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Ph.D. Thesis

Drug permeation study through biological membrane barriers

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ARTICLES RELATED TO THE PH.D. THESIS

I. Miklós Resch, Béla Resch, **Eszter Csizmazia**, László Imre, János Németh, Piroska Révész, Erzsébet Csányi: *Permeability of human amniotic membrane to ofloxacin in vitro.* Invest. Ophth. Vis. Sci. (2010) 51, 1024-1027

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II. **Eszter Csizmazia**, Mária Budai-Szűcs, István Erős, Zsolt Makai, Piroska Szabó-Révész, Gábor Varju, Erzsébet Csányi: *Thermoanalytical method for predicting the hydration effect permanency of dermal semisolid preparations.* J. Therm. Anal. Calorim. (2010) 102, 313–316

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III. **Eszter Csizmazia**, Gábor Erős, Ottó Berkesi, Szilvia Berkó, Piroska Szabó-Révész, Erzsébet Csányi: *Ibuprofen penetration enhance by sucrose ester examined by ATR-FTIR in vivo*. Pharm. Dev. Technol. doi: 10.3109/10837450.2010.508076

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IV. Miklós D Resch, Béla E Resch, **Eszter Csizmazia**, László Imre, János Németh, Piroska Révész, Erzsébet Csányi: *Drug Reservoir Function of Human Amniotic Membrane*. J. Ocul. Pharmacol. Th. *(accepted for publication)*

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V. **Eszter Csizmazia**, Gábor Erős, Ottó Berkesi, Szilvia Berkó, Piroska Szabó-Révész, Erzsébet Csányi: *Penetration enhancer effect of Sucrose laurate and Transcutol on Ibuprofen.* J. Drug Deliv. Sci. Tec. *(accepted for publication)*

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ABSTRACTS

I. Miklós D. Resch, Béla E. Resch, **Eszter Csizmazia**, László Imre, János Németh, Piroska Révész, Erzsébet Csányi: *Permeability of human amniotic membrane to ofloxacin in vitro ,* SOE 2009 The 17th Congress of the European Society of Ophthalmology 13-16 June 2009 Amsterdam (FP-BAS-041)

II. **Csizmazia Eszter**: *Félszilárd készítmények stratum corneumra gyakorolt hatásának vizsgálata*, Magyar Tudomány Ünnepe, Szegedi Akadémiai Bizottság Székháza, 2009. november 10. Szeged

III. **Csizmazia Eszter**, Budai-Szűcs Mária, Erős István, Makai Zsolt, Szabóné Révész Piroska, Csányi Erzsébet: *Új típusú dermális gyógyszerhordozó rendszerek bőrhidratációra gyakorolt hatása,* Congressus Pharmaceuticus Hungaricus XIV. 2009. november 13-15. Budapest, Gyógyszerészet 53. 2009: Suppl. 1, S91-S92. p. (P-47.)

IV. Berkó Szilvia, **Csizmazia Eszter**, Szabóné Révész Piroska, Csányi Erzsébet: *Új típusú alapanyagok és trendek a bőrápolásban,* Congressus Pharmaceuticus Hungaricus XIV. 2009. november 13-15. Budapest, Gyógyszerészet 53. 2009: Suppl. 1, S88. p. (P-38.)

V. **Eszter Csizmazia**, Mária Budai-Szűcs, András Fehér, Zsolt Makai, Piroska Szabó-Révész, Erzsébet Csányi: *Effect of new types of dermal delivery vehicles on skin hydration and barrier function.* Skin Forum 11th Annual Meeting 6-7 July 2010 Edinburgh, Scotland (P45.)

VI. Berkó Szilvia, Erős Gábor, **Csizmazia Eszter**, Csányi Erzsébet: *Egy új in vivo modell bemutatása hatóanyagok bőrön keresztüli penetrációjának vizsgálatára* Gyógyszerkémiai és Gyógyszertechnológiai Szimpózium 2010. október 4-5. Velence

VII. Csányi Erzsébet, **Csizmazia Eszter**, Berkó Szilvia: *Hatóanyagok bőrön keresztüli penetrációjának vizsgálati lehetőségei I., In vitro, ex vivo modell,* „XVI. Országos Gyógyszertechnológiai Konferencia" és „VIII. Gyógyszer az Ezredfordulón Konferencia" 2010. október 20-22. Siófok (EA-05)

VIII. Berkó Szilvia, Erős Gábor, **Csizmazia Eszter**, Csányi Erzsébet: *Hatóanyagok bőrön keresztüli penetrációjának vizsgálati lehetőségei II., In vivo egér modell,* „XVI. Országos Gyógyszertechnológiai Konferencia" és „VIII. Gyógyszer az Ezredfordulón Konferencia" 2010. október 20-22. Siófok (EA-06)

IX. **Csizmazia Eszter**, Erős Gábor, Berkesi Ottó, Berkó Szilvia, Csányi Erzsébet: *Hatóanyagok bőrön keresztüli penetrációjának vizsgálati lehetőségei III., FT-IR alkalmazása,* „XVI. Országos Gyógyszertechnológiai Konferencia" és „VIII. Gyógyszer az Ezredfordulón Konferencia" 2010. október 20-22. Siófok (EA-07)

X. **Csizmazia Eszter**: *FTIR alkalmazása a bőr szerkezeti változásainak vizsgálatához* Magyar Tudomány Ünnepe, Szegedi Akadémiai Bizottság Székháza, 2010. november 17. Szeged

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ABBREVIATIONS

AM	amniotic membrane
API	active pharmaceutical ingredient
ATR-FTIR	Attenuated Total Reflection-Fourier Transform Infrared
BSC	Biopharmaceutics Classification System
C_d	drug concentration of the donor phase
CSS	cultured skin substitute
DTG	derivative TG
EI	enhancer index
FTS	full thickness skin
HBS	human bioengineered skin
HLB	hydrophilic-lipophilic balance
HSE	heat-separated epidermis
HSS	human skin substitute
IBU	Ibuprofen
J	steady state flux
K_p	permeability coefficient
LLC	lyotropic liquid crystal
LSE	living skin equivalent
MES	maximal evaporation speed
NSAID	Non-Steroidal Anti-Inflammatory Drug
OFL	Ofloxacin
PA	1,2 propandiol-alginate
PBS	Phosphate buffer solution
PE	penetration enhancer
PTR1	Pemulen^{TM} TR1
Q	cumulative amount of API permeated per cm^2
RHE	reconstructed human epidermis
SC	stratum corneum
SE	Sucrose ester
TEWL	transepidermal waterloss
TG	thermogravimetry
T_{lag}	lag time
TR	Transcutol

1. INTRODUCTION

The topical drug administration routes (dermal, transdermal, buccal, nasal, vaginal, rectal, ophthalmological...etc.) have had an increasing role in the pharmaceutics, recently. The chief reason for the success of the topical drug delivery systems to date is the avoidance of hepatic "first-pass" metabolism, leading to increased drug bioavailability, and the decrease of drug peak concentration -observed after orally administrated drugs- which causes reduced side effects. They have the advantages of predictable and extended duration of activity, patient-modulated delivery, elimination of multiple dosing schedules, thus enhancing patient compliance [1].

Non-Steroidal Anti-Inflammatory Drugs (NSAID) are often used for the treatment of chronic musculoskeletal injuries (e.g. rheumatoid arthritis, osteoarthritis) [2]. However, they can cause gastrointestinal mucosal damage which may result in ulceration and/or bleeding. Therefore, there is a great interest in developing preparations for topical application to ensure the good transdermal permeation of the active pharmaceutical ingredients (API) into the inflamed joint and muscle. This administration route can eliminate the oral side effects, allowing faster pain relief and providing relatively consistent drug levels at the application site for prolonged periods [3, 4].

The main problem associated with the topical drug administration is that only a small number of APIs is suitable for overcoming the biological barriers in the body. The skin and the mucosa have principal function to act as a barrier against extraneous materials and the loss of tissue water. **Skin** is one of the best biological barriers known to man. Its outermost layer, which is in direct contact with the environment, the stratum corneum (SC) has a crucial role in this protection. Due to its special and strictly ordered structure and its excellent diffusional resistance, it makes the transdermal delivery of APIs difficult or frequently impossible [5].

Optimization of drug delivery through human skin is an important and innovative research area in the modern therapy. The nature of the barrier, the balance between the physicochemical properties of the membrane and the drug, the technologies available for the pharmaceutical scientists to facilitate transdermal transport should be taken into consideration. Ideal transdermal candidates are characterized by their low molecular weights and their relatively high therapeutic potencies [6, 7]. Adequate solubility is required, it should be lipophilic enough to help the penetration in the SC domains, but hydrophilic enough to distribute in the tissues of the epidermis. When these criteria are met, the transdermal route is useful for molecules with poor oral bioavailability and short biological half-life [8].

Recently other human biological membranes (buccal and nasal mucosa, lung tissue, cornea... etc.) have been used besides the skin for drug penetration experiments. A new and interesting area is the study of the human amniotic membrane's permeability.

Amniotic membrane (AM) transplantation has become frequently used in ocular surface surgery, but it may create a barrier for topically administered drugs to reach the corneal tissues. The pharmacokinetic impact of the amniotic membrane, however, has not been exactly explored in the literature yet.

It is necessarily challenging a task for the pharmaceutical scientists to find a solution for enhancing penetration in respect of API absorption. There is a considerable interest in the various enhancement strategies and techniques in the literature. The aim is to find and choose from the numerous ways the one which acts the most efficiently and safely without causing irreversible harmful alteration in the membrane structure.

2. LITERATURE SURVEY

2.1. Skin as biological barrier

The skin is one of the best biological barriers and it is also the largest organ of the human body with a total weight of more than 3 kg and a total area of 1.5-2 m^2. The skin consists of three main morphological layers (Figure 1):

- subcutis,
- dermis or cutis and
- epidermis.

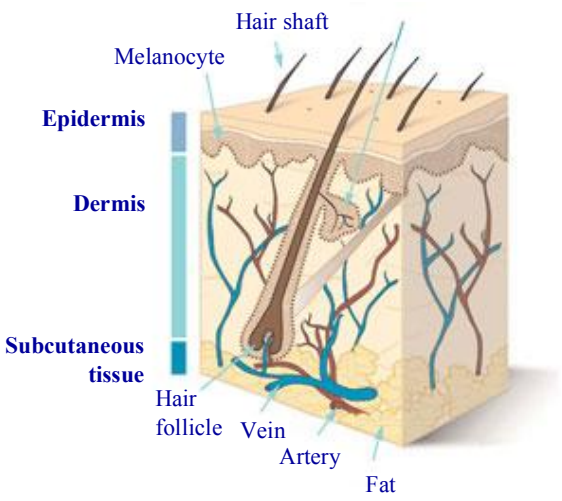


Figure 1 The main parts of the skin [9]

The **subcutis** is the bottommost layer of the skin and is composed of fatty tissue predominantly. This layer provides a thermal barrier and a mechanical cushion and stores readily available high-energy chemicals.

The **dermis** is the thickest layer of the skin (1-2 mm), but it contains fewer cells. This skin region is mechanically stabilised by an interwoven network of fibrous proteins (collagen, elastin and reticulin). The dermis also shelters the blood and lymph capillaries, nerve endings, glands (sebaceous and sweat) and hair follicles. Moreover, this part of the skin is responsible for the biochemical and biological degradation of materials transported across the skin [10].

The **epidermis** is 20-200 µm thick and in permanent contact with the environment. Due to its exposure to the harsh surroundings, the epidermis, despite its chemical robustness,

must be completely renewed at least once every month to maintain its optimum protective properties [1]. The epidermis contains keratinocytas in 90%. In this layer there could be distinguished five strata by the differentiation of these cells (Figure 2):

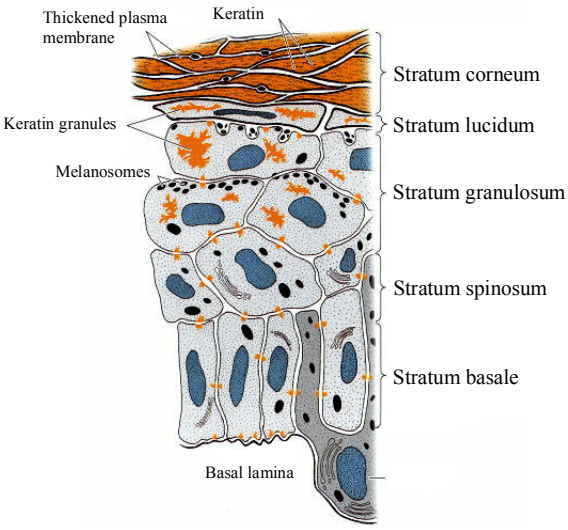


Figure 2 The different cells in the layers of the epidermis [11]

The **stratum corneum** (SC) or horny layer is the outermost, very thin (10-15 μm) layer of the epidermis, it contributes over 80% to the skin permeability resistance. It consists of a few dozen flat and partly overlapping, non-viable, cornified, almost non-permeable cells, so-called corneocytes. Corneocytes are very tightly packed and attached to each other through desmosomes. The intercellular space contains specialised multi-lamellar lipid sheets with variable ultrastructures that are covalently attached to the corneocytes membranes. Most lipids in the SC are non-polar. Particularly prominent are more than a dozen ceramide species contributing ≈50% to the total lipid mass, free fatty acids, triglycerides, cholesterol and cholesterol esters. The horny layer structure was pictorially described in the "brick and mortar" model, in which the embedded corneocytes represent the "bricks" and the intercellular lipids are the "mortar" [1, 12].

2.1.1. Drug permeation routes

Due to the special and strictly ordered structure of the SC and its excellent diffusional resistance, it makes the transdermal permeation of APIs difficult or frequently impossible. Skin permeation of drugs through the skin includes the diffusion through the intact epidermis and through the skin appendages, i.e. hair follicles and sweat glands, which form shunt

pathways through the intact epidermis. However, these skin appendages occupy only 0.1% of the total human skin surface and the contribution of this pathway is usually considered to be small. The physicochemical properties of the compound, as well as the used formulation, are the main factors influencing the choice of pathway. Originating from the structure of the SC, two permeation pathways are possible through the intact SC: (a) the transcellular route crossing through the corneocytes and the intervening lipids, and (b) the intercellular lipid route between the corneocytes (Figure 3).

