Ukrainica Bioorganica Acta 1 (2011) 17—21

Ukrainica Bioorganica Acta

www.bioorganica.org.ua

Influence of cholesterol and its esters on skin penetration *in vivo* and *in vitro* in rats and mice

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Summary. Major lipids are ceramides, cholesterol and free fatty acids whose stratum corneum lipid matrix plays a key role in mammalian skin barrier function. The effect of the cholesterol esters on the penetration of the stratum corneum *in vivo* and *in vitro* were studied in the corazol test *in vivo* and *in vitro* in rats and mice and the effect of cholesterol esters on the fluidity of the liposome's lecithin were studied by the fluorometric method. This study shows that inclusion of cholesterol esters to this transdermal delivery system (TDS) increased the permeability of the stratum corneum for 7-brom-5-(2'-chlor)phenyl-1,2-dihydro-3H-1,4-benzodiazepine-2-on. Cholesterol esters inclusion to the liposomes increased their fluidity. Thus cholesterol esters can be effective enhancers for transdermal delivery.

Keywords: cholesterol esters, transdermal, liposomes, skin, *in vitro*, *in vivo*.

Introduction. Cholesterol and its esters are important constituents of the cellular membranes and play a fundamental role in biological processes [1, 2]. These sterol affects membrane permeability, lateral lipid organization, signal transduction and membrane trafficking. Large amounts of cholesterol present in the membrane of myelin filament, erythrocytes, hepatocytes [3] and skin [4]. Cholesterol is the stratum corneum major sterol. Cholesterol an essential component of the stratum corneum lipid membranes contributes to the layer's stability, fluidity and promotes the liquid condensed state in the lipid mixtures, containing unsaturated and saturated diacyl chains [5, 6].

Cholesterol and its esters can be used as penetration enhancers for transdermal delivery [7].

We investigated the effects of cholesterol and its esters on the transdermal delivery of 7-brom-5-(2'-chlor)phenyl-1,2-dihydro-3H-1,4-benzodiazepine-2-on *in vivo* and *in vitro* in rats and mice.

Materials and methods. 7-brom-5-(2'-chlor)-phenyl-1,2-dihydro-3H-1,4-benzodiazepine-2 (phenazepam) and ^{14}C (at 2C*) was synthesized in the department of Medical Chemistry of AV Bogatsky Physico-Chemical Institute of NAS of Ukraine [8]. Cold phenazepam was utilized *in vivo* and ^{14}C-phenazepam *in vitro*.

We utilized transdermal delivery enhancers: cholesterol, cholesteryl capronate (C5), cholesteryl pelargonate (C8), cholesteryl caprinate (C9), cholesteryl undecilate (C10), cholesteryl laurate (C11), cholesteryl tridecilate (C12), cholesteryl miristinate (C13), cholesteryl palmitate (C15), cholesteryl stearate (C17) (TCI America).

Outbred white mice (5 animals/group) of both sexes, weight 18-22 g *in vivo* were studied. The animal ethics committee of the Odessa National University (Ukraine) approved the study.

The transdermal delivery system base consisted of water, polyvinyl alcohol, glycerol, PEO-400 and 1.2-propylene glycol (4:2:1:1:2) and 10 % enhancer was added to the base. Phenazepam

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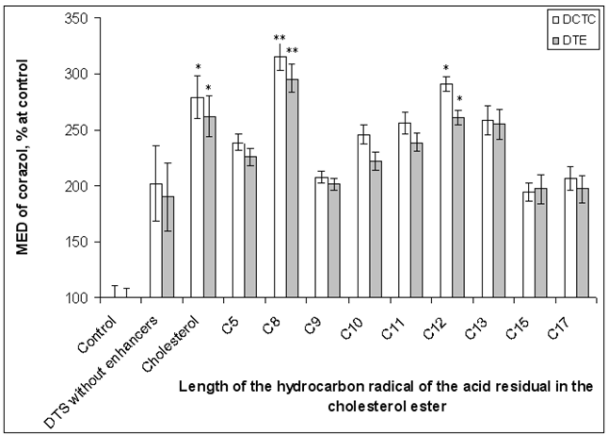

