

## THE IMPACT OF LIPOSOMES ON TRANSDERMAL PERMEATION OF NAPROXEN – *IN VITRO* STUDIES

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**Abstract:** The possibility of applying liposomes as a topical drug delivery system is still a matter of intensive research. The purpose of this study was to determine the suitability of liposomes as carriers of naproxen and to prove their impact on the effectiveness of transdermal permeation of an active substance. The study was conducted with the use of Franz Diffusion Cell System by comparing the efficacy of a preparation containing 20% of phosphatidylcholine (PC) and 10% of naproxen with reference preparations, i.e., a formulation containing 10% of naproxen without PC and the commercial product Naproxen 10%, gel. The largest transdermal penetration flux of naproxen and the highest efficacy of naproxen permeation were obtained for the formulation containing 10% of naproxen and 20% of PC. The study of the influence of liposomes size and topology on the transdermal diffusion of naproxen (large unilamellar vesicle, LUV, multilamellar vesicle, MLV) showed that there was no statistically significant difference in the flux or total amounts of transdermally diffused naproxen between compared formulations. In conclusion, liposomes present in a formulation double the efficacy of the transdermal permeation of naproxen *in vitro* compared to reference preparations containing no carriers. Better permeation effect of a formulation was not related to the liposome type (LUV or MLV).

**Keywords:** naproxen, liposomes, phosphatidylcholine, transdermal permeation.

The most popular forms of administration for naproxen (non-steroidal anti-inflammatory drug – NSAID) (1) are oral and transdermal. Oral administration is limited by the potential risk of adverse effects (AE) affecting primarily the gastrointestinal tract (2, 3). Therefore, the tolerance for classical oral forms of NSAIDs (including naproxen) is often poor and in some patients their use may lead to many unwanted symptoms, such as: nausea, vomiting, indigestion, pain, diarrhoea and constipation (4), as well as gastric ulcer and gastrointestinal bleeding (5). Because of these adverse effects, associated with the oral NSAIDs delivery, alternative routes of naproxen administration were introduced, such as transdermal preparations. The transdermal route is more and more often used for products containing NSAIDs in the local treatment of pain associated with muscle and joint inflammation (6). Contemporary pharmaceutical technology has

enabled topical drug administration to provide systemic effects (7). Transdermal systems of drug administration have many advantages compared to traditional routes, including small but long-lasting and stable release of an active substance to the system and fewer adverse effects associated with medication. In 1994, Singh et al. showed that local concentration of NSAIDs, including naproxen, in the subcutaneous tissues is proportional to the transdermal permeation rate of the active substance (6). However, due to the protective function of the skin, there are in its structure many barriers restricting possible ways of drug delivery to and through the skin. Therefore, many strategies are still under investigation with the aim to increase the potential of the transdermally delivered substances to permeate the skin.

There are two main reasons for low efficacy of transdermal preparations containing naproxen: poor

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solubility of naproxen in the aqueous phase (water solubility approximately 15.9 mg/L at 25°C,  $\log P = 3.18$  (8, 9), which explains why it is frequently used in preparations in a form of crystals, and high resistance of the stratum corneum (SC) of the skin to external factors. The efficacy of a naproxen containing product could be ameliorated by improving the solubility of naproxen in a formulation and by applying the, so called, permeation enhancers. However, substances used as permeation enhancers, e.g.: surfactants, organic solvents or substances altering skin permeability, give rise to adverse effects themselves (10). Transdermal drug permeation enhancers may cause local irritation or erythema, therefore, intensive search is carried on for efficient and safe carrier systems (11-13). Different nanoparticles have been tested, which could be used as a transdermal carrier system. For example, Kirjavainen et al. have shown that phospholipids (liposomes) used as a carrier system increase the level of estradiol, progesterone and propranolol penetration to the lipid layer of SC (14). There are many advantages of applying liposomes since phospholipids which build liposomes are non-toxic, non-immunogenic, biocompatible and biodegradable. Yokomizo et al. showed that phospholipids containing unsaturated fatty acids are strong promoters of transdermal permeation for some topically applied drugs (15), while Valjakka-Koskela et al. proved that phospholipids and other co-solvents (ethanol and propylene glycol) help to increase the transdermal penetration flux of naproxen (16). Other studies evaluated the influence of phospholipids and unsaturated fatty acids on the increased transdermal permeation of naproxen (17, 18). It was shown that liposomes built of phospholipids containing unsaturated fatty acids induced increased transdermal drug permeation and its distribution into tissues to higher degree than its accumulation in the skin.