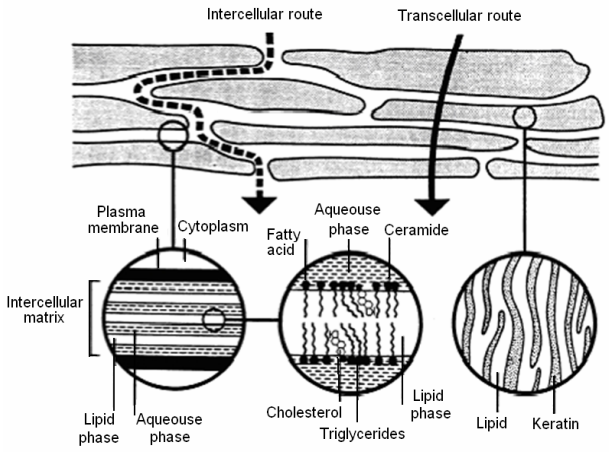


Figure 3 The ordered structure of the SC and the penetration routes [13]

Under normal conditions, the transcellular route is not considered as the preferred way of dermal invasion, the reason being the very low permeability through the corneocytes and the obligation to partition several times from the more hydrophilic corneocytes into the lipid intercellular layers in the SC and vice versa. The transcellular pathway can gain in importance when a penetration enhancer is used, which increases the permeability of the corneocytes by altering the keratin structure [14].

The intercellular microroute is considered to be the predominantly used pathway in most cases. Resulting from the bilayer structure, the intercellular pathway provides hydrophilic and lipophilic regions, allowing more hydrophilic substances to use the hydrophilic and more lipophilic substances to use the lipophilic route. In addition, it is possible to influence this pathway by certain excipients in the formulation. Many enhancing techniques aim to disrupt or bypass this special molecular architecture [15].

2.1.2. Possible ways for drug penetration enhance

It is generally accepted that the best drug candidates for transdermal administration must be nonionic, have low molecular weight (<500 Da) and adequate solubility ($logP_{octanol/water:}$ 1–4) within the lipid domains of the SC to permit diffusion through this domain whilst still having sufficient hydrophilic nature to allow partitioning into the viable tissues of the epidermis [8]. Given these operating parameters, the number of drug candidates which fit the criteria may seem low. Nevertheless, with the development of novel technologies, such constraints may be overcome [16].

Overcoming the effective SC barrier and reaching the deeper regions, targeting the API to the location of the inflammation (muscle and joint) is an important and widely studied problem in the treatment of musculoskeletal disorders. The most frequently used strategies for improving the drug penetration are summarized in the Table 1 below:

Table 1 The optimization strategies for enhanced transdermal drug delivery [7]

Optimizing transdermal delivery	
Drug-vehicle interactions	• Drug/prodrug • Chemical potential • Ion pairs/Coacervates • Eutectic systems
Vesicles and particles	• Liposomes, niosomes, transferosomes, microemulsion and nanoemulsion, solid lipid nanoparticles • High velocity particles
Stratum corneum modification	• **Hydration** • **Chemical enhancers**
Stratum corneum bypass or removal	• Microneedle array • Ablation • Follicular delivery
Electrically assisted methods	• Ultrasound • Iontophoresis • Electroporation • Magnetophoresis • Photomechanical wave

The literature considers drug-vehicle interactions and the role of vesicles and particles. Electrically assisted methods show considerable promise. A possible way can be the removal, bypass or modification of the SC. From among the numerous facilities, the latter will be detailed and discussed in my thesis. Penetration enhance by alteration of the SC can be achieved in two ways: by increasing the skin hydration and by using of chemical penetration enhancers [7].

The water content of the SC is around 15-20% of the dry weight but can vary according to the humidity of the external environment [17]. Increasing the **skin hydration** is the most widely used and safest method to increase skin penetration of both hydrophilic and lipophilic permeants [18]. Water within the SC could alter permeant solubility and thereby modify partitioning from the vehicle into the membrane. In addition, increased skin hydration may swell and open up the compact structure of the horny layer leading to an increase in penetration [7, 8]. So it is required to develop well-moisturizing transdermal delivery systems.

The most extensively investigated enhancement strategy involves the use of chemicals that compromise the skin's barrier function and consequently allow the entry of otherwise poorly penetrating molecules into the deeper layers. One possible classification of the **chemical penetration enhancers** (PE) is via the lipid-protein-partitioning concept.

Lipid action

The enhancer disrupts SC lipid organization, making it permeable. The essential action increases the drug's diffusion coefficient. Many enhancers act mainly in this way (e.g. Azone, terpens, fatty acids, DMSO and alcohols). It was assumed that such enhancers would penetrate into the skin and mix homogeneously with the lipids. Some solvents (e.g. DMSO, ethanol) may also extract lipids, making the horny layer more permeable through forming aqueous channels.

Protein modification

Ionic surfactants, decylmethylsulphoxide and DMSO interact well with keratin in corneocytes, opening up the dense protein structure, making it more permeable and thus increasing diffusion coefficient. However, the intracellular route is usually not important in drug permeation, although drastic reduction to this route's resistance could open up an alternative pathway, but it may be irreversible.

Partitioning promotion

Many solvents enter the SC, change its solution properties by altering the chemical environment, and thus increase the partitioning of the second molecule into the horny layer. Propylene glycol is widely employed, particularly to provide synergistic mixtures with molecules such as Azone, oleic acid and terpens to raise the horny layer concentration of these enhancers [7].

The mechanisms of action proposed for commonly used chemical penetration enhancers are listed below [3, 16]:

- **Sulfoxides** (DMSO) (1) increase lipid fluidity, (2) interact with keratin and (3) promote drug partitioning.
- **Alcohols** (Ethanol, benzyl alcohol) (1) Low-molecular-weight alkanols (C≤6) may act as solubilizing agents. (2) More hydrophobic alkanols may extract lipids from the SC, leading to increased diffusion.
- **Polyols** (Propylene glycol (PG), polyethylene glycol (PEG)) may solvate α-keratin and occupy hydrogen bonding sites, reducing drug-tissue binding.
- **Amides** (Urea) hydrate the SC, have a keratolytic effect, and create hydrophilic diffusion channels. Cyclic amide (Azone) (1) affects the lipid structure of SC, (2) increases partitioning and (3) increases membrane fluidity. Azone, the first molecule to be synthesised so as to act as a skin penetration enhancer.
- **Fatty acids** (oleic acid) decrease the phase transition temperatures of the lipid, increasing motional freedom or fluidity of lipids.
- **Fatty acid esters** (isopropyl myristate) have a direct action on the SC, permeating into the bilayers, increasing fluidity. Aliphatic: increase diffusivity in the SC and/or the partition coefficient.
- **Anionic surfactants** (Sodium lauryl sulphate) alter the barrier function of the SC, allowing removal of water-soluble agents that normally act as plasticizers.
- **Cationic surfactants** (Cetyl trimethyl ammonium bromide) adsorb at interfaces and interact with biological membranes, causing damage to skin.
- **Nonionic surfactants** (Polysorbate, Myrj, Span) emulsify sebum, enhancing the thermodynamic activity of drugs.

- **Terpens** (D-Limonene) (1) increase the diffusivity of drugs within the SC due to the disruption of the intercellular lipid barrier (2) open new polar pathways within and across the SC.

The protective function of the human SC imposes physicochemical limitations to the type of molecules that can traverse the barrier. That is why the use of an appropriate penetration enhancer is particularly important in the development of transdermal delivery systems in order to increase the membrane permeation rate of API without damaging the skin and causing toxicity [18, 19].

The ideal characteristics of PEs include the following: Be both pharmacologically and chemically inert, be chemically stable, have a high degree of potency with specific activity and rapid onset. They should work undirectionally, i.e. should allow therapeutic agents into the body whilst preventing the loss of endogenous material from the body. The activity and duration of their effect should be both predictable and reproducible. They should have reversible effect on skin properties, when they are removed from the skin, barrier properties should return both rapidly and fully. The ideal penetration enhancer should be appropriate for formulation into diverse topical preparations, thus should be compatible with both excipients and drugs, should be non-irritant, non-allergenic, non-phototoxic, and should be odourless, tasteless, colourless, cosmetically acceptable, and inexpensive, be readily formulated into dermatological preparations [16, 18].

In this respect, an interesting possibility is offered by **Transcutol®** (diethylenglycol monoethyl ether), which is a powerful solubilizing agent for a number of APIs due to its miscibility with both polar and non-polar solvents. Transcutol (TR) seems to be very attractive as a penetration enhancer for various APIs due to its non-toxicity, biocompatibility with the skin and humectant properties [20, 21]. TR can absorb water from the skin thus increasing the water content of the SC and swelling its intercellular lipids without alteration of their multiple bilayer structure, consequently decreasing the diffusional resistance [22, 23]. TR acts as by altering the solubility of a permeant in the skin [24].

Sucrose esters (SE), a new generation of nonionic type of surfactants, are promising penetration enhancers as well. They have a sugar substituent, sucrose, as the polar head group and fatty acids as apolar groups. In recent years SEs have been in focus in the pharmaceutical field because of their wide range of HLB values and because they consist of natural origin parts (sucrose and fatty acid), they are non-toxic and they damage the barrier less than other surfactants [25]. Their big advantage is the biodegradability in the body via initial ester

hydrolysis. The unsubstituted sucrose esters degrade rapidly [26], so they are also suitable for dermal and transdermal applications. There are only few literature data about the applicability of SEs as permeation enhancers through the skin or mucosa till now [27, 28]. Although all types of tested SEs increased the drug release. This effect and the *in vitro* absorption process depend on the HLB value and the C atom number of the fatty acid chain of SEs [29]. Structural parameters, such as chain length, degree and position of unsaturation, and the nature of substituents can influence the ability of surfactants to act as skin penetration enhancers. It is well accepted that a linear alkyl chain of 12 carbon atoms maximizes the effect of a surfactant on membrane permeability. The C12 chain has intermediate oil/water solubility and is able to penetrate the lipid bilayer. Among a series of polyoxyethylene ethers, the lauryl (C12) ether was reported to be the most effective enhancer for Ibuprofen [30].

2.1.3. Examination methods of the drug penetration

Vertical Franz Diffusion Cells model is regarded as the most valid *in vitro* model for evaluating drug penetration from semisolid preparations [31, 32]. Basically, a donor and an acceptor compartment are separated by a membrane (animal, human or artificial origin). The most widely used skin permeation model membranes are collected in the Figure 4.

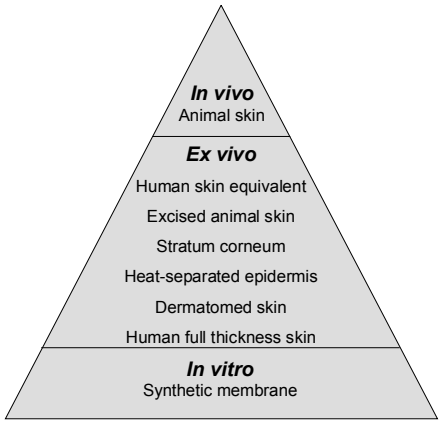


Figure 4 Model membranes for permeation examinations

The permeation examinations were performed predominantly through cellulose acetate **synthetic membrane** in the earlier years. Generally, these membranes are reported to be more permeable than biological membranes and are non-discriminatory to the characteristics of diffusant molecule. These membranes have been used for quality control release studies.

Presently, various biological membranes are used more extensively than synthetic membranes [33]. Because it is not enough to make *in vitro* membrane diffusion measurements by testing newly developed transdermal preparations. It is also indispensable to examine penetration process through the skin to know not only the diffused amount of API, but also to study their interaction with the skin and the incidental reservoir function of the SC. Moreover this method is suitable also for verifying the acting mechanism of various penetration enhancers, if they act as a result of increasing the diffusion process, influencing the solubility and the thermodynamic activity of API, or if they interact with the skin, causing a temporary structure alteration in the lipid bilayer.

The experiments could be performed via excised human **full thickness skin (FTS)**, which consists of SC, viable epidermis and dermis (2000-3000 μm thick). If FTS is used it must be borne in mind that *in vivo* the permeating molecule would not have to traverse the dermal layer in all cases. The vasculature at the dermo-epidermal junction can rapidly clear the diffusant reaching this interface, therefore, *ex vivo* experiments are best conducted, for certain diffusants, without the dermis in place. To overcome this problem, **dermatomed skin** (SC, viable epidermis and a part of the dermis: 400-500 μm thick) sectioned by dermatomisation technique can be used [34, 35]. Complete removal of the dermis may be achieved by several mechanical, thermal, and chemical techniques. Most commonly, the epidermal–dermal junction is split by heat-separation technique at 60°C for 30–120 sec [36]. The **heat-separated epidermis (HSE)** has a thickness of approximately 20-200 μm. In the last decade the number of publications in the literature about penetration via human epidermis increased twofold (Figure 5).

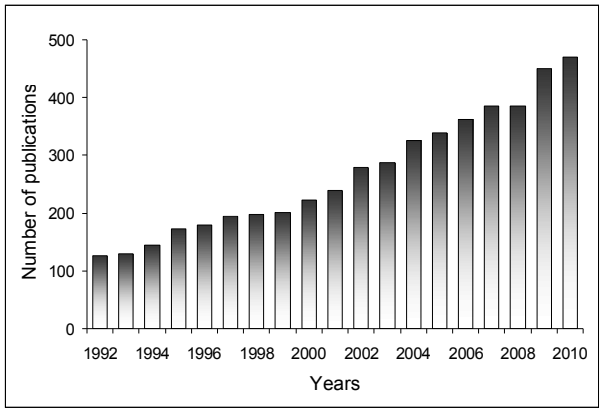


Figure 5 Number of publications with keywords “human epidermis” and “penetration” in the Science Direct

In some cases only the **stratum corneum** (10-15 μm) is required for experimental work. The cornified skin layers may be isolated by digestion of the connective epidermal tissue using trypsin in buffer solutions of pH=7.4. This may be achieved by complete immersion of full thickness skin in trypsin solution or by placing the heat-separated epidermis for 24 h at 37 °C on a filter paper soaked with the enzyme preparation [15, 37].

Although the best and most relevant is the human skin, because of its limited availability, together with an increasing need to test the bioavailability of dermal products in pharmaceutics, as well as risk assessment of chemicals, has promoted the search for other models. In the literature several **animal skin** models have been suggested as a substitute for human skin. Excised skin of rat, hairless rat [38], hairless mouse [4], pig [39, 40] and guinea pig are used usually as *ex vivo* models to predict percutaneous penetration in humans. However, the SC of the animals differs in the thickness, in the number of corneocyte layers, hair density, water content, lipid profile, and morphology from the human SC [41]. It has been described that percutaneous penetration in monkey [42] and in pig [43, 44] may closely predict that in human. However, such large animals are less available and more expensive than smaller laboratory animals [45]. Thus, the skin of rodents is widely applied. Due to the lack of fur some authors suggested the use of hairless rats [46], guinea pigs [47] and mice [48] several years ago. Hairlessness is an important point since it has been revealed that the number of folliculi, opening diameter and follicular volume play a major role in drug penetration [49, 50]. Although it has been reported that murine skin is more permeable to certain drugs than human tissue [51], hairless mice are frequently applied in penetration studies [52, 53].