Fig. 1. Anticonvulsive activity of phenazepam by inclusion cholesterol and its esters in the TDS. DCTC — dose of corasole for inducing clonic-tonical convulsions of experimental animals; DTE — dose of corasole for inducing tonical extension of experimental animals; Cholesterol — TDS with cholesteryl as penetration enhancer; C5 — TDS with cholestreryl capronate as penetration enhancer; C8 — TDS with cholesteryl pelargonate as penetration enhancer; C9 — TDS with cholesteryl caprinate as penetration enhancer; C10 — TDS with cholesteryl undecilate as penetration enhancer; C11 — TDS with cholesteryl laurate as penetration enhancer; C12 — TDS with cholesteryl tridecilate as penetration enhancer; C13 — TDS with cholesteryl miristinate as penetration enhancer; C15 — TDS with cholesteryl palmitate as penetration enhancer; C17 — TDS with cholesteryl stearate as penetration enhancer.
Data were expressed as means ±SEM, n=5
**p<0.05; **p<0.01 vs. TDS without enhancers*

concentration was 0.4 mg/cm^2 (1.7 mg/g TDS for ^{14}C-phenazepam) in TDS.

TDS ([C]=0.4 mg/cm^2) were applied to the shaved back area of mice for 2 hours. Pharmacodinamics were measured in terms of changes of the minimal effective dose (MED) of corasole for inducing clonic-tonical convulsions (DCTC) and tonical extension (DTE) of experimental animals.

For percutaneous penetration *in vitro* we used young rats (1.5-2 month) stratum corneum. The stratum corneum was separated after tripsinization (1.5 % of the trypsin (250 units/mg), 4 ˚C, 24 h and 3 h, 37 ˚C).

In vitro permeability studies were performed using stationary glass cells. The TDS (1 cm^2, containing 0.4 mg/cm^2 ^{14}C-phenazepam) or control (1 cm^2 TDS) was applied to stratum corneum and mounted in the cell (reservoir volume 12 ml area exposed to donor compartment 12.6 cm^2). The receptor medium was 10% PEG-400 in water. The receptor solution was maintained at 37±1 ˚C by surrounding water-jacket and stirred by magnetic rod operated on a magnetic stirrer.

Aliquots of the receptor fluid were withdrawn periodically up to 24 h and samples were analyzed by Canberra PACKARD TRICARB 2700.

Fluidity of the liposomal membranes measured membranous probe method by a spectrofluorimeter «Varian Carry Eclipse», by changes of pyrene fluorescence. Liposome's consisted of pyrene, enhancer's delivery and lecithin (molar ratio 1:10:100) dissolved in chloroform. The chloroform was distilled off. We added water to the solid which was intensively mixed. The emulsion was treated by ultrasound (10 min, frequency 22 KHz). The liposome's concentration in the solution was 0.8 g/l.

Data were expressed as mean±SEM; n — refers to the number of experiments. Statistical differences between two mean data were determined by Student's test. The least squares method was used for calculating linear regression. $P<0.05$ and $p<0.01$ was considered to be statistically significant.

Results and discussion. The transdermal permeability of phenazepam studied with TDS contained cholesterol and cholesterol esters *in vivo* (Fig. 1).

The inclusion of the cholesterol in TDS increased permeability of phenazepam across the barrier. This effect in terms of pharmacologic response is to increase anticonvulsive activity of phenazepam (DCTC and DTE from 202 and 190 % for «clear» TDS to 316 and 296 %** for TDS with cholesteryl pelargonate and 291 and 261 %* or TDS with cholesteryl tridecilate, accordingly. $p<0.05$; $p<0.01$ vs. «clear» TDS).

Cholesterol esters enhance the permeability by increase of the radical length from 5 to 8 atoms of carbon. Cholesteryl caprinate with 9 atoms of carbon in the radical decrease enhance permeability as compared with capronate (5C) and pelargonate (8C). Esters with longer radical (>9) increased activity by enhance permeability (max activity for 13C). One possible mechanism may be differences in conformation of cholesterol esters in lipid layers. Differences of the radical length determine changes of disposition molecules of esters in the lipid layer and a change of the membrane fluidity.