Liposomal delivery systems (LDSs) are carrier systems which encapsulate or attach a hydrophobic or hydrophilic substance (e.g., a drug) and thereby change its pharmacokinetic properties. The use of phospholipids in pharmaceutical preparations and their influence on the skin has been discussed in many publications (19-21). Liposomes may impact the kinetics of a given drug in many ways, which largely depend on composition and physicochemical properties of liposomes (22-24). When applied to the skin, liposomes may either increase the local concentration of a drug by streaming the active substance into target tissues, or decrease the intensity of side effects, or increase the amount of the active substance to be delivered to the system (25, 26).

Four major mechanisms have been described for the liposomal enhancement of the transdermal permeation of an active substance. They are: the impact on the diffusion of a free drug, the impact on increased transdermal permeation, the impact on the fusion with SC, and, the most controversial one, the impact on the permeation of intact liposomes (together with the active substance encapsulated within liposome) (25-27). The mechanism of liposomal influence on drug activity may depend on size, structure and composition of liposomes, and also on the route of administration (occlusive or nonocclusive technique) (23).

The purpose of the study conducted and presented here was to demonstrate the suitability of liposomes as naproxen carriers.

## MATERIALS AND METHODS

### Materials

The permeability of formulations being compared was tested *in vitro* using the Franz diffusion cell system on the pig ear skin, which is well recognized substitute for human skin (the test material was obtained from animals from the Institute of Animals of the Wrocław University of Environmental and Life Sciences; according to Resolution 22/2006 of the National Ethic Committee for Experimental Animals of 7 November 2006, § 1 p. 2b no local ethic committee's consent is required to conduct such experiments).

The permeation efficacy of the drug from various formulations was compared for the experimental formulation containing 10% of naproxen and 20% of phosphatidylcholine (PC) in a form of liposomes and for the reference formulations: a naproxen product having the same composition but no PC and the commercial product Naproxen 10%, gel. The study of the effect of size and topology of liposomes on the efficacy of permeation was tested comparing the rate of transdermal diffusion of naproxen from a formulation containing calibrated, large unilamellar vesicles (LUV) of  $125 \pm 10$  nm diameter, PDI = 0.150 and from a formulation containing multilamellar vesicles (MLV).

### Reagents

The following reagents were used: phosphatidylcholine (PC) (Phospholipon 90NG) from Phospholipid (Colony, Germany), propylene glycol (PGly) from DOW Europe (Frankfurt a/M, Germany), naproxen from Zhejiang Charioteer Pharmaceutical (Shanghai, China), phosphate buffer

(PBS) from Sigma-Aldrich (Steinheim, Germany). The remaining reagents (acetonitrile,  $\text{KH}_2\text{PO}_4$ , orthophosphoric acid, ethanol) were analytically pure and they were obtained from Chempur (Piekary Śląskie, Poland).

#### Test formulations containing naproxen

1. 10% of naproxen, 20% of PC, 20% of PGly, 50% of PBS; 2. 10% of naproxen, 20% of PGly, 70% of PBS; 3. a commercial gel, containing 10% of naproxen (Hasco-Lek, Poland); 4A. 2.8% of naproxen, 20% of PC (MLV), 20% of PGly, 57.2% of distilled water; 4B. 2.8% of naproxen, 20% of PC (LUV), 20% of PGly, 57.2% of distilled water.