In the recent years, substantial efforts have been made to develop an alternative model to human skin. This is primarily due to the low availability of human skin and increasing worldwide concerns over animal testing. In the future the **human skin equivalents** can offer solution. Human skin substitutes (HSS), human bioengineered skin (HBS), cultured skin substitutes (CSS), living skin equivalents (LSE) and reconstructed human epidermis (RHE) provide a new possibility. Various types are under investigation to serve as membranes in permeation experiments: *Epiderm* (MatTek, USA), *Skinethic* (Skinethik, France) *Episkin*® (L'Oreal, France) [15, 54, 55].

Although *ex vivo* tests are frequently used and approved methods [56] and their application is strongly encouraged in order to reduce the need for both animal and human studies, they show some imperfections. The majority of *ex vivo* models are not able to test the metabolism of drug molecules in the skin [57]. Thus, ***in vivo* examinations** cannot be spared in

pharmacological and dermatological studies. Many different types of *in vivo* methods have been developed. Nevertheless, in contrast to the Franz type diffusion cell, these techniques do not provide possibility for the exact quantitative measurement of penetration. Accordingly, there might be a need for broadening the toolbox of models available for permeation studies.

Dorsal skin fold chamber is an accepted and sophisticated experimental model to study the microcirculation under different conditions, to test the biocompatibility of different materials [58] and to examine angiogenesis [59] and wound healing [60]. This chamber is tolerated by the animals even for weeks and it has been used in a considerable number of experimental studies within the field of physiology and pathophysiology for more than twenty years [61].

We developed a novel modified version of this experimental setup which seemed to provide effective means for *in vivo* examination of transdermal permeation. Two holding stitches were inserted in the dorsal midline and a moderate tension was exerted by them in order to form a skin fold. Two symmetrical titanium frames (IROLA GmbH, Schonach, Germany) were then implanted to sandwich the extended double layer of the skin (Figure 6a). A circular full dermal thickness wound was formed on one side of the skin fold (Figure 6b).

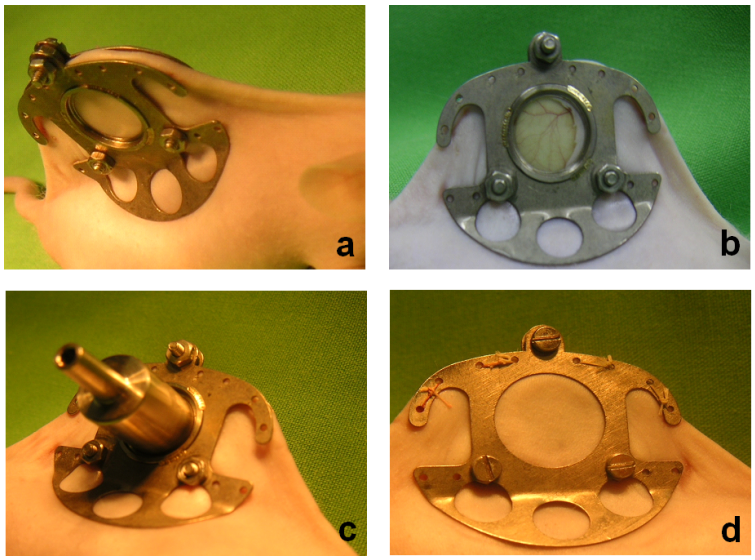


Figure 6 The novel mouse model

A metal cylinder of stainless steel with the volume of 1 ml was fixed into the window of the titanium frame on the wounded site (Figure 6c). The opposite, non-wounded site (Figure 6d) served for the application of the study formulation. In our model the formation of the circular wound made the double-layered skin fold one-layered on the area of the cylinder with the

acceptor phase. Thus, the drug placed onto the non-wounded site had to penetrate via one layer of epidermis, dermis and a thin layer of striated muscle, respectively, prior to entering the acceptor buffer.

2.2. Other biological membrane barriers

The Franz diffusion cell system can also provide an applicable model for drug penetration experiments for other biological membranes besides the skin, such as buccal, nasal mucosa, lung tissue, cornea. A new research area is the permeability study of the amniotic membrane.

Amniotic membrane (AM) is the innermost avascular layer of the placenta consisting of an epithelium, a basement membrane, and a stromal layer [62]. AM transplantation has become frequently used in ocular surface surgery and has been found to be beneficial in a number of ocular surface diseases including persisting epithelial defects, perforating or non-perforating corneal ulcers [63, 64, 65], alkali burns [66], pterygium [67] and band keratopathy [68] and after excimer laser keratectomy [69]. In all cases topical antibiotic and anti-inflammatory treatment is essential after amniotic membrane transplantation. The ocular penetration of locally administered medicaments is known; the API concentration can be measured in corneal tissues [70] and aqueous humour [71, 72, 73]. However, amniotic membrane, especially in cases of multilayer transplantation, may create a barrier for topically administered drugs to reach the corneal tissues. The pharmacokinetic impact of amniotic membrane, however, has not been exactly explored in the literature yet.

3. AIMS

To overcome the biological barriers safely and reversibly while enabling the penetration of API is a fundamental problem in the field of topical drug delivery. The aim of my thesis was to investigate the drug permeation through various biological and synthetic membranes.

1. In the first part of my Ph.D. work the transdermal drug penetration was studied. The following aims can be summarized:

- to develop semisolid vehicles,
- to examine their effect on the skin hydration and on the barrier properties of the stratum corneum (TransEpidermal WaterLoss measurements) *in vivo*,
- to perform thermoanalytical measurements in order to find a connection between the water binding mechanism and the hydrating effect of the samples,

- to study the rheological properties of Ibuprofen containing preparations,
- to investigate drug release and diffusion with a Franz diffusion cell through synthetic membrane *in vitro*,
- to examine drug permeation with the Franz cell method using excised human epidermis *ex vivo* and to examine the effect of different penetration enhancers,
- to study alterations in the stratum corneum structure at molecular level and to elucidate the mechanism of the penetration enhancers by ATR-FTIR spectroscopy combined with tape stripping method *in vivo*.

2. The second part of my Ph.D. research was to study the permeability of the human amniotic membrane. The aim was:

- to examine the barrier and reservoir function of the AM to Ofloxacin eye drops, a widely used topical antibiotic in ocular surface disease after AM transplantation using the Franz diffusion cell *in vitro* and *ex vivo*.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Preparation of semisolid vehicles

Various types of semisolid drug delivery systems were developed and investigated, such as lamellar lyotropic liquid crystals, polymeric emulsifier containing gel-emulsion and hydrogel, alginate containing hydrogel and oil dispersion, Carbopol based hydrogel. They were compared with two commonly used oil/water creams (Ph.Hg.VII.) containing an anionic or nonionic emulsifier generally used in everyday dermatological practice.

Lyotropic liquid crystals (LLCs) contained Polyethoxylated 40 hydrogenated castor oil (BASF, Cremophor RH40, official in USP/NF) as emulsifier (Table 2). The water phase of the systems was purified water (Ph.Eur.6.), the oil phase was Isopropyl myristate (Ph.Eur.6.). In our experiment we developed lamellar lyotropic liquid crystals, because their structure demonstrates the greatest similarity to the lipid bilayer of the SC. LLCs are expected to be ideal dermal vehicles with the ability to integrate into the structure of the SC and restore it [74-76].

The **polymeric emulsifier containing gel-emulsion** and **hydrogel** was prepared with the use of Pemulen™ TR1 (PTR1) (S&D Chemicals Ltd, Hungary), which served as an emulsifier and a gel-forming agent, too (Table 2). This is a cross-linked block copolymer of poly(acrylic acid) and hydrophobic long-chain methacrylates. It is able to stabilize o/w emulsions because its short lipophilic part integrates into the oil droplets whilst its long hydrophilic part forms a micro-gel around the droplets arresting their fusion. The aqueous phase was purified water. In the case of the gel-emulsion the oil phase was neutral oil (Ph.Eur.6., Miglyol 812) and the neutralizing agent was Triethanolamine (Ph.Eur.6.) [77, 78].

For the formulation of **1,2 propandiol-alginate** (PA) **containing hydrogel** and **oil dispersion** purified water and neutral oil were used (Table 2).

In order to ascertain whether oil as an occlusive ingredient has an influence on skin hydration, we investigated LLCs containing 10% and 60% of oil, we formulated an oil-free and an oil containing system with PTR1, and as PA is ready to incorporate a small amount of oil without added surfactant, PA oil dispersion was also prepared.

The **Carbopol based hydrogel** was formulated with the use of Carbopol 971 (S&D Chemicals Ltd, Hungary), purified water and Triethanolamine (Table 2).

The two traditional **o/w creams** were different in respect of the emulsifiers. The Unguentum hydrophilicum anionicum (Ph.Hg.VII.) was formed with an anionic emulsifier (Sodium lauryl sulphate) while the Unguentum hydrophilicum nonionicum (Ph.Hg.VII.) included a nonionic emulsifier (Polysorbate 60).

Table 2 The content of the examined preparations

Constituents (%)	LLC1	LLC2	PTR1 gel	PTR1 gel-emulsion	PA gel	PA oil dispersion	Carbopol 971 gel
Cremophor RH40	60	30	-	-	-	-	-
Isopropyl myristate	10	60	-	-	-	-	-
PTR1	-	-	0.2	0.2	-	-	-
1,2 propandiol-alginate	-	-	-	-	5	4.5	-
Miglyol 812	-	-	-	30	-	10	-
Carbopol 971	-	-	-	-	-	-	3
Triethanolamine	-	-	0.1	0.1	-	-	0.6
Purified water	30	10	99.7	69.7	95	85.5	96.4

4.1.2. Ibuprofen containing hydrogels

A Non-Steroidal Anti-Inflammatory Drug (NSAID), the **Ibuprofen** [(RS)-(2,4 isobutylphenyl) propionic acid] (Figure 7) was chosen as API. It is widely used in the treatment of musculoskeletal injuries, but there is a great interest in developing its topical dosage forms to avoid its gastrointestinal side effects and to reach fast pain relief and anti-inflammatory effect directly at the inflamed region (joint, muscle) [4].

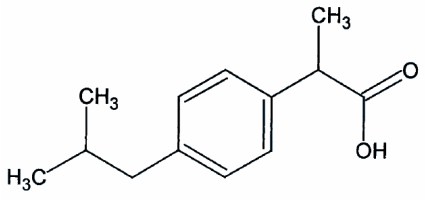


Figure 7 Chemical structure of Ibuprofen

The racemic mixture is regarded as a non-selective cyclooxygenase (COX)-inhibitor. The S(+)-enantiomer was found to be a selective COX-1 inhibitor while R(-)-Ibuprofen has little pharmacodynamic efficacy [79]. According to the present regulations, the Ibuprofen (IBU) is a BCS class II drug, showing high permeability and pH-dependent solubility. Its molecular weight is 206.28, its pK_a is in the range of 4.5-4.6 and its logP value is 3.68 [80]. IBU is considered as one of the safest NSAIDs available. It is not the best potent API in this group, but it offers the best balance between safety and therapeutic effect [81].

Transcutol® (TR, Diethylene glycol monoethylether, Gattefossé and S&D Chemicals Ltd, Hungary) (Figure 8a) was used as a penetration enhancer excipient.

Sucrose laurate (D-1216, Mitsubishi-Kagaku Foods Co, Tokyo, Japan) was the other tested penetration enhancer, which has sucrose, as the polar head group and 12 C atom long lauryl acid as the apolar group (Figure 8b). Its HLB value (16) is considerably high [82].

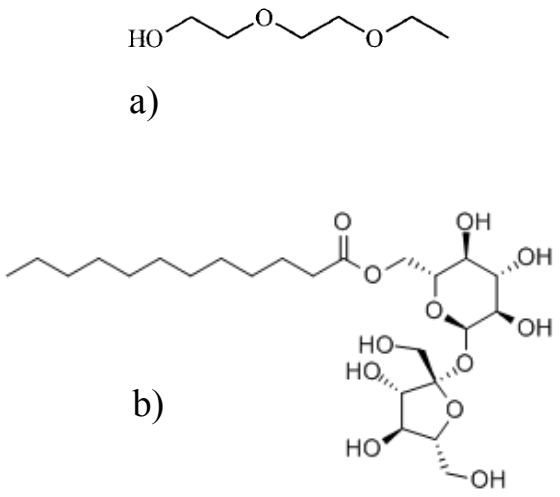


Figure 8 The chemical structure of **a)** Transcutol and of **b)** Sucrose laurate

A penetration enhancer free hydrogel (**IBU gel**) was prepared by the following procedure. 5% Ibuprofen (Sigma, St Louis, USA) was dissolved in Polyethylene glycol 400 (20%) (Hungaropharma Ltd., Hungary) and this solution was added to Carbopol 971 (S&D Chemicals Ltd, Hungary) hydrogel. The pH value was adjusted by adding Triethanolamine (7%) (Hungaropharma Ltd., Hungary). Similar compositions were prepared by using 10% Transcutol® (**IBU-TR gel**) or 2.64% Sucrose laurate (**IBU-SE gel**) (Table 3).

Table 3 Composition of the API containing samples

Constituents (%)	IBU gel	IBU-TR gel	IBU-SE gel
Ibuprofen	5	5	5
Polyethylene glycol 400	20	10	20
Transcutol	-	10	-
Triethanolamine	7	7	7
3% Carbopol 971 gel	68	68	-
SE containing Carbopol 971 gel	-	-	68

4.1.3. Ophthalmological preparation for amniotic membrane permeability experiments

300 mg of 3% commercially available Ofloxacin (OFL) eye drops (Floxal®; Dr. Mann-Pharma, Berlin, Germany), a widely used topical antibiotic preparation in ocular surface disease were used for the *in vitro* and *ex vivo* permeation and drug release studies.

4.2. Methods

4.2.1. Skin hydration and transepidermal waterloss measurements

Nowadays a variety of bioengineering techniques has also been employed to provide a non-invasive and more objective evaluation of the skin, such as measurements of skin moisture content and the TransEpidermal WaterLoss (TEWL). The **Corneometer® CM 825** (Figure 9a) is the mostly used instrument worldwide to determine the hydration level of the skin surface, mainly the SC [83, 84]. The investigation is based on capacitance measurement of a dielectric medium.

The **Tewameter® TM 300** (Figure 9b) (all devices by Courage and Khazaka Electronic GmbH, Cologne, Germany) is the most accepted measuring device for the assessment of the TEWL. This is the most important parameter for the evaluation of the barrier function of the skin. The probe measures the density gradient of the water evaporation from the skin indirectly by the two pairs of sensors inside the hollow cylinder [85, 86].

a) b)

Figure 9a) The Corneometer and **b)** The Tewameter [87]

Fifteen healthy subjects (11 females and 4 males) of ages between 22 and 51 years without any known dermatological diseases or allergy participated in the experiment. Informed consent was obtained from all volunteers and the study was approved by the local ethics committee (Regional and Institutional Human Medical Biological Research Ethics Committee at the University of Szeged). During the test period, the subjects were not allowed to use any other skin care products on their hands. To prove the standard circumstances, all the skin tests were performed after the adaptation of the volunteers to room conditions (30 min at 23–25 °C and 40–50% RH). During the experiment the samples were applied on the dorsal hand of all the subjects. The electrical capacitance of the SC and the TEWL value were determined before and after the sample application. The measured values were compared with the values detected on the non-treated skin. The changes in moisturizing level and in TEWL were expressed in percentage [88-90].

