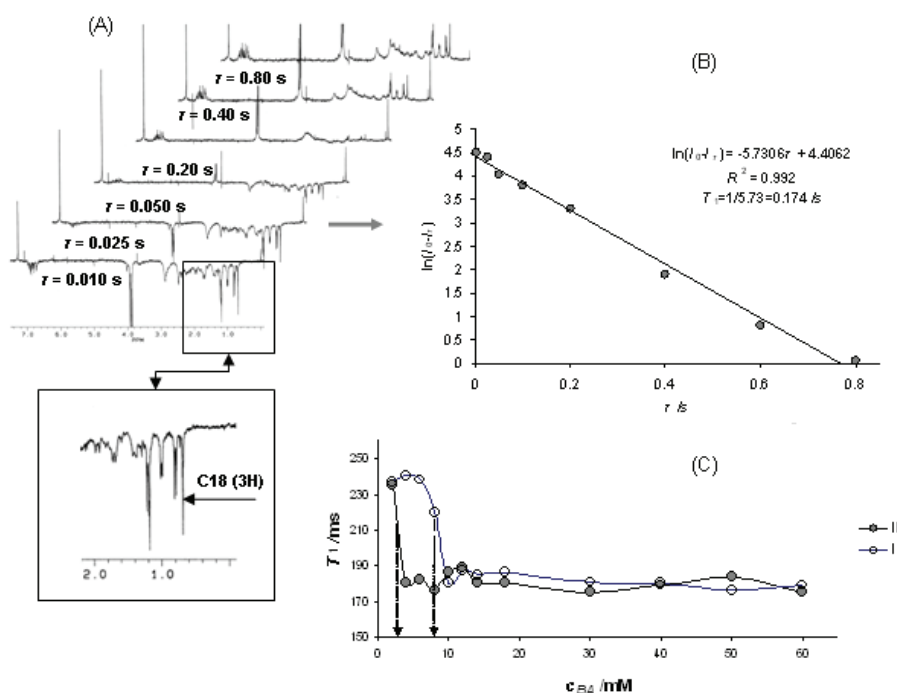

Fig. 4. A) ^{1}H-NMR spectra of a mixture of 7-oC (60 mM in CDCl$_3$) and verapamil (4 mM) obtained by the $180°$-τ-$90°$-AQC method. Each spectrum was recorded at different times τ after the inversion ($180°$); B) change of the area of the signal of the C18 group of 7-oD as a function of the time after the inversion; C) relaxation time T_1 for the protons of the C18 methyl group of 7-oD as a function of the concentration c_{BA} (I in the absence and II in the presence of verapamil).

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sults in a decrease of the relaxation rate (decay of the T_1 value), *i.e.*, there is a slower return of the spin system to the Bolcman equilibrium state. For solutions in deuterated chloroform, this can be explained by the Oakenfull Model[19] of bile acid aggregates, according to which the bile acid monomers are connected by hydrogen bonds *via* the *α*-sides of the steroid ring system, forming reverse micelles in chloroform (Fig. 5A). By simulating the molecular dynamics, Partay *et al.*[20] confirmed the possibility of the existence of the Oakenfull model for cholic and deoxycholic acids. If verapamil (4 mM) was also present in the deuterated chlo-
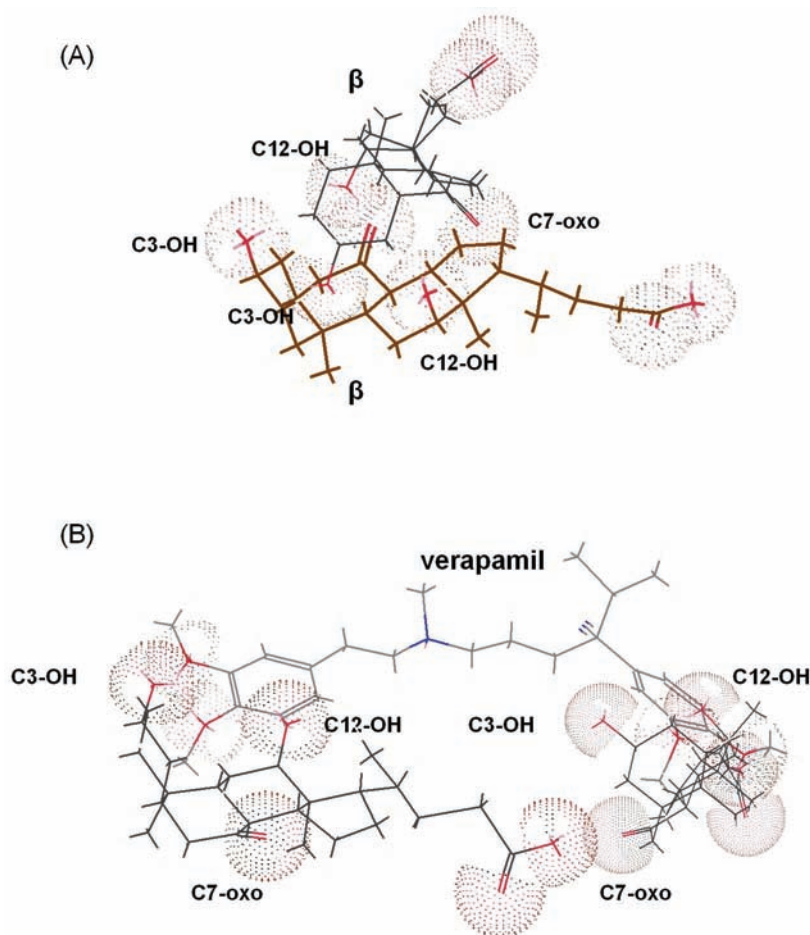