#### Methods

##### The preparation and characteristics of liposomes

In order to prepare the formulation containing naproxen, the adequate amount of lipid (depending on the test formulation) was dissolved in propylene glycol. The solution was stirred intensively at 50°C. Then, powdered naproxen was added. A clear, light-yellow solution was obtained and mixed with water/buffer. This produced multilamellar vesicles (MLV) which were then calibrated through two polycarbonate membranes with pore diameter of 100 nm (Nucleopore, Whatman, England) using the Avestin Emulsiflex C50 extruder (Avestin, Ottawa, Canada) to obtain large unilamellar vesicles (LUV) of  $125 \pm 10$  nm diameter, PDI = 0.150. The size of liposomes was determined using the dynamic light scattering technique (Zetasizer Nano S, Malvern Instruments, Malvern, England).

##### The transdermal diffusion testing (diffusion method)

The Franz diffusion test was used to measure the transdermal permeation of naproxen (PermeGear, Hellertown, USA). The Franz diffusion cell system is composed of the donor and acceptor compartments, and the skin is attached between them. The skin patches were cut out from the dorsal area of a pig's ear. They were physically removed from defrosted pig ears using a scalpel. The skin was then cut into smaller patches of approximately 1 cm in diameter, and hair was removed using scissors. The quality and intactness of each skin patch was checked by measuring impedance ( $\text{k}\Omega/\text{cm}^2$ ) according to the method described in detail in (28). The patches for which the value of impedance was greater than or equal to 27  $\text{k}\Omega$  (MT 4090 LCR Meter, Motech Instruments, Tainan, Taiwan) were used for further tests. Patches so prepared were stored at  $-20^\circ\text{C}$  and defrosted

immediately before testing. Round skin fragments were gently placed on the receptor compartment of the diffusion cell so that the epidermis was facing the donor compartment. The receptor compartment was filled with PBS and the cells were then incubated at 32°C (which corresponded to the temperature of human skin) using an external water coat. After 30 min of conditioning of the pig skin with receptor solution, approximately 50 mg of the test formulation was applied onto the donor compartment of each cell using a plastic syringe. The receptor solution was continuously stirred throughout the experiment by a magnetic stirrer at 400 rpm. After 20 h of incubation, the diffused cells were carefully disassembled. Transdermal permeation of naproxen was calculated on the basis of its concentration in the acceptor compartment. The accuracy of calculations was confirmed by determining the total recovery of naproxen from each compartment. Only these results were used for further calculations for which the total recovery of naproxen was from 90 to 100%. Each experiment with Franz diffusion cells was performed using 9 cells for each formulation.

##### HPLC analysis

The concentration of naproxen in the acceptor compartments was determined spectrophotometrically, using the high performance liquid chromatography. The HPLC analysis was carried out using the Agilent 1200 system chromatograph (Agilent Technologies) with a UV-VIS (254 nm) detector and a column thermostat (50°C). The stationary phase was a chromatographic column filled with LiChrospher® 100, 250 × 4.6 mm, RP-18 and pore diameter of 5  $\mu\text{m}$  from Merck (Germany). The mobile phase was composed of a mixture of 10 mM  $\text{KH}_2\text{PO}_4$  pH 2.0 and acetonitrile 58 : 42 (v/v), injected in isocratic conditions onto the column at the rate of flow of 1.5 mL/min.

The method was validated for specificity, linearity, precision, accuracy, robustness, limit of detection and quantitation (29). The accuracy of the method was between 96.8–101.3% (average 99.1%, RSD = 1.5%) and the calibration curve was linear ( $R^2 = 0.99$ ) over naproxen concentrations ranging from 35.8 to 86.4  $\mu\text{g}/\text{mL}$ . The limit of detection (LOD) and limit of quantitation (LOQ) were 7.81 ng/mL and 26.00 ng/mL, respectively. The method was found to be specific, precise, accurate, and reproducible.