4.2.2. Thermoanalytical investigations

Thermogravimetry (TG) allows verifying the microstructure of various semisolid preparations through studying indirectly the water binding mechanisms in them [78]. This property is very informative in predicting at what rate they can release their water content [91]. By this analytical method, different forms of incorporated water (free, bulk, bound and interlamellar) could be detected, which were delivered from the preparations in different times, ensuring longer hydration on the skin. The knowledge of the proportion of various types of water is also important to get information about the structure, which has a strong effect on drug release from pharmaceutical formulations [92, 93].

The thermogravimetric analysis was carried out using a **MOM Derivatograph-C** (MOM GmbH, Hungary) instrument. Samples were weighed (40–50 mg) in platinum pans (No.4). The reference was an aluminium oxide containing pan. Two types of measurements were performed. At a slow heating rate the samples were heated from 25 to 120 °C at 1 °C min^{-1}, at a fast heating rate the systems were heated from 25 to 200 °C at 10 °C min^{-1}. TG (mass loss % versus temperature) and derivative TG (DTG) curves were plotted. Each study was repeated three times.

4.2.3. Rheological measurements

The rheological profile of the samples was studied by **PaarPhysica MCR101 rheometer** (Anton Paar GmbH, Austria). A cone-plate measuring device was used in which the cone angle was 1 degree and the thickness of the sample was 0.046 mm in the middle of the cone. The measurements were carried out at 25 °C. Flow curves of the different samples were determined. The shear rate was increased from 0.1 to 100 1/s (up curve), and then decreased from 100 to 0.1 1/s (down curve). The shearing time was 300 s in case of both segments.

4.2.4. Drug diffusion and permeation investigations

Franz diffusion cell method

Membrane diffusion and permeability studies were performed with a vertical **Franz diffusion cell** system (Hanson Microette TM Topical & Transdermal Diffusion Cell System, Hanson Research Corporation, USA).

0.30 g of sample was placed as a donor phase on Porafil membrane filter (cellulose acetate, the pore diameter was 0.45 μm, Macherey-Nagel GmbH & Co. KG, Germany) in itself (*in vitro*) and on heat-separated epidermis or AM (*ex vivo*) supported with Porafil membrane filter.

With the use of AM other method was also performed. AM pieces were soaked in 10 ml of 3% commercially available Ofloxacin ophthalmic solution (Floxal eye drops) in Petri dishes for 1 (Group 1), 2 (Group 2) and 3 (Group 3) hours. After soaking, AM pieces were placed on membrane filters soaked in Phosphate buffer solution (PBS pH=7.4±0.15) for 20 min.

The effective diffusion surface area was 1.767 cm^2. PBS was used as an acceptor phase to ensure sink conditions. The rotation of the magnetic stir bar was set to 450 rpm. Experiments were performed at 37±0.5 °C. The skin permeation was examined over 24 (*in vitro*) and

48 hours (*ex vivo*), the period of the transamniotic permeability measurements was 1.5 hours and the reservoir function of the AM was investigated over 7.5 hours. Samples of 0.8 ml were taken from the acceptor phase by the autosampler (Hanson Microette Autosampling System, Hanson Research Co., USA) and replaced with fresh receiving medium [94]. The quantitative measurement of Ibuprofen and Ofloxacin was carried out with an UV spectrophotometer (Unicam Heλios α Thermospectronic UV-spectrophotometer v4.55, UK) at a wavelength of λ=263 nm and λ=287 nm, respectively. The mean values (±SD) were calculated from five parallel measurements each time.

Preparation of heat-separated epidermis (HSE)

Excised human skin from Caucasian female patients who had undergone abdominal plastic surgery was used. This was approved by the by the local ethics committee. Immediately after excision, the subcutaneous fatty tissue was removed (Figure 10a) and the skin was stored frozen at -20 °C. For the *ex vivo* permeation study the skin was thawed, the epidermis was separated from the underlying dermis using the heat-separation technique based on a procedure reported by Kligman and Christophers [36]. Individual portions were immersed in water bath at 60 °C for 90-120 s (Figure 10b). After removing the skin from the water bath, it was placed SC side up on a filter paper, the epidermis (comprising SC and viable epidermis) was gently removed from the underlying dermis using forceps (Figure 10c). The latter was discarded and the epidermal membrane was floated onto the surface of PBS (pH=7.4±0.15) at least 20 min and it was placed on supporting Porafil membrane [95, 96]. To make sure of the integrity of the epidermis, stereomicroscopic images ("Leica & Zeiss" image processing and analysis system) were taken. Only intact skin was used for the measurements.

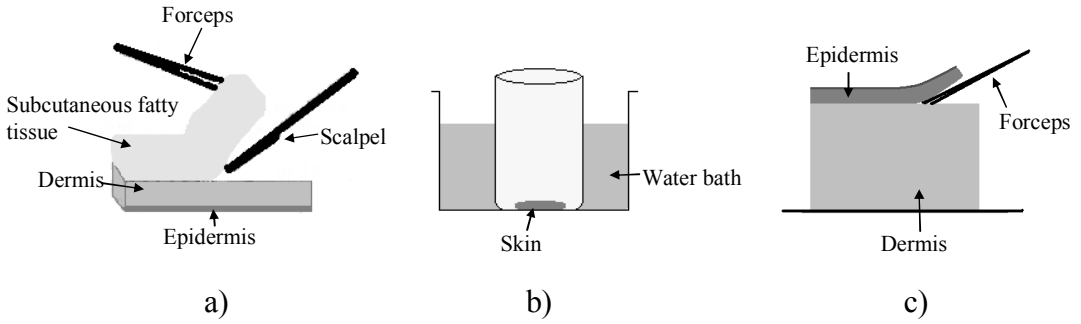


Figure 10a)-c) Steps of removing the epidermis from the underlying dermis

Preparation of human amniotic membrane (AM)

The research was approved by the Institutional Human Experimentation Committee and adhered to the tenets of the Declaration of Helsinki. Amniotic membrane obtained by elective cesarean section was separated from the chorion as soon as possible, 1 hour after delivery at the latest, by blunt dissection and was rinsed with PBS (pH=7.4±0.15). AM pieces of 25 mm in diameter (with the epithelial side up) were placed on cellulose acetate membrane filters. Two groups were created according to the preservation technique of the amniotic membrane, as follows: (1) with fresh amniotic membranes (no preservative), amniotic membranes with membrane filters were used within 6 hours of preparation; (2) with cryopreserved amniotic membranes, AM pieces on filter membranes were frozen at -20 °C, and neither antibiotic nor preservative was added to the medium.

Data analysis

Permeation parameters were obtained from the cumulative amount of API permeated per cm^2 versus time plots (Q). The steady state flux (J) -representing the absorption rate per unit area- and lag time (T_{lag}) -symbolising the time of delay which describes the first contact of the drug with the skin surface until a steady state flux- were obtained from the slope and X-interception value of the linear portion, respectively. The permeability coefficient (K_p) was calculated according to Fick's first law of diffusion, based on the steady state flux and the applied drug concentration (C_d) of the donor phase [96].

$$K_p=J/C_d \qquad \text{Eq.1.}$$

To characterize the effect of the penetration enhancers, enhancer index (EI) was calculated by dividing the K_p of the hydrogels with enhancer after 48 h by the K_p of the enhancer free IBU gel after 48 h [98].

$$EI=K_{p(\text{with enhancer})}/K_{p(\text{without enhancer})} \qquad \text{Eq.2.}$$

Statistical analysis

Student's *t*-test was performed to see any significant difference in the diffused, permeated or released amount (Q) of API (IBU, OFL) between the control and test preparations or between the AMs. Differences were regarded as significant, with $p<0.05$.

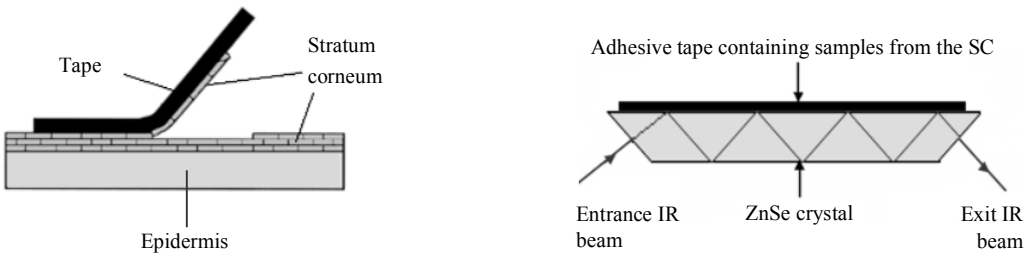
4.2.5. ATR-FTIR spectroscopy

ATR-Fourier Transform infrared spectroscopy

The Fourier Transform Infrared (FTIR) spectroscopic measurement by Attenuated Total Reflection (ATR) method is a powerful tool for studying the structure of the SC at molecular level [99-102], characterizing its lipid [103], protein and water content [104, 105], examining the drug penetration into the skin [106, 107] and the biochemical modifications induced by the penetration and the influence of various penetration enhancers on the SC barrier function [108-110]. The big advantage is that this technique enables the elucidation of the extent and mechanism of percutaneous penetration enhancement non-invasive *in vivo*. However, the information obtained pertains only to the immediate layer in contact with the ATR crystal. Information from the deeper regions of the SC can only be obtained through sequential tape stripping [111].

Tape stripping method

The experiments were performed on 15-week-old male SKH-1 hairless mice (body weight: 35–41 g). The procedure and protocol applied, were approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged in advance. The animals were anesthetized with a mixture of ketamine (90 mg/kg bw) and xylazine (25 mg/kg bw) intraperitoneally. The skin of hairless mice was treated with the formulations. With the use of adhesive tape (D-Squame® Skin sampling discs, CuDerm Corporation, Dallas, USA) samples were collected from the uppermost layer of their dorsal skin 30 min after the application of the preparations (Figure 11a). Due to the possibility of surface contamination and to remove the excess of the preparation, the first tape was discarded. The stripping procedure was repeated for up to 18 strips recording an IR spectrum after each third tape strip (Figure 11b). The spectrum of the non-treated skin was also recorded [112, 113].



a) b)

Figure 11a) The tape stripping method **b)** The adhesive tape on the ZnSe crystal [13]

All FTIR measurements were performed by an **Avatar 330 FTIR spectrometer** (Thermo Nicolet, USA) equipped with a horizontal ATR crystal (ZnSe, 45°). Spectra were recorded between 4000 cm^{-1} and 400 cm^{-1} at 4 cm^{-1} optical resolution, and 32 scans were co-added. The spectra of three parallel samples, gained from three different animals, were recorded and corrected with the spectrum of the tape. In order to obtain a reference spectrum of the API, a KBr pellet containing 0.5 mg IBU was prepared and used. The spectra of the preparations and pure PEs were also recorded.

5. RESULTS AND DISCUSSION

5.1. Investigation of the developed semisolid vehicles

Investigating and testing the API free vehicles was the first step of transdermal delivery systems development. Their effect on the skin should be taken into consideration. They should be non-irritating, non-sensitizing and they should not disrupt the special lipid bilayer in the SC. Further requirements include good consistency, aesthetic appearance and moisturizing effect since the appropriate hydration state of the SC helps to hold the barrier function of the skin and can also promote drug penetration. The effect of the formulations on the skin was investigated by the *in vivo* test, the duration of the hydration effect and the water binding mechanisms were studied by thermoanalytical measurements.

5.1.1. *In vivo* skin tests and thermogravimetric prediction method for hydration effect

The moisturizing effect and the influence on the skin barrier of the developed drug delivery systems were tested on healthy volunteers *in vivo*. However, these investigations are time consuming, sometimes risky and require ethical approval. Hence, there is a need to find a proper method for predicting the hydration effect of various formulations, hereby decreasing the number of *in vivo* human tests. The thermogravimetry could be an appropriate method for this purpose.

During the investigation with derivatograph the samples lost water due to heating, the TG curves show this weight loss. In the case of the fast heating rate they could be divided by the DTG curves into sections, which indicate different types of water spaces. More peaks could be distinguished in the DTG curves of the preparations with a complex structure (LLCs, PTR1 gel-emulsion, PA oil dispersion, o/w creams) (Figure 12), where the water is supposed to be bound through various binding mechanisms. One peak may correspond to free water at about 100 °C, which is able to diffuse to the surface at a faster rate than the evaporation process itself, hence the process represents free surface evaporation. The other peak at about 140 °C may correspond to the residual water being strongly bound within the system [92]. Besides the free water, the LLCs also contain interlamellar water incorporated between the

hydrophobic layers, the o/w creams contain interlamellar and bulk water as well. The PTR1 gel-emulsion has a special structure, a part of the water is in a very strong bond around the oil droplet (micro-gel water), which is indicated by the second DTG peak, while in the PTR1, PA and Carbopol 971 gel the entire amount of water is bound but not so strongly as in the PTR1 gel-emulsion and PA oil dispersion, which is shown by the only DTG peak at 120 °C.