Fig. 5. A) The Oakenfull model of the aggregate (reverse micelle) of the 7-oC dimer (7-oC molecules are mutually bonded by the hydrogen bonds between the C12–OH group of the one and C7–oxo group of the other molecule); B) aggregate between the verapamil molecule and 7-oC (hydrogen bonds are formed between the verapamil methoxy group and the C3– and C12–OH groups of 7-oC, as well as between the C7–oxo group of the one and carboxylic function of the other molecule of the bile acid).

roform, then the function $T_1 = f(c_{BA})$ has an abrupt jump after the 2 mM concentration of 7-oC (Fig. 4C, curve II), which suggests that interaction between the bile acid molecule and the drug occurred. A study of the molecular models indicates that the formation of hydrogen bonds between the verapamil methoxy groups and the C3 (*i.e.*, C12) OH group of the bile acid in the aggregate of verapamil and 7-oC are sterically possible. For such an aggregate, the energy optimization shows that the distance between the carboxylic group of one and the C7 oxo group of the other bile acid molecule may also allow the formation of hydrogen bonds (Fig. 5B). The fact that curve II, even at higher 7-oC concentrations, did not return to its initial value (237 ms) although the CDCl$_3$ contained only 4 mM verapamil, may mean that verapamil has a catalytic role in the formation of the Oakenfull aggregates at higher bile acid concentrations. Namely, in the verapamil–bile acid aggregate, due to the mutual orientation and proximity (entropy effect), the 7-oC molecules may form hydrogen bonds between themselves, thus releasing verapamil molecules.

Verapamil had no influence on the course of the function $T_1 = f(c_{BA})$ when 3,7,12-toC was the tested bile acid.

Depot experiment

The verapamil concentration in chloroform was significantly higher (*t*-test, $p < 0.05$) compared to the control value when the chloroform contained the methyl ester of 7-oC (methyl esters of bile acids are used to prevent the passage of bile acids to an aqueous solution). On the other hand, the verapamil concentration did not differ from the control value when the chloroform contained the methyl ester of 3,7,12-toC (Table II). Therefore, the presence of methyl ester of 7-oC decreased the rate of verapamil transfer from chloroform (model of the membrane, *i.e.* of the epithelial cell) to the flowing buffer pH 7.4 (model of the capillary); thus, the existence of the depot effect was confirmed.

TABLE II. Verapamil concentration (mM) after 3 h of transport from the aqueous medium to chloroform; concentrations of bile acid methyl esters in chloroform: 18 (c_1) and 36 mM (c_2)

Bile acid	c_1	c_2
Control	2.02±0.16	
7-oC	5.41±0.43	7.85±0.39
3,7,12-toC	2.09±0.05	2.12±0.07

CONCLUSIONS

If the concentration of Na-7-oC is below its *CMC* value, the adsorption of verapamil hydrochloride is enhanced compared to the control. In deuterated chloroform, an abrupt change on the curve $T_1 = f(c_{BA})$ in the presence of verapamil appears at a 7-oC concentration of 2 mM (in the absence of verapamil it appears after 8 mM), which indicates the existence of interaction between 7-oC

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and the investigated drug. In the depot experiment, the presence of the methyl ester of 7-oC prevents the transition of verapamil from chloroform to the aqueous buffer solution. Therefore, in the considered physico-chemical model systems, of the two tested bile acids only 7-oC showed a significant effect on verapamil, which indicates that a bile acid molecule has to contain OH groups in its steroid skeleton for interaction with verapamil. Namely, a prerequisite for the formation of hydrogen bonds with the proton-accepting methoxy groups of verapamil is that the bile acid molecule must have proton donors. In addition, the absence of an effect of 3,7,12-toC on verapamil confirms in an indirect way the importance of the hydrogen bonds that are formed between the tested drug and 7-oC molecules.

ИЗВОД

УТИЦАЈ НАТРИЈУМОВИХ СОЛИ 3α,12α-ДИХИДРОКСИ-7-ОКСО-5β-ХОЛАНСКЕ И 3,7,12-ТРИОКСО-5β-ХОЛАНСКЕ КИСЕЛИНЕ НА ВЕРАПАМИЛ-ХИДРОХЛОРИД У БИОФИЗИЧКО–ХЕМИЈСКИМ МОДЕЛ ЕКСПЕРИМЕНТИМА

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Познато је да одређене жучне киселине испољавају промоторно деловање на неке лекове. Нарочита пажња се посвећује жучним киселинама код којих су ОН групе у стероидном скелету супституисане оксо групама, пошто се код ових деривата смањује хемолтички потенцијал (мембранотоксичност). У раду је испитиван утицај Na-соли 3α,12α-дихидрокси-7--оксо-5β-холанске киселине (7-оC) и 3,7,12-триоксо-5β-холанске киселине (3,7,12-toC) на адсорпцију верапамил-хидрохлорида на активном угљу (модел ћелијске мембране). Праћен је ефекат верапамила на функцију зависности спин-решетка релаксационог времена T_1 (протон C18 ангуларне групе молекула жучне киселине) од концентрације жучне киселине у деутерисаном хлороформу (модел липидне фазе ћелијске мембране). Такође је испитивана могућност појаве депо ефекта верапамила уколико су жучне киселине 7-оC и 3,7,12-toC (у облику метил естра) присутне у хлороформу. Нађено је да значајан ефекат у овим експериментима има само 7-оC, док 3,7,12-toC не показује деловање у односу на контролне вредности испитиваних параметара. Ово указује на то да жучна киселина мора имати ОН групе везане за стероидно језгро да би могла испољавати ефекат на физичко-хемијске параметре верапамила.

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