The results were statistically analyzed using Student *t*-test and Snedecor F test. The statistical significance of difference for the Snedecor F test was estimated at  $p = 95\%$  (31).

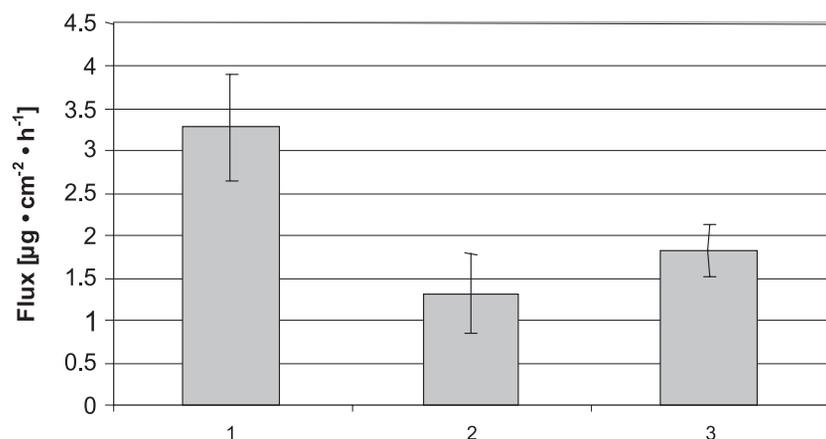


Figure 1. Average values of naproxen flux for three test formulations. Error posts indicate standard deviation. 1 – formulation containing 10% of naproxen and 20% of PC; 2 – formulation containing 10% of naproxen and no PC; 3 – commercial product Naproxen 10%, gel

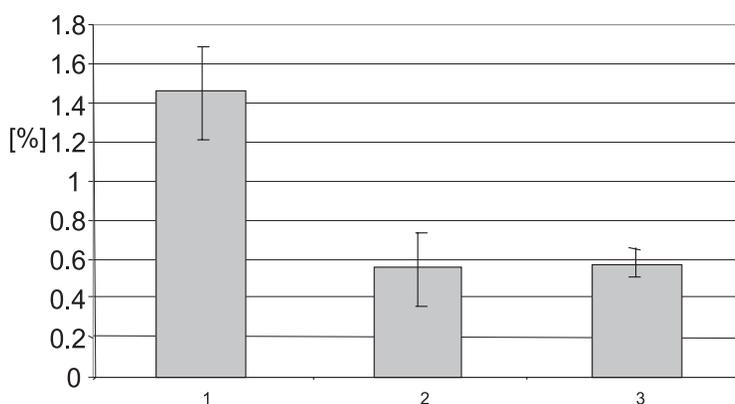


Figure 2. The fraction of the active substance transported across the skin under the experimental conditions, expressed as a percentage of naproxen applied to the skin. 1 – formulation containing 10% of naproxen and 20% of PC; 2 – formulation containing 10% of naproxen and no PC; 3 – commercial product Naproxen 10%, gel

## RESULTS

### The influence of liposomes on the transdermal permeation of naproxen

The influence of liposomes on the naproxen transport across the skin of pig's ear was analyzed by comparing for various formulations the amount of the active substance that passed through the skin from the test formulations to the acceptor compartment of the diffusion cell.

Results were presented as the total amount of naproxen [ $\mu\text{g}$ ] transported across  $1 \text{ cm}^2$  of skin as a function of time (flux). The average values of naproxen flux obtained in 9 repetitions of the experiment are presented in Figure 1.

The greatest transdermal flux of naproxen was obtained for the formulation containing 10% of naproxen and 20% of PC. When compared to other formulations, the difference was statistically significant.