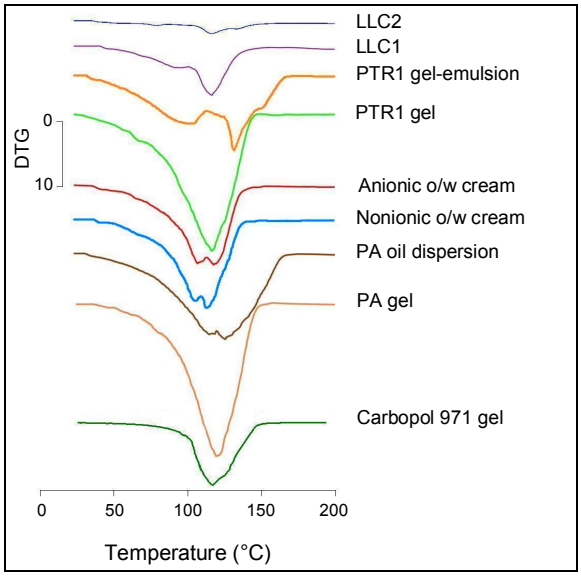
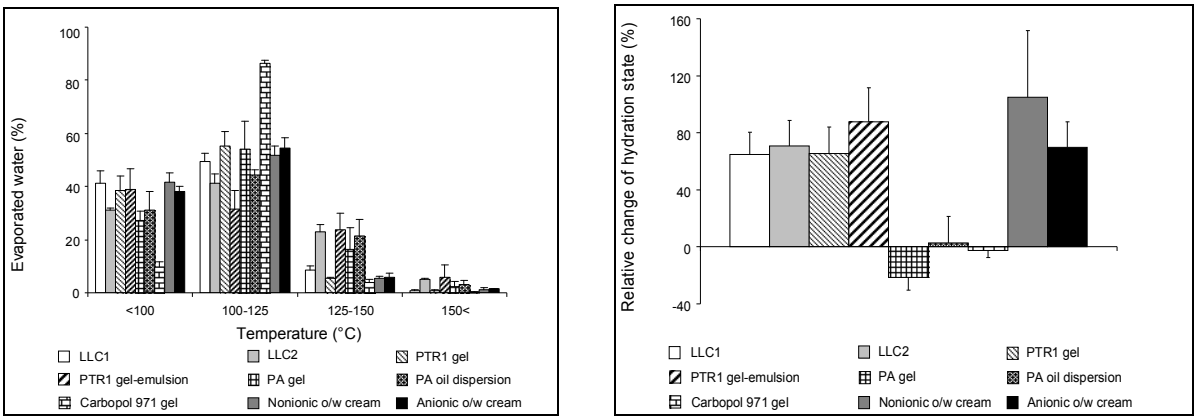


Figure 12 The DTG curves of the investigated preparations

From the measured weight loss it was calculated how many percents of water evaporated from the preparations in the specified temperature ranges (Figure 13a).



a) b)

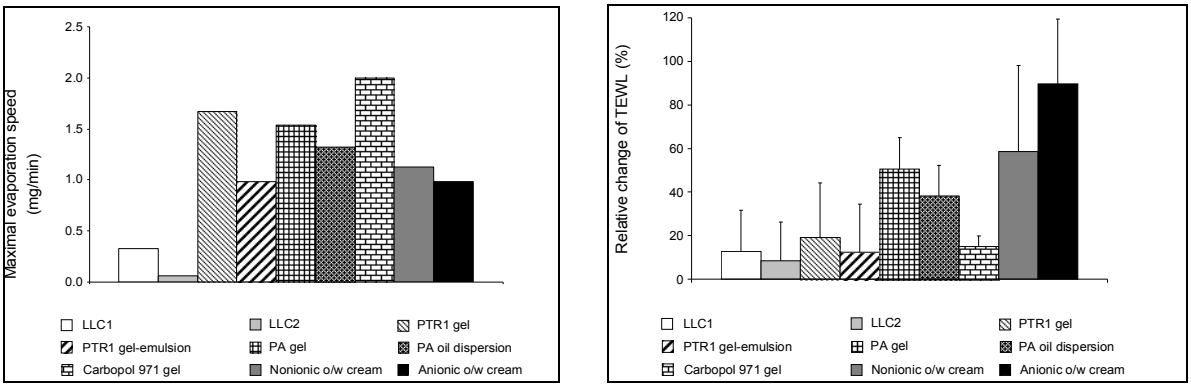
Figure 13a) Percentage weight loss values over specified temperature ranges

b) Mean values of the changes in the skin hydration of the volunteers

It could be established clearly that the samples with more differentiated curves deliver the differently bound water in several steps, resulting in a lasting moisturizing effect. It could be observed that the oil containing PTR1 gel-emulsion, PA oil dispersion and the higher oil containing LLC hold approximately 5% water even over 150 °C. They have a relevantly better water binding capacity than the oil free samples. The reason for it could be that the oil arrests the evaporation. The occlusive effect of the oil was proved by corneometric measurements on the human skin, too, since the oil containing PTR1 gel-emulsion and the PA oil dispersion showed a better moisturizing effect than the oil-free samples (Figure 13b).

In order to examine the whole water content of the systems, a slow heating rate was used. A tangent was fitted to the TG curve in the peak of the DTG curve. From the slope of this tangent the maximum evaporation speed (MES) (mg/min) could be calculated. The steeper the curve is, the faster the moisture evaporates from the preparation.

The lowest MES values were shown by the LLCs (Figure 14a) and the least transepidermal waterloss was found also in the case of the LLCs (Figure 14b). The reason for it could be, on the one hand, their lamellar structure with strong water bonds. Namely, they contain a major proportion of incorporated water concentrated in the layers between the hydrophilic domains. On the other hand, their lamellar structure is very similar to the human SC lipid bilayer, thus they are able to integrate into the structure of the skin and to restore its barrier function, arresting the TEWL [114].



a) b)

Figure 14a) The maximum evaporation speed

b) Mean values of the changes in the TEWL of the investigated samples

A remarkable difference was found between the oil containing and oil-free samples with respect to MES and TEWL changes. This could be explained with the more complex structure

of the oil containing PTR1 gel-emulsion and PA oil dispersion, which is made possible by the presence of water bound with different strengths in these preparations.

The ideal semisolid drug delivery system ensures a good, lasting moisturizing effect and retards the evaporative water loss. It can be concluded from the results of our investigations that the oil containing systems with a complex structure (LLC1, LLC2, PTR1 gel-emulsion) satisfy these requirements the most.

The reason for their excellent moisturizing effect can be, on the one hand, that oil as an occlusive ingredient forms a protecting film on the skin surface, thereby arresting water loss. On the other hand, thanks to their complex structure (lamellar, micro-gel structure), they have a strong water binding capacity and they can deliver the differently bound water in several steps. These observations were also supported by thermogravimetric measurements as well as by corneometric and TEWL measurements on healthy volunteers.

The results of the *in vivo* moisturizing tests are in accordance with the results of the thermoanalytical measurements. Thus thermogravimetry can be used for characterizing the microstructure and water binding capacity of semisolid vehicles, from which the rate of water release and the permanency of the skin hydrating effect can be concluded. Therefore thermogravimetry seems to be a possible predicting method for the characterization of the hydrating effect of different semisolid preparations. It could be used for screening the potential drug delivery systems cost and time effectively, reducing the number of *in vivo* tests.

5.2. Study of the Ibuprofen containing hydrogels

A NSAID (Ibuprofen) was incorporated in the Carbopol 971 based hydrogel. Rheological measurements were performed in order to study the consistency of the API containing samples and to compare their rheological properties to the API free vehicle, whether the Ibuprofen modified on it. In addition, drug diffusion and penetration experiments were carried out with TR and SE containing IBU gels through synthetic membrane and human epidermis. These investigations were completed with FTIR analysis in order to verify the acting mechanism of these two penetration enhancers and to monitor the API in the SC layers.

5.2.1. Rheological characterization

Rheology is the study of how matters deform and flow under the influence of external forces. This deformation is strongly influenced by the inner structure, in this way rheological investigations are useful tools to describe different materials [115]. In case of flow curves shear stress was measured as the function of increasing and decreasing shear rate.

It could be established, that the structure of the IBU containing samples (IBU gel, IBU-SE gel, IBU-TR gel) could be deformed slightly more easily than the structure of the IBU free Carbopol 971 gel. However, the API incorporation in the hydrogel did not influence the viscosity of the formulation remarkably (Figure 15).

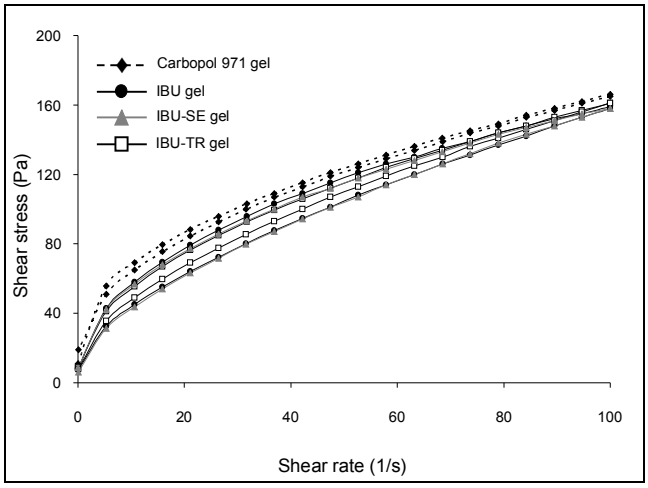


Figure 15 The flow curves of the Carbopol 971 gel and the IBU containing formulations

5.2.2. Drug diffusion and permeation examinations

The aim was to study how the two penetration enhancers, Transcutol and Sucrose laurate influence the diffusion and penetration of IBU *in vitro* and *ex vivo*. Figure 16 shows the cumulative amount per unit area of IBU diffused through synthetic membrane from the different formulations against time. It could be established that IBU diffusion from the IBU gel without any penetration enhancer was the most intensive in the first 6 hours. The diffused amount of API was 6276.33±715.14 μg/cm^2. After that the diffusion process slowed down and after the 10th hour a steady state could be seen. Higher values were found in the case of the gel containing Transcutol as penetration enhancer (IBU-TR gel) than in the case of the penetration enhancer free IBU gel. The diffused amount was 7504.85±499.32 μg/cm^2 after 6 hours. After the 3rd hour the difference between the IBU amount diffused from IBU gel and IBU-TR gel was statistically significant ($p<0.05$). Based on our investigations, Transcutol increased the diffusion of IBU.

On the basis of the synthetic membrane diffusion measurements, the IBU diffusion was hampered from the sucrose ester containing gel (IBU-SE gel). In the first 6 hours a lower amount (4561.45±656.55 μg/cm^2) was found than in the case of the IBU gel and the curve did not have a plateau. From the characteristics of the curve it could be established that the IBU-SE gel releases API slower than the other two formulations.

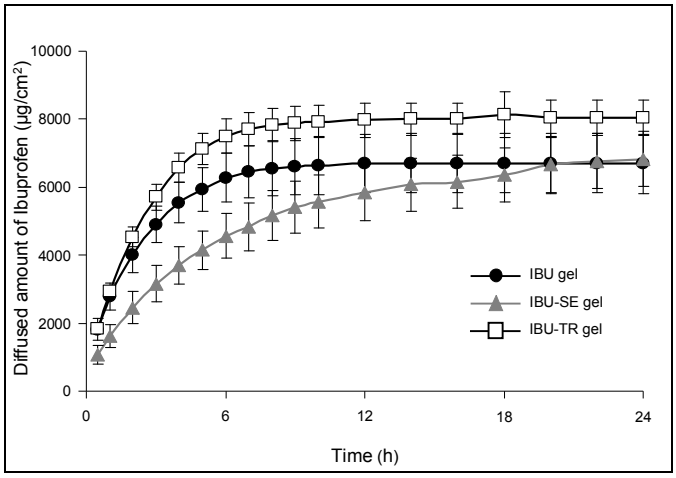


Figure 16 The cumulative diffused amount of Ibuprofen through synthetic membrane

Figure 17 presents the permeated amount of IBU across excised human epidermis. Different results were obtained with the *ex vivo* permeation experiments than with the *in vitro* membrane diffusion measurements. The sucrose ester containing gel increased the API

permeation through skin notably compared to the enhancer free gel, but the Transcutol containing gel did not enhance the IBU permeation. Moreover, an overall transdermal permeation decrease could be found.

This could be explained by the different acting mechanism of the two enhancers. SE may cause a temporary slight alteration in the skin structure, its long hydrocarbon chains may interact with the lipids of the SC, reducing the barrier function [116]. That is why SE influences the skin penetration *ex vivo* and does not act on the synthetic membrane *in vitro*.

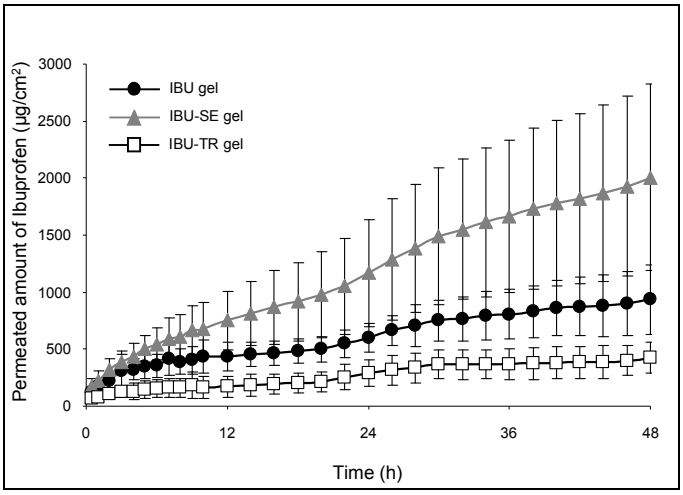


Figure 17 The cumulative permeated amount of Ibuprofen through human epidermis

TR acts by increasing the solubility of the penetrant in the barrier. It works as a humectant, absorbs water, thus increasing the water content of the skin and the donor compartment. The change in the donor composition can influence the solubility and the thermodynamic activity of API [22]. However, TR has also been reported to increase the skin accumulation of topically applied compounds without a concomitant increase in transdermal penetration. The reason for the decreased *ex vivo* skin permeation could be the API depot in the SC. The depot effect is created by the swelling of SC intercellular lipids without the alteration of their multiple bilayer structure. These swollen lipids then retain API, especially lipophilic compounds, such as IBU, and the increased skin accumulation of API results in a decrease in the transdermal permeation *ex vivo* [117].

The flux values (J) and the permeability coefficients (K_p) confirm the above results (Table 4). The highest values were found with the IBU-TR gel *in vitro* and with the IBU-SE gel *ex vivo*. The lag time was the shortest in the case of the IBU-SE gel and the longest in the case of the IBU gel after 48 hours. The enhancer index characterizes the enhancer effect of the

penetration enhancers. By comparing the enhancer indexes it could be established that TR is an effective diffusion increaser for IBU through synthetic membrane, but it could not enhance its skin penetration. On the basis of our investigations, SE is a more effective enhancer for IBU than TR. SE has promoted the skin penetration of IBU 2.15-fold.

Table 4 Permeation parameters of Ibuprofen

	Diffusion of Ibuprofen through synthetic membrane after 24 hours			Permeation of Ibuprofen through human epidermis after 48 hours		
	IBU gel	IBU-TR gel	IBU-SE gel	IBU gel	IBU-TR gel	IBU-SE gel
Cumulative amount of IBU (%)	78.15±9.30	94.58±6.25	79.97±9.71	10.87±3.58	4.97±1.60	20.43±6.70
J (μg/cm^2/h)	1047.30	1340.50	748.61	15.38	7.23	37.44
K_p*10^{-3} (cm/h)	5.57±0.66	6.70±0.40	5.70±0.61	0.39±0.11	0.18±0.37	0.84±0.27
T_{lag} (h)	1.54	1.12	1.11	12.82	10.33	5.60
Enhancer index (EI)	1.00	1.20	1.02	1.00	0.45	2.15

From our study it seems that it is not enough to make *in vitro* membrane diffusion measurements by testing newly developed transdermal preparations. It is also indispensable to examine permeation through the skin to know not only the diffused amount of API, but also to verify the acting mechanism of penetration enhancers, if they act as a result of increasing the diffusion process, influencing the solubility and the thermodynamic activity of API, or if they interact with the skin, causing a temporary structure alteration in the lipid bilayer. Our investigations show that Sucrose laurate seems to be an appropriate and effective penetration enhancer for Ibuprofen.