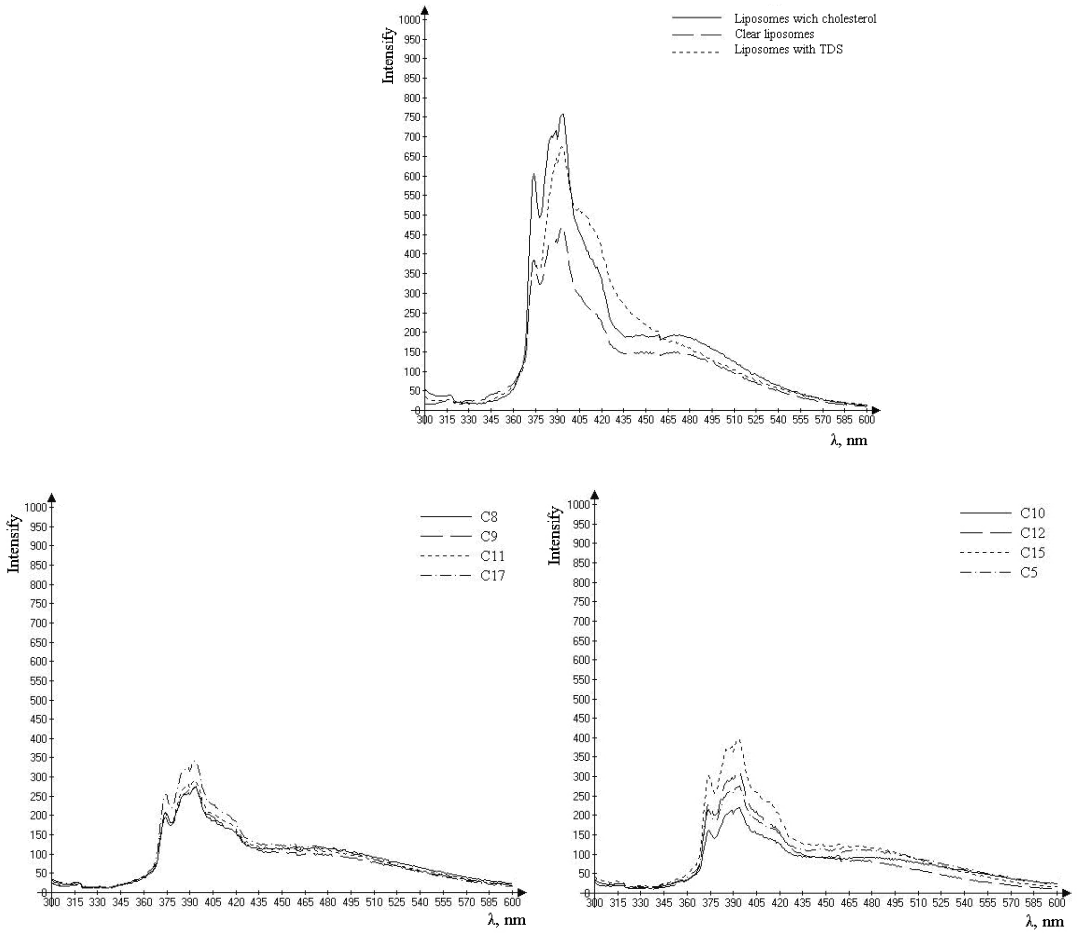

Fig. 2. Intensify of the pyrene fluorescence in the lecithin liposome by inclusion the cholesterol and cholesterol esters. C5-C17 — liposomes with cholesterol esters; Liposomes with TDS — liposomes with TDS components (polyvinyl alcohol, glycerol, PEO-400 and 1.2-propylene glycol); Clear liposomes — liposomes without TDS components.

The influence of cholesterol and its esters at the permeability of isolated stratum corneum for ^{14}C-phenazepam *in vitro* are in table 1.

The *in vitro* data differs from that *in vivo*. We explain this in terms of absence of the regulatory metabolic system in the stratum corneum *in vitro*. Cholesterol has a sealing effect to the lipid bilayers [6, 13, 14], however, the cholesterol increased permeability *in vivo* in our investigation.