The efficacy of transdermal permeation of naproxen can also be expressed as the ratio of the amount of naproxen transported across the skin to the amount of naproxen applied to the skin (in %). The average values obtained in 9 repetitions were:  $1.45 \pm 0.4\%$  for formulation (1),  $0.55 \pm 0.18\%$  for formulation (2), and  $0.58 \pm 0.07\%$  for formulation (3). The formulation (1) which contained liposomes was found to be much more efficient than the other two, and the difference was statistically significant (Fig. 2).

Table 1. The flux and the fraction of transdermally diffused naproxen to the total amount of naproxen applied to the skin.

Naproxen formulation	Flux [ $\mu\text{g} / \text{cm}^2 / \text{h}$ ]	Percentage [%]
(A)	$4.58 \pm 2.83$	$6.35 \pm 2.82$
(B)	$3.95 \pm 2.13$	$4.88 \pm 1.91$

(A) – formulation containing MLV liposomes; (B) – formulation containing LUV liposomes

The results have shown that the efficacy (measured as the permeability rate) of the preparation containing 10% of naproxen with the addition of liposomes was two times higher than that of the test reference preparations containing no PC.

#### The influence of the liposome size on the transdermal permeability of naproxen

The influence of the liposome size on the transdermal diffusion of naproxen was tested by comparing the efficacy *in vitro* of two formulations containing 2.8% of naproxen and 20% of PC, where one formulation contained large unilamellar vesicles (LUV) (4B) and the other multilamellar vesicles (MLV) (4A). The average values from the repeated experiments, expressed as a fraction of naproxen applied to the skin which passed to the receptor compartment was  $6.35 \pm 2.82\%$  for formulation A and  $4.88 \pm 1.91\%$  for formulation B. The results have shown that there is no statistically significant difference between both formulations when the fluxes or the amounts of transdermally diffused naproxen are compared (Table 1).

## DISCUSSION

Transdermal delivery of systemic drugs is a way to avoid factors which impact the gastrointestinal absorption of an active substance, such as pH, food or motor behavior of the gastrointestinal tract. Moreover, skin application of a drug is advantageous for drugs with little bioavailability as the so called first-pass effect can be eliminated (31). Unfortunately, transdermal drug administration is problematic as skin permeability for both hydrophobic and hydrophilic substances is small due to the fact that skin is the natural protective barrier (31). The major barrier for exogenic substances is the external epidermal layer (SC) (32). It is built of a highly ordered lipid structure localized between corneocytes (33). Therefore, in order to increase the intensity of the transdermal permeation of drugs, and to increase the efficacy of these drugs, a variety of modifiers are used enhancing skin permeability (14).

Naproxen is an anti-inflammatory drug sparingly soluble in water. As the gradient of concentration between the formulation and epidermis is low, the transport of naproxen across the skin after topical application is also small. Low gradient is caused by low concentration of naproxen in the formulation. The majority of commercial formulations contain naproxen in the form of crystals that are unable to pass through the skin barrier (no results are presented here). One of possibilities of improving the transdermal transport of naproxen is to increase its solubility. This can be done by using complex macromolecular mixtures containing for example cyclodextrins, hydrophilic polymers (34), amino acids (35) or polyvinylpyrrolidone (PVP) (36, 37). In their work, Zerrouk et al. have tested the ability of PVP and chitosan to increase the solubility and absorption of naproxen. They showed that both these ingredients increased the solubility of the drug (27, 38).

The use of micelles, microemulsion or liposomes as drug carriers largely contributes to the increased stability and bioavailability of the drug and reduces the risk of adverse effects. Micelles and microemulsions have already been used to enhance naproxen solubility. Poloxamer gel 409 (PF-127) significantly increased solubility of naproxen by spontaneous formation of micelles containing naproxen. Due to this effect the amount of naproxen in a non-crystalline form significantly increases resulting in the rise of its effective concentration, which should facilitate the transdermal permeation. This effect is a linear function of PF-127 concentration (16). In another example, Correa et al. showed that better solubility of naproxen in the presence of an emulsion stabilized by hexadecyltrimethylammonium bromide, containing isopropyl myristate or butyl stearate, is directly proportional to the volume of the microemulsion phase and that it depends on pH (39).