5.2.3. FTIR analysis

FTIR is an excellent non-invasive complementary method for studying the skin penetration *in vivo*. It provides information about the API, water, lipid, protein content of the SC and about the structure alterations in the special bilayer.

FTIR analysis was performed in order to confirm the above written assumptions about the acting mechanisms of Transcutol and the sucrose ester. It was studied whether the penetration

enhancers improve the IBU penetration into the skin or they cause API accumulation in the SC and if they generate harmful modification in the special lipid bilayer structure of the SC and protein conformation in the corneocytes.

Figure 18 shows the spectra of the pure IBU (6), pure TR (4), pure SE (5) and the applied preparations (1-3). The band of the C-C ring vibration could be seen at 1512 cm^{-1} in the spectrum of the IBU, which is considered as the most discriminative band and was used for the identification of the IBU in the SC [118]. The preparations have peaks at 1395 and 1562 cm^{-1}, which correspond to the symmetric and asymmetric carboxylate vibrations. The most characteristic bond of the SE assigned to the ester group (can be seen at 1728 cm^{-1} in spectrum 5).

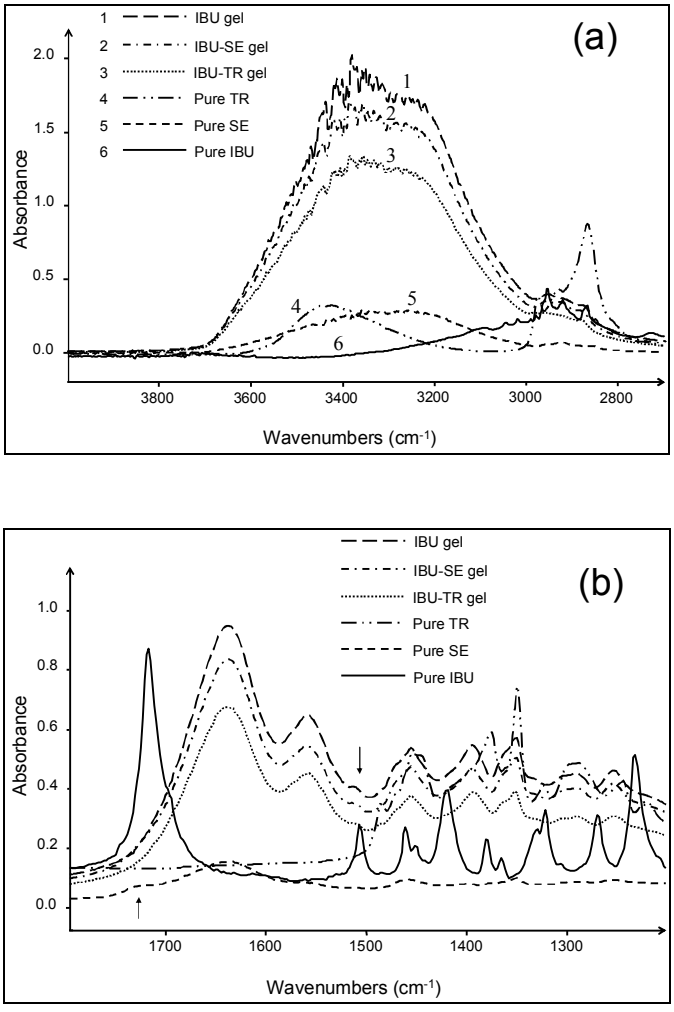


Figure 18a)-b) FTIR spectra of the preparations and the pure materials

The most characteristic bonds and absorption maximums in the skin are summarized in the Table 5 [105]:

Table 5 The principal absorbing species in the skin

Molecular source		Absorption maximum (cm^{-1})	Physiologic parameter
Water	[OH]	3000-3500	SC hydration
Lipids	[CH_3]	2960; 2870	SC lipids
	[CH_2]	2920; 2850	
Proteins	[C=O] amide I	around 1645	SC proteins
	[N-H] amide II	around 1545	

Figure 19 shows the IR spectra of the non-treated (10), the IBU gel (8), the IBU-TR gel (9) and IBU-SE gel (7) treated SC after the 6th tape stripping.

The spectra feature strong absorbance peaks in the region of 3000-3500 cm^{-1} after the treatment with the preparations (Figure 19a), which come from water, because the band of the O-H stretchings of the TR and SE are too weak and IBU has no absorbance peak in this range (Figure 18a). So, all the three hydrogels can increase the water content of the SC. The IBU-TR gel caused similar moisturization on the skin as the IBU gel, but the IBU-SE gel had most well-hydrating effect.

The hydrocarbon chains of lipids give asymmetric and symmetric CH_2 stretching vibrations at 2920 and 2850 cm^{-1}, and asymmetric and symmetric CH_3 stretching vibrations at 2960 and 2870 cm^{-1}, respectively (Table 5). Any extraction of the lipids by enhancer results in a decrease of peak height and area. We found that the SC uptook the lipophilic hydrocarbon based components of the preparations, so the absorbance peak near 2850 cm^{-1} increased in all cases, but the most remarkable change was observed after the treatment with the IBU-SE gel (Figure 19a). Some enhancers may fluidise the SC lipids, which can be noted from the shift of CH_2 stretching peaks to higher wavenumbers (trans to gauche conformation) and an increase in peak width [94, 98]. Minimal shifts were observed in all cases.

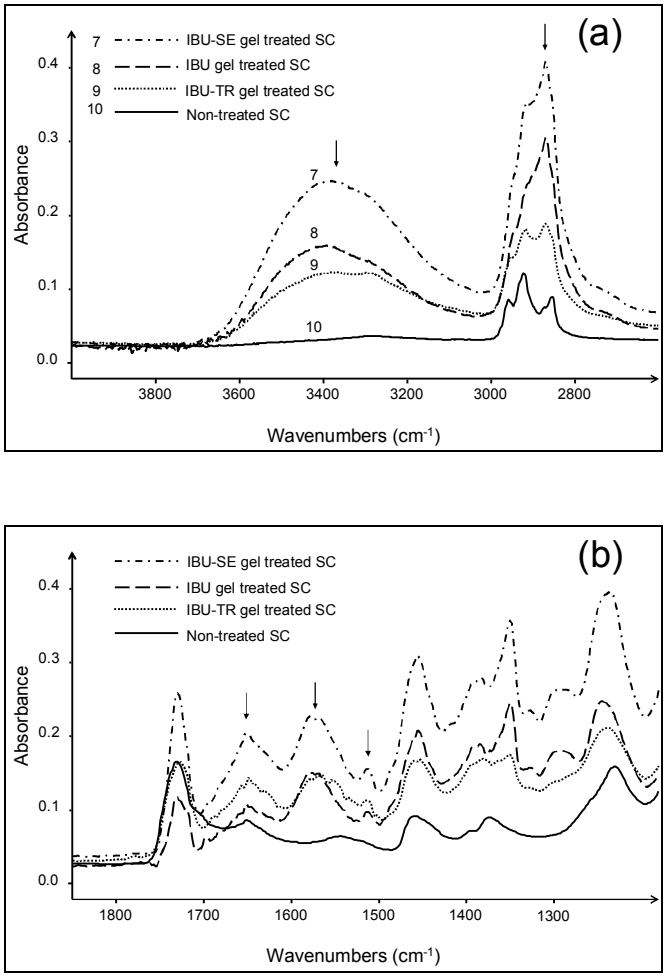


Figure 19a)-b) FTIR spectra of the non-treated SC and the SC treated with the IBU, IBU-TR and IBU-SE gel

The absorption bands at around 1650 cm^{-1} (amid I) and 1550 cm^{-1} (amid II) are typical protein bands which arise mainly from C=O stretching and N-H bending vibrations. These frequencies are sensitive to the conformation of proteins present in the SC. We found the amid I peak near 1650 cm^{-1}, which did not shift due to the treatment, so the preparations also caused only minimal changes in the protein structure (Figure 19b). The amid II peak range, however, showed strong overlapping bands around 1570 cm^{-1} in the case of the treated SC, which can be assigned to the carboxylate modes of Carbopol and the IBU itself produced by the neutralization of the preparations by Triethanolamine.

A higher peak was found near 1728 cm^{-1} in the spectrum of IBU-SE gel treated SC compared to the other spectra. It is caused by the strong C=O band of the SE.

The sign of the IBU could be successfully detected in the treated skin at 1512 cm^{-1}.

Table 6 lists the relative absorbance values at 3392 cm^{-1}, which are proportional to the water content of the SC, and at 1512 cm^{-1}, which correspond to the amount of IBU penetrated into the SC layers. It was found that the upper layers of the SC were the most hydrated, and the water content decreases with the number of the tape strips. The application of the IBU gel and the IBU-TR gel caused similar moisturization on the skin in each layer. However the IBU-SE gel had remarkable hydration effect. It increased the water content of the SC to 5.66-fold in the outermost layer and to 2.63-fold even in the deepest region.

Table 6 Intensities in the different layers of the treated stratum corneum

	Absorbance at 3392 cm^{-1} (ν_{O-H})				Absorbance at 1512 cm^{-1} (IBU)			
Number of tape strips	Non-treated SC	Treated with IBU gel	Treated with IBU-TR gel	Treated with IBU-SE gel	Non-treated SC	Treated with IBU gel	Treated with IBU-TR gel	Treated with IBU-SE gel
3. strip	0.0407	0.1968	0.2070	0.2305	0.0544	0.1403	0.2114	0.1495
6. strip	0.0371	0.1507	0.1253	0.2374	0.0484	0.0970	0.1231	0.1568
9. strip	0.0329	0.1119	0.1111	0.1808	0.0530	0.0847	0.1107	0.1351
12. strip	0.0267	0.0849	0.0793	0.1509	0.0434	0.0706	0.0997	0.1143
15. strip	0.0321	0.0720	0.0805	0.0931	0.0486	0.0540	0.1028	0.0812
18. strip	0.0264	0.0673	0.0550	0.0695	0.0498.	0.0699	0.0794	0.0742

Table 6 also represents the distribution of the API in the different SC layers after the various treatments. The amount of IBU decreases in deeper layers in all cases. After the IBU gel application, the most of the API is in the outermost layer of the skin. IBU penetrates into the deeper layers with difficulty. Furthermore, it could be assessed that both penetration enhancers increased the API amount in the skin. Extremely high absorbance was measured in the case of the IBU-TR gel application in the first layer. It could confirm our assumption about the TR and API accumulation in the SC. Thus this investigation gave an explanation for the low permeated amount by the permeation experiments. The IBU-SE gel treatment achieved a higher IBU content in each layer compared to the IBU gel treatment, in spite of the same API content in both preparations. Moreover, the IBU distribution in the different strata is the most uniform in this case.

Thus it can be ascertained that all of the preparations caused only minimal modification in the lipid and the protein structure, promoting the skin hydration and therefore also the penetration of IBU. TR causes IBU accumulation in the skin, hereby assuring a sustained effect. The

degree of the moisturization and penetration was the most intense in the case of the IBU-SE gel treatment. It has been proven that SE acts as an effective and non-irritating hydration and penetration enhancer for IBU through the skin.

5.3. The permeability of human amniotic membrane

The ocular tolerance of artificial substances (such as hydrogels or therapeutic contact lenses) can be problematic, but amniotic membrane is well tolerated and absorbed or integrated by corneal tissues [119]. It provides a new possibility in the treatment of corneal ulcers, pterygium and band keratopathy, however, its barrier capacity and pharmacokinetic properties have not been described in the literature yet. So there is a great interest in studying these parameters.

By the *in vitro* measurements the synthetic membrane alone created a barrier for the penetration of Ofloxacin. OFL was detectable in the acceptor phase as early as 1 min after instillation, when 30.47±11.30 μg/cm^2 was calculated. A gradual increase of the diffused amount could be observed within 90 min, when 142.38±14.50 μg/cm^2 Ofloxacin was diffused in the acceptor phase. Figure 20 shows the cumulative amount of the permeated OFL through the synthetic membrane (positive control), fresh AM and through cryopreserved AM (in negative controls no OFL could be detected.)

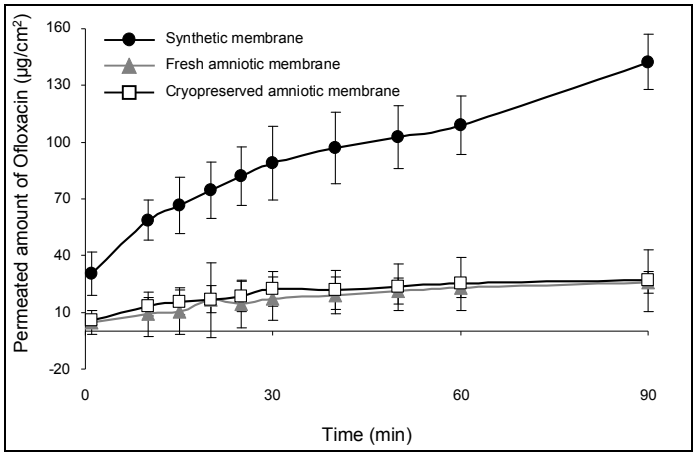


Figure 20 Transamniotic permeation of Ofloxacin from ophthalmic solution

In the case of the fresh and cryopreserved amniotic membranes, the amount of permeated OFL was lower than in the case of the artificial membrane. Compared with positive control, both membranes showed significant differences at all time points. At 30 min 17.09±11.40 μg/cm^2 of OFL permeated the freshly prepared amniotic membrane, and 22.20±9.17 μg/cm^2 permeated the cryopreserved amniotic membrane compared with 89.05±19.40 μg/cm^2 penetrant in the filter membrane alone. At 90 min the penetration rates of

OFL were 25.68±5.66, 26.82±16.39 μg/cm^2, and 142.38±14.50 μg/cm^2, respectively. The difference was not significant between freshly prepared and cryopreserved amniotic membranes at any time point. It is very advantageous, since the use of cryopreserved AM is preferred in the practice, because in this way transplantation need not be connected to another operation.

We concluded that the Franz diffusion cell system provides an applicable model also for transamniotic drug penetration studies for water-based solutions. The barrier effect of amniotic membrane on OFL penetration could be demonstrated and measured by the model. It has been shown that the amniotic membrane reduces OFL penetration and that cryopreservation does not play a significant role in the permeability of amniotic membrane.

So AM acts as a barrier to topical antibiotics, but a reservoir function is also supposed. Our other aim was the quantitative pharmacokinetic evaluation of drug release from pre-treated AM *ex vivo*. Amniotic membrane was soaked in Ofloxacin solution for different durations (Group 1: 1 h, Group 2: 2 h and Group 3: 3 h). The clinically relevant question was the determination of the minimal period, which provides the best reservoir capacity of amniotic membrane.