We propose that *in vivo* we have enzymatic transformation of the cholesterol. Enzymatic system absent in the isolated stratum corneum, therefore cholesterol fully blocked permeability of stratum corneum for phenazepam *in vitro*. Cholesterol esters influence packing order in the lipid layers and cause increased stratum corneum permeability.

Cholesteryl laurate has maximal influence at the phenazepam permeability across stratum corneum (22-fold as compared with TDS without enhancer's delivery). This data differs from the *in vivo* experiment data. This can be explain by the lack of metabolic processes in the stratum corneum *in vitro*. Cholesteryl stearate increased permeability across stratum corneum (11-fold as compared with TDS without enhancer's delivery). The data *in vitro* correlate well with the data *in vivo*.

We utilized membrane probes for evaluation of membrane fluidity. As the changes of the fluidity of the membrane lipids takes place a corresponding change of the fluorescence of the membrane probe in the lipid layers [18, 19].

Spectrums of the pyrene fluorescence in the liposome phospholipids (by inclusion the cholesterol and cholesterol esters) are illustrated in Fig. 2.

Stratum corneum, major barrier, isolates internal body medium from the external environment and is a polymorphous mass consisting of the keratin and lipids. The lipid composition consists of ceramides, cholesterol, cholesteryl sulphate and free fatty acids [9]. Cholesterol and cholesterol esters have many functions. Skin

Table 1

The rate of the phenazepam permeability across isolated stratum corneum with use the cholesterol and cholesterol esters

Time, h.	Quantity of penetrate phenazepam, mcg/cm^2										
	«Clear» TDS	Chol.	C5	C8	C9	C10	C11	C12	C13	C15	C17
1	1.42 ± 0.02	0	1.45± 0.02	0.57± 0.01	1.43± 0.02	0.65± 0.02	2.87± 0.01	3.25± 0.06	0.65± 0.01	0.66± 0.03	0.55± 0.01
2	1.50 ± 0.03	0	1.57± 0.02	1.27± 0.02	1.64± 0.02	1.35± 0.01	2.99± 0.02	3.83± 0.05	0.70± 0.03	1.61± 0.02	2.03± 0.01
3	1.68 ± 0.02	0	1.84± 0.03	1.78± 0.04	1.72± 0.04	1.40± 0.01	7.74± 0.04*	4.78± 0.05	0.78± 0.03	2.55± 0.04	4.13± 0.03*
6	1.91 ± 0.02	0	2.24± 0.02	4.16± 0.06*	1.93± 0.02	1.63± 0.02	14.42± 0.01**	5.55± 0.07*	0.89± 0.01	3.93± 0.03*	11.56± 0.16**
18	2.75 ± 0.03	0	4.90± 0.05*	11.17± 0.08**	3.04± 0.05	4.46± 0.02*	67.55± 0.18**	7.61± 0.08*	2.87± 0.02	10.20± 0.09*	33.50± 0.18**
24	3.79 ± 0.03	0	5.30± 0.04*	12.98± 0.06**	3.65± 0.04	6.64± 0.04*	82.39± 1.5**	10.14± 0.07*	6.08± 0.03	14.94± 0.16**	44.04± 0.21**

*«Clear TDS» — TDS without penetration enhancer; Chol — TDS with cholesterol as penetration enhancer; C5 — TDS with cholestreryl capronate as penetration enhancer; C8 — TDS with cholesteryl pelargonate as penetration enhancer; C9 — TDS with cholesteryl caprinate as penetration enhancer; C10 — TDS with cholesteryl undecilate as penetration enhancer; C11 — TDS with cholesteryl laurate as penetration enhancer; C12 — TDS with cholesteryl tridecilate as penetration enhancer; C13 — TDS with cholesterol miristinate as penetration enhancer; C15 — TDS with cholesteryl palmitate as penetration enhancer; C17 — TDS with cholesteryl stearate as penetration enhancer. Data were expressed as means ±SEM, n=3. *p<0.05; **p<0.01 vs. control.*