The purpose of the study presented here was to obtain a naproxen containing formulation for transdermal administration with the efficacy better than traditional ointments or gels. In this context, drug

efficacy is understood as its ability to pass through the skin of pig ear, which was tested using the Franz diffusion cell method. In order to improve solubility of naproxen and to enhance the skin permeability liposomes were added to the formulation base.

Liposomes are more and more frequently used as efficient drug carriers. They have also been the subject of studies which have recently gained in intensity. The structure of liposomes is such that they encapsulate drug particles inside a vesicle or build particles into their membrane. In addition to that, liposomes as skin modifiers enhance the transdermal transport of an active substance and skin absorption, which makes them efficient and popular drug carriers. There are many examples in the literature of the influence of liposomes on the enhanced transdermal permeation and absorption of different active substances (40–42). In the studies discussed here, the transdermal transport of naproxen in the presence of liposomes was two times higher compared to the reference formulations.

These experiments were conducted using liposomes built of natural phosphatidylcholine. The most recent studies on this type of liposomes have shown that in the majority of cases the classic liposomes fail to pass into the lower parts of skin intact, and that their activity is rather limited to the superficial layers of SC (43). We suggest three possible mechanisms of liposomal activity used in our studies: (i) liposomes improve solubility of naproxen, (ii) liposomes act directly on SC by lowering the natural permeability barrier, and (iii) phospholipids form a thin film on the skin surface, which facilitate the transport of the drug from the formulation into the skin.

The transport of an active substance from liposomes across the skin is determined by such factors as: the composition of lipids, the lamellar structure and the liposomal surface charge. Little is known about the effect the liposome size has on the transdermal permeability and absorption of drugs. The correlation between these parameters has not yet been established. Du Plessis et al. compared effects of topical administration of lipophilic substances: cyclosporine and cholesterol sulfate in the presence of liposomes of various size (44). They showed that medium size liposomes (300 nm, 600 nm) are better absorbed to the SC than the small ones (60 nm), but do not permeate to the deeper skin layers. Michel et al. have proved that there was an insignificant difference in the amount of the lipophilic drug that passed through the skin when SUVs ((small, unilamellar vesicles, < 50 nm) or MLVs (> 130 nm) were used as a carrier (34). The studies of the influ-

ence of the liposome size on the transport of hydrophilic substances were conducted by Šentjurc et al. (45). Their results show that the transdermal transport of a drug is independent of the size of liposomes, irrespective of the type or composition of the test liposomes and niosomes, as long as their diameter is less than 200 nm. For smaller nanoparticles, transport is significantly decreased. They also proved that small liposomes are unstable and decompose immediately upon contact with various surface types (44). Other studies, conducted by Verma et al. focused on the effect the size of carrier particles has on the transport of two marked fluorescent substances across the human skin. Their data confirmed that liposomes of 120 nm diameter improve the transport of carboxyfluorescein to the skin compared to larger liposomes (24).

The results above clearly show that the type of liposomes added to the formulation (MLV or LUV) is insignificant as far as the effect on the transdermal transport of naproxen is concerned. The *in vitro* studies showed that the formulation containing naproxen encapsulated in liposomes built of phosphatidylcholine significantly improved the transdermal transport of the drug. Under the experimental conditions, the amount of naproxen that passed through the skin was two times greater compared to the reference formulation containing the same amount of naproxen but no liposomes. Probably the same effect will be obtained *in vivo*, but further studies still need to be conducted. It is suggested that the mechanism of liposomal activity is complex and multileveled. This could be explained by the fact that liposomes act as co-solvents and/or skin modifiers. A complete understanding of the actual mechanism of these phenomena should help to design new and more efficient formulations containing naproxen.

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