Figure 21 summarizes cumulative released OFL. In all groups the rapid increase of released OFL could be observed from 1 min until 120 min. From 120 min to 450 min the amount of released OFL showed a slower increasing pattern. Released OFL in Group 1 was significantly lower than in Group 2 after 90 min (19.4±10.4 μg/cm^2, 51.6±20.7 μg/cm^2 respectively). In Group 3 cumulative drug release was higher than in Group 1 at all time points. Between Groups 2 and 3 no significant difference could be demonstrated only at 1 min time point (11.2±2.4 μg/cm^2, 22.1±7.5 μg/cm^2).

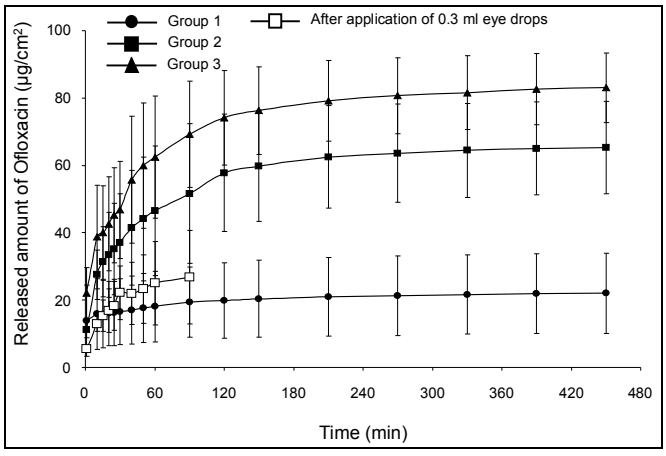


Figure 21 Ofloxacin release from amniotic membrane after soaking in Ofloxacin solution

The impact of amniotic membrane soaking time was also evaluated by analyzing the released OFL per cm^2 in one minute (Table 7).

Table 7 Ofloxacin release in the various time periods

Time points (min)	Group 1 (μg/cm^2/min)	Group 2 (μg/cm^2/min)	Group 3 (μg/cm^2/min)
1	24.5625	19.7791	39.0242
10	0.3878	3.2072	3.2673
15	0.0100	1.3668	0.4717
20	0.0726	0.7341	0.9202
25	0.1055	0.5962	0.8996
30	0.0881	0.6558	0.5554
40	0.0923	0.7871	1.5850
50	0.0947	0.4657	0.7488
60	0.0777	0.4161	0.4428
90	0.0790	0.3052	0.4003
120	0.0313	0.3620	0.2862
150	0.0276	0.1192	0.1256
210	0.0174	0.0803	0.0859
270	0.0087	0.0323	0.0444
330	0.0101	0.0247	0.0266
390	0.0086	0.0170	0.0304
450	0.0031	0.0069	0.0127

Drug release was the highest in the first 15 min of the experiment. In Group 1 drug release per minute decreased faster, than in Groups 2 and 3. A further gradual decrease of drug release was observed after 15 min, but even at the end of the observation period, some drug release could be demonstrated: 0.0031 μg/cm^2/min, 0.0069 μg/cm^2/min, 0.013 μg/cm^2/min respectively. In the acceptor phase of the control group no OFL could be detected.

Our experiments demonstrated that the reservoir capacity is dependent on the duration of soaking. It could be shown, that amniotic membrane can store more OFL in general, when soaking time is increased form 1 to 2 h. The further increase of the soaking period to 3 h did not result in a further significant increase of the released drug amount in general. If 2 and 3 h results are compared, we can assume, that the released OFL is greater than in the 1 h group, but the difference is significant from 90 min only at 2 h, but already at 10 min in the 3 h

group. Results of 1 h soaking are quite similar to our previous experiments, when 0.3 ml OFL solution was added to the untreated amniotic membrane.

It could be concluded, that significant OFL reservoir capacity of a single human amniotic layer could be demonstrated *ex vivo* in a Franz diffusion cell system. *Ex vivo* experiments showed, that amniotic membrane comprises a dual pharmacokinetic impact on topically administered OFL: a barrier and a drug reservoir function. Amniotic membrane acted as an OFL slow release device for up to 7 h *ex vivo*. Drug release was dependent on the duration of the pre-treatment of AM. 2 h of pre-treatment with OFL was found to be necessary for the complete fill up of the reservoir capacity of amniotic membrane. The clinical relevance of our findings might be that the individual pre-treatment of amniotic membrane could increase the beneficial effects of amniotic membrane transplantation especially in the early postoperative period, when usually the frequent application of eye drops is necessary [120].

6. SUMMARY

The aim of my research work was to study the transdermal drug permeation process and to overcome this biological barrier safely and reversibly and to examine the permeability and the reservoir function of the human amniotic membrane.

1. In the frame of this work various types of semisolid drug delivery vehicles were developed and prepared, such as lyotropic liquid crystal, gel-emulsion, different hydrogels and creams. They were tested first *in vivo* in order to ascertain their ***effect on skin hydration*** and the ***barrier function*** of the stratum corneum. Then thermoanalytical examination was performed in order to study their ***water binding capacity*** and to conclude the ***permanency of the moisturizing effect***.

- We established that the samples with a complex structure could deliver the differently bound water in several steps, resulting in a lasting moisturizing effect.
- Based on our results, the thermogravimetry seems to be a possible predicting method for the characterization of the hydrating effect of semisolid preparations. It could be used for screening the potential drug delivery systems cost and time effectively, reducing the number of *in vivo* tests.

A NSAID (Ibuprofen) was incorporated in the Carbopol 971 based hydrogel. In order to improve the diffusion and permeation facility through the skin, two penetration enhancer excipients were tested (Transcutol and Sucrose laurate) in this composition. Rheological investigations were carried out to get information about ***consistency*** of the formulations. Drug diffusion measurements were performed to study the API ***liberation*** from the vehicle and the ***diffusion*** through synthetic membrane. Drug ***penetration*** and ***permeation*** experiments were executed through excised human epidermis *ex vivo*.

- It could be assessed, that the API incorporation into the vehicle did not influence the viscosity of the drug delivery system notably.
- By comparing the enhancer indexes of Transcutol and Sucrose laurate it could be established that TR is an effective diffusion increaser for IBU through a synthetic membrane, but it could not enhance its skin penetration.
- On the basis of our investigations, SE is a more effective enhancer for IBU than TR. SE has promoted the skin penetration of IBU relevantly.

- From our study it seems that it is not enough to make *in vitro* membrane diffusion measurements by testing newly developed transdermal preparations. It is also indispensable to examine permeation through the skin to know not only the diffused amount of API, but also to study the drug penetration into the SC, its interaction with the skin or its incidental accumulation and to verify the acting mechanism of penetration enhancers.

FTIR analysis was performed in order to confirm the ***acting mechanisms*** of the Transcutol and the sucrose ester.

- This measurement combined with the tape stripping technique seems to be a powerful non-invasive method for studying the SC hydration, detecting the structure alteration of the lipid bilayer, examining the changes in the protein conformation, monitoring the API penetration and verifying the acting mechanism of penetration enhancers.
- It could be ascertained, that the TR causes Ibuprofen accumulation in the SC, hereby ensuring a sustained effect.
- It has been proven that SE acts as an effective and non-irritating hydration and penetration enhancer for IBU through the skin.

2. The ***permeability*** of the human amniotic membrane to Ofloxacin eye drops was also studied in my research work using the Franz diffusion cell. As a summary of the experimental results it can be concluded that:

- The Franz diffusion cell system provides an applicable model also for transamniotic drug permeation studies.
- Our experiments showed that amniotic membrane comprises a dual pharmacokinetic impact on topically administered API: a barrier and a drug reservoir function as well.
- The clinical relevance of our findings might be that amniotic membrane can act as an Ofloxacin slow release device and can increase the beneficial effects of amniotic membrane transplantation especially in the early postoperative period, when usually the frequent application of eye drops is necessary.

Modelling, understanding and characterizing the penetration and permeation process of drugs through various biological membrane barriers is essential in order to predict the *in vivo* behaviour of an API or the success of a penetration-enhancing method.

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ANNEX

I.

Permeability of Human Amniotic Membrane to Ofloxacin In Vitro

Miklós D. Resch,[1] *Béla E. Resch,*[2] *Eszter Csizmazia,*[3] *László Imre,*[1] *János Németh,*[1] *Piroska Révész,*[3] *and Erzsébet Csányi*[3]

Purpose. The aim of this study was to develop a model to investigate the permeability of the amniotic membrane (AM) to ofloxacin eye drops, a widely used topical antibiotic in ocular surface disease after AM transplantation.

Methods. AM pieces on cellulose acetate filter membranes were mounted in a vertical Franz-diffusion cell system equipped with an autosampler. In vitro release of 300 mg of 3% commercially available ofloxacin ophthalmic solution was determined by quantitative absorbance measurement carried out with a UV spectrophotometer (wavelength, 287 nm). Freshly prepared and cryopreserved AMs were compared. Filter membranes without AM served as positive controls.

Results. Ofloxacin was detectable in the acceptor phase 1 minute after instillation, and a gradual increase of concentration could be detected in a period of 90 minutes in all groups. At 30 minutes 3.35% ± 2.23% of ofloxacin penetrated the freshly prepared AM, 4.35% ± 1.8% through cryopreserved AM compared with 17.52% ± 3.91% filter membrane alone. At 90 minutes, penetration rates of ofloxacin were 5.04% ± 1.11%, 5.26% ± 3.21%, and 27.91% ± 3.05%, respectively. Difference ($P > 0.05$; *t*-test) was not significant between freshly prepared and cryopreserved AMs. Compared with control, both membranes showed significant differences ($P < 0.05$; *t*-test) at all time points.

Conclusions. The in vitro model of the Franz-diffusion cell system was found to be applicable for drug permeability studies of human amniotic membranes to water-based solutions. The filter membrane and AM were permeable to a water-based solution of ofloxacin. Significant barrier function of the AM could be measured in ofloxacin permeability. Cryopreservation did not influence the permeability of the AM. (*Invest Ophthalmol Vis Sci.* 2010;51:1024–1027) DOI:10.1167/iovs.09-4254

Amniotic membrane (AM) is the innermost avascular layer of the placenta consisting of an epithelium, a basement membrane, and a stromal layer.[1] AM transplantation has become frequently used in ocular surface surgery and has been found to be beneficial in a number of ocular surface diseases including persisting epithelial defects, perforating or nonperforating corneal ulcers,[2–4] alkali burns,[5] pterygium,[6] and band keratopathy[7] and after excimer laser keratectomy.[8]

In all cases topical antibiotic and anti-inflammatory treatment is essential after amniotic membrane transplantation. Ocular penetration of topically administered medicaments are known; for example, the concentration of ofloxacin was measured in corneal tissues[9] and aqueous humor.[10–12]

Amniotic membrane, especially in cases of multilayer transplantation, creates a barrier for topically administered drugs to reach the corneal tissues. The pharmacokinetic impact of amniotic membrane, however, has not exactly been explored yet. The aim of our study was to develop a model to investigate the permeability of amniotic membrane with eye drops already routinely used in clinical ophthalmologic practice or under development. To test the pharmacokinetic capability of our model, our objective was to examine the transamniotic pharmacokinetics of ofloxacin, a frequently used broad-spectrum topical antibiotic in ocular surface disease.[13]

Methods

Amniotic Membrane Preparation

The research was approved by the Institutional Human Experimentation Committee and adhered to the tenets of the Declaration of Helsinki. Amniotic membrane obtained by elective cesarean section was separated from the chorion as soon as possible, 1 hour after delivery at the latest, by blunt dissection and was rinsed with PBS (pH 7.24). Amniotic membrane pieces of 25 mm in diameter (with the epithelial side up) were placed on cellulose acetate membrane filters of the same size (Porafil; Macherey-Nagel GmbH & Co. KG, Düren, Germany) with pore diameters of 0.45 μm. Two groups were created according to the preservation technique of the amniotic membrane, as follows: with fresh amniotic membranes (no preservative), amniotic membranes with membrane filters were used within 6 hours of preparation; with cryopreserved amniotic membranes, AM pieces on filter membranes were frozen in PBS (pH 7.24) at −20°C, and neither antibiotic nor preservative was added to the medium.

In Vitro Drug Permeability Studies

In vitro permeability studies were performed with a vertical Franz-diffusion cell (Fig. 1) system (Microette Topical and Transdermal Diffusion Cell System; Hanson Research, Chatsworth, CA) containing six cells.[14–16] In both groups AM on membrane filters were mounted to Franz diffusion cells. The donor phase contained 300 mg of 3% ofloxacin eye drops (Floxal; Dr. Mann-Pharma, Berlin, Germany), which was placed on the amniotic membrane. The effective diffusion surface was 1.767 cm^2. PBS was used as an acceptor phase. Rotation of the stir-bar was set to 450 rpm. Experiments were performed at 37°C ± 0.5°C water bath. Position and condition of AM was continuously checked.

Samples of 0.8 mL were taken from the acceptor phase by the autosampler (Microette Autosampling System; Hanson Research) after 1, 10, 15, 20, 25, 30, 40, 50, 60, and 90 minutes and were replaced

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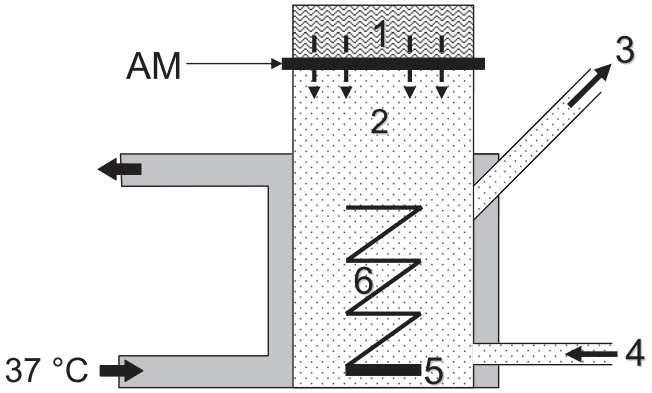

FIGURE 1. Schematic drawing of amniotic membrane mounted in the Franz cell. The donor compartment (1) above contains ofloxacin. The compartment below is the acceptor phase (2), from which samples are taken through the sampling port (3), to the acceptor phase replacing port (4). The acceptor compartment is surrounded with a water jacket kept at 37°C. At the bottom of the acceptor phase, a stir-bar (5) and a helix mixer (6) are rotated magnetically.

with fresh receiving medium. From each group, 10 Franz cells were set.