penetration can be enhanced by application of TDS with cholesterol esters. This can be explaind by several mechanisms. The inclusion of cholesterol esters to lipid layers produce destabilization. Cholesterol esters and fatty acids as opposed to phospholipids and ceramides can rapidly penetrate inside the lipid matrix [10, 11]. Cholesterol esters hydrolyzed to cholesterol and free fatty acids in the skin. Cholesterol esters and free fatty acids induce changes at the fluidity, packing density, temperature of phase transition [5, 12]. Influence of cholesterol on the structure and function of stratum corneum depends on many factors. Inclusion of cholesterol to membranes stimulates an increase of density and decreased fluidity [6, 13, 14]. Cholesterol molecules have vertical position in the plain of lipid layers, its hydroxyl groups turn to the water phase and the hydrocarbon component turn to lipid layers [15, 16].

Cholesterol induces the transfer from lamellar phase to gel phase and decreases amount of fixed water in the stratum corneum *in vivo* [4]. When the cholesterol adds to the stratum corneum from outside (TDS), it activates cholesterol sulphatase and transforms into the cholesterolsulphate. It has the following important functions — proliferation and desquamation of keratinocytes, control of water's balance, etc [5, 17]. Cholesterolsulphate concentration increase stimulates to intensify hydration the boundary of corneocytes-intercellular matrix, increases desquamation of corneocytes and permeability of the stratum corneum.

Cholesterol esters increased membrane fluidity in our studies. We observed the maximal fluidization of the lipid environment in the presence of cholesteryl laurate, cholesteryl pelargonate, cholesteryl undecilate and cholestryl capronate. The intensify of pyrene fluorescence decreased at 1,5-2-fold in comparison with liposome without esters.

Some differences in these results from results *in vitro*, may be explained by the presence of the large quantity ceramides and by the decrease of the phospholipids in the stratum corneum. Cholesterol esters with medium and short acid radical increase fluidity of the phospholipids membranes, but this we can not observe for ceramides membranes.

The cholesterol increase intensify of fluorescence to 1,76-fold, this is evidence about the decrease of the fluidity and come to an agreement with our previous studies and data of another investigations [6, 13, 14]. TDS components (polyvinyl alcohol, glycerol, PEO-400 and 1,2-propylene glycol) increase the intensify of fluorescence by incorporation to the liposome (1,1-fold), also.

Conclusion. We studied the influence of the cholesterol and its esters on the permeability of stratum corneum and the fluidity of the liposomes and showed that cholesterol and cholesterol esters can be use as enhancers of the skin

permeability. We suggest the mechanism of action of the cholesterol and cholesterol esters relate to the stratum corneum *in vitro* and *in vivo* and showed the difference of cholesterol action *in vitro* and *in vivo*. We increased membrane fluidity by the addition of cholesterol esters that confirm our theory of the cholesterol esters action.

Acknowledgement. We would like to thank PhD Pavlovsky V.I. (Department of Medical Chemistry of AV Bogatsky Physico-Chemical Institute of NAS of Ukraine) for synthesis of ^{14}C-phenazepam.

Надійшла в редакцію 21.03.2011 р.

Вплив холестерину і його естерів на проникність шкіри *in vivo* та *in vitro* у пацюків та мишей

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Резюме. Ліпідний матрикс рогового шару відіграє важливу бар'єрну роль у шкірі ссавців. Основні ліпіди, які входять до його складу, — цераміди, холестерин та жирні кислоти. Вивчено вплив естерів холестерину на проникність рогового шару *in vitro* та *in vivo* за допомогою коразолового тесту та комірок Франсу. Вплив естерів холестерину на плинність лецитинових ліпосом вивчено флуорометричним методом. Показано, що включення естерів холестерину до складу трансдермальних терапевтичних систем підвищує проникність рогового шару для 7-бром-5-(2'-хлор)феніл-1,2-дигідро-3Н-1,4-бенздіазепін-2-ону. Включення естерів до складу ліпосом призводить до підвищення плинності останніх. Продемонстровано, що естери холестерину можуть бути ефективними підсилювачами крізьшкірної проникності.

Ключові слова: естери холестерину, трансдермальний, ліпосоми, шкіра, *in vitro, in vivo*.

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