In vitro release of samples containing 300 mg of 3% ofloxacin eye drops was determined by quantitative absorbance measurement carried out with a UV spectrophotometer (Thermospectronic UV spectrophotometer, v 4.55; Unicam Helios, Cambridge, UK) at a wavelength of $\lambda = 287$ nm. Before quantitative ofloxacin UV-spectrophotometry calibration was performed, ofloxacin solution was prepared using PBS buffer solution (pH 7.24). This solution was scanned over a range of 200 nm to 500 nm in the spectrum mode. On the absorption diagram (Fig. 2), the highest peak from spectra at wavelength 287 nm was selected for the measurements of ofloxacin. For the quantitative measurements of ofloxacin, different concentrations in the range of 1.0 to 16.0 μg/mL solutions were prepared with PBS buffer solution. The UV- spectrophotometric calibration curve was constructed by plotting the absorbance values at 287 nm versus concentration of the solution. The calibration curve was found to be linear with the correlation coefficient (r) 0.9999; the regression equation was $y = 0.07978x$.

Statistical Analysis

Drug permeabilities of freshly prepared and cryopreserved amniotic membranes were compared with each other and with controls. Filter membranes without amniotic membrane served as positive controls. Negative control meant adding 300 mg PBS without ofloxacin in the donor compartment. Independent sample t-tests were performed applying spreadsheet software (Excel; Microsoft, Redmond, WA). Differences were regarded as significant, with $P \leq 0.05$.

RESULTS

Model

Vertical Franz-diffusion cells provided sufficient fixation of the amniotic membranes; significant displacement of the amniotic membrane was observed in two cases, and no filter membrane decentration was found (these cases were excluded). Amniotic membranes were intact at the beginning and at the end of the experiment.

Drug Release

Filter membrane alone created a barrier for the penetration of ofloxacin. Ofloxacin was detectable in the acceptor phase as early as 1 minute after instillation, when 5.98% ± 2.23% of the original concentration was measured. A gradual increase of concentration could be observed within 90 minutes, when 27.91% ± 3.05% ofloxacin concentration could be detected in the acceptor phase. Table 1 summarizes the cumulative amount of the penetrated ofloxacin, and Figure 3 depicts the percentages (mean ± SD) of penetrated ofloxacin in the acceptor phase in groups 1 and 2 and in positive controls (in negative controls, no ofloxacin could be detected.)

In fresh and cryopreserved amniotic membranes, the percentages of penetrated ofloxacin were lower than in positive control. Compared with control, both membranes showed significant differences ($P < 0.05$, t-test; Table 1) at all time points. At 30 minutes, 3.35% ± 2.23% of ofloxacin penetrated freshly prepared amniotic membrane, and 4.35% ± 1.8% penetrated cryopreserved amniotic membrane compared with 17.52% ± 3.91% penetrance in the filter membrane alone. At 90 minutes, the penetration rates of ofloxacin were 5.04% ± 1.11%, 5.26% ± 3.21%, and 27.91% ± 3.05%, respectively. The difference ($P > 0.05$, t-test) was not significant between freshly prepared and cryopreserved amniotic membranes at any time point.

DISCUSSION

Ofloxacin is one of the most commonly used commercial preparations of topical fluoroquinolones.[13] In ocular surface disease, topical broad-spectrum antibiotic administration is essential, and the use of amniotic membrane with appropriate topical antibiotic treatment may induce faster wound healing and less corneal scarring.[2] Transcorneal penetration of ofloxacin was investigated in healthy and pathologic corneas as well.

According to the in vivo examinations of Beck et al.,[17] who examined healthy corneal permeability, ofloxacin achieved in aqueous humor the minimum inhibitory concentration (MIC_{90}) values of the frequently occurring Gram-positive and Gram-negative bacteria. Beck et al.[17] examined aqueous samples of 224 patients with healthy corneas undergoing cataract surgery and found good transcorneal penetration after multiple modes of application. Their results were confirmed by Cekic et al.,[18] who also examined the penetration of fluoroquinolones through healthy corneas. It has been shown that the route of ofloxacin administration can influence aqueous concentra-

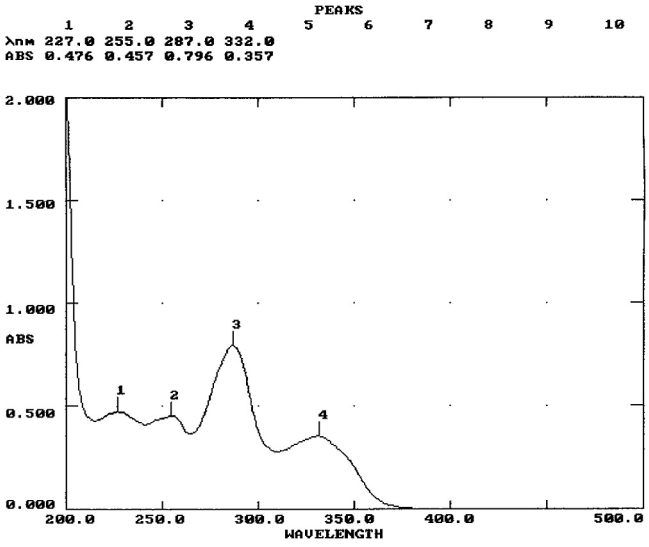

FIGURE 2. Absorption diagram of ofloxacin. The highest peak from spectra at a wavelength 287 nm was selected for quantitative measurement of ofloxacin concentration.

TABLE 1. Summary of Penetrated Cumulative Amounts (μg) of Ofloxacin via 1 cm^2 Amniotic Membrane

Time after Administration (min)	Control (filter)		Fresh AM		Cryopreserved AM		*P* (fresh AM filter)	*P* (cryopreserved AM filter)	*P* (fresh cryopreserved AM)
	Mean	SD	Mean	SD	Mean	SD			
1	30.5	11.3	4.8	6.4	5.6	2.2	0.0105	0.0331	0.9953
10	58.7	10.8	9.0	11.8	13.0	4.7	0.0051	0.0000	0.5795
15	66.5	14.7	10.6	12.4	15.4	6.5	0.0081	0.0001	0.5609
20	74.7	14.8	16.7	19.8	16.9	7.2	0.0305	0.0003	0.9319
25	81.9	15.4	14.5	12.4	18.3	8.0	0.0153	0.0006	0.7005
30	89.0	19.4	17.1	11.4	22.2	9.2	0.0188	0.0014	0.6680
40	97.0	19.0	18.8	9.8	22.0	10.2	0.0267	0.0034	0.8347
50	102.8	16.6	21.1	6.9	23.3	12.3	0.0488	0.0169	0.9486
60	109.0	15.4	22.8	5.1	25.0	13.9	0.0451	0.0429	0.8860
90	142.4	14.5	25.7	5.7	26.8	16.4	0.0311	0.0245	0.7425

Independent sample *t*-test results demonstrate significant differences, where $P < 0.05$.

tions.[19] Several modes of application were compared. Some patients received eye drops three times at 2-hour intervals on the day before surgery and three drops at 1-hour intervals on the day of surgery. Other patients received nine drops at 15-minute intervals on the day of surgery only. In all application modes, ofloxacin was detectable in the anterior chamber.

Besides normal corneas, abnormal corneas were also evaluated in a multicenter randomized study by Healy et al.,[10] when 0.3% ofloxacin ophthalmic solution was administered twice (15 and 10 minutes) before penetrating keratoplasty. In corneal stromal tissues and aqueous humor samples, ofloxacin could be detected by high-performance liquid chromatography (HPLC). Transcorneal penetration was examined in corneas with different noninflammatory abnormalities. It was demonstrated that ofloxacin penetration also offers a sufficient concentration in the anterior chamber in healthy and pathologic corneas as well.

Ofloxacin quantitative concentration analysis can be performed by UV-spectrophotometry and HPLC. Both methods were found equally accurate in the case of ofloxacin.[20] There was no significant difference between the two techniques. UV-spectrophotometry is also an accepted method, specifically in quantitative ofloxacin measurements by Srividya et al.[21] and Fegade et al.[22]

UV-spectrophotometry is a less expensive technique than HPLC and is proven to be same exact.[23] Other authors have found slightly different values of absorption maximum: Srividya et al.[21] at 290 nm, Fegade et al.[22] at 300 nm, and Chavanpatil et al.[24] at 291 nm. The absorption curves can change with the pH and with the instrument used. By automated sampling, continuous measurements could be performed. Franz cells seem to be an applicable model for the examination of amniotic membrane permeability.

Amniotic membrane permeability was originally examined by Kovács et al.[25] to investigate fetomaternal transport. Later, when the amniotic membrane was introduced in ocular surface surgery, the impact of amniotic membrane on membrane transport gained a new perspective of interest. Immunohistochemical and electron microscopic examination of corneas after amniotic membrane transplantation surgery demonstrated that amniotic tissues can integrate the corneal tissues.[26,27] It can be supposed that intracorneal amniotic membrane integration can affect the transcorneal pharmacokinetics of topical drugs.

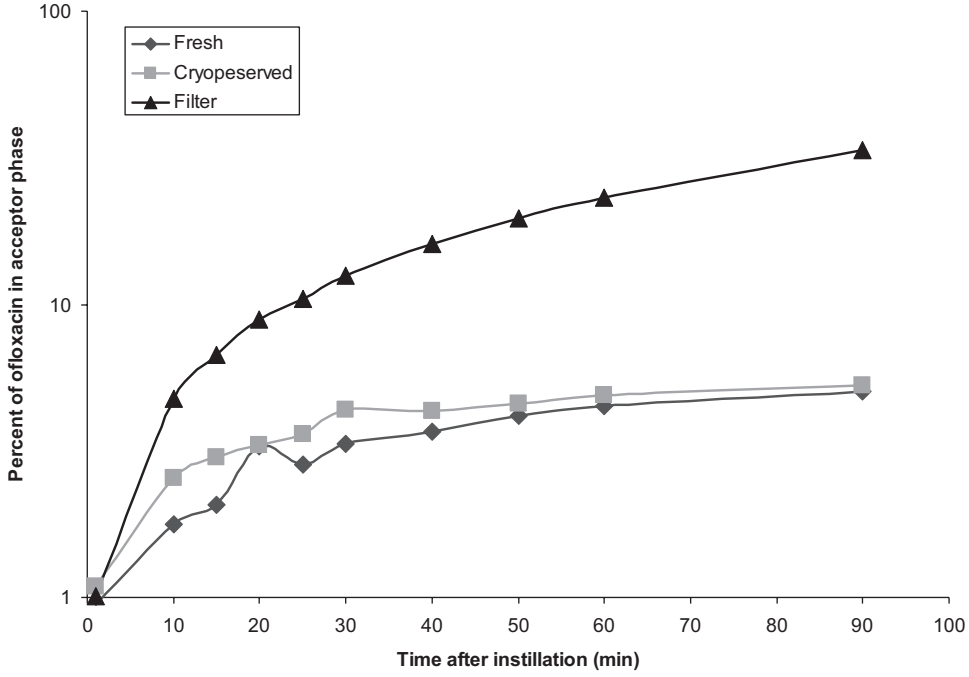

FIGURE 3. Diagram showing the time course of ofloxacin penetrated through amniotic membrane into the acceptor phase when 3% topical ofloxacin eye drops were used. One hundred percent indicates that the total amount was filled into the donor phase of the Franz cell.

Kim et al.[28] evaluated the effect of amniotic membrane on the concentration of ofloxacin in the cornea, aqueous humor, and tears in vivo on the rabbit cornea. They concluded that amniotic membrane transplantation interferes with the ocular penetration of topical ofloxacin in normal rabbit corneas but enhances ofloxacin penetration in corneas with epithelial defects after the administration of ofloxacin four times every 15 minutes. Diamond et al.[29] reported, after four drops of ofloxacin (and three other types of fluoroquinolones) at 2-minute intervals in 12 patients undergoing corneal transplantation, that the corneal concentration of ofloxacin from resected cornea was significantly higher than that of ciprofloxacin and norfloxacin.

In vivo several factors can have impact on transamniotic drug penetration. O'Brien et al.[30] reported that inflammation corneal deepithelialization enhances the ocular penetration of topical antibiotics. Cryopreservation may also affect the amniotic epithelium structure and thus drug permeability. We found, however, that cryopreservation does not have any impact on the permeability of amniotic membranes in vitro.

Healy et al.[10] and Robert and Adenis[31] reported that transcorneal penetration of most drugs, including the fluoroquinolones, occurs primarily by passive diffusion and is correlated in a positive manner with the drug's aqueous solubility and degree of lipophilicity. The thickness variability of fresh or cryopreserved amniotic membranes may explain the SD values in Table 1.

We conclude that the Franz-diffusion cell system provides an applicable model for transamniotic drug release studies for water-based solutions. The barrier effect of amniotic membrane on ofloxacin penetration could be demonstrated and measured by the model. It has been shown that the amniotic membrane reduces ofloxacin penetration and that cryopreservation does not play a significant role in the permeability of amniotic membrane.

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II.

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Thermoanalytical method for predicting the hydration effect permanency of dermal semisolid preparations

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Abstract Our aim was to develop potential dermal drug delivery systems (DDSs) with a good and lasting moisturizing effect. Lyotropic liquid crystals (LLCs), gel-emulsions and hydrogels were investigated by means of thermogravimetry, which can give information about the structure of these preparations, and we could study the water binding mechanisms indirectly in them. We found that the preparations with a complex structure and strong water bonds hydrate the skin well and lastingly by in vivo tests. Since the thermoanalytical results correlate with the in vivo test results, this method could be suited for predicting the moisturizing effect of the vehicles and provide the possibility to select the potential semisolid DDSs for in vivo tests cost and time effectively.

Keywords Gel-emulsion · Lyotropic liquid crystal · Skin hydration · Thermogravimetry · Water bond

Introduction

Thermoanalytical examinations have an increasingly greater role in the structural investigations of different pharmaceutical substances and dosage forms, especially in the case of solid dosage forms, but they have also been used successfully in the examination of semisolid systems [1–4].

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Thermogravimetry allows verifying the microstructure of various semisolid preparations [lyotropic liquid crystals (LLCs), gel-emulsions, hydrogels] through studying indirectly the water binding mechanisms in them [5]. This property is very informative in predicting at what rate they can release their water content [6]. By this analytical method, different forms of incorporated water (free, bulk, bound and interlamellar) could be detected, which were delivered from the preparations in different times, ensuring longer hydration on the skin. The knowledge of the proportion of various types of water is also important to get information about the structure, which has a strong effect on drug release from pharmaceutical formulations [7, 8].

Our study had multiple aims: (1) to develop semisolid dermal drug delivery systems (DDSs) with a good moisturizing effect which do not increase the transepidermal water loss (TEWL); (2) to examine the structure of our new developed preparations and their water binding mechanisms by thermogravimetric measurements; (3) to perform in vivo hydration and TEWL tests; (4) to find a connection between the moisturizing effect of the developed preparations and their structure. The studied samples were LLCs [9], gel-emulsions [10] and hydrogels.

Experimental

Materials

Lyotropic liquid crystals (LLCs) contained polyethoxylated 40 hydrogenated castor oil (BASF, Cremophor RH40 official in USP/NF) as emulsifier (Table 1). The water phase of the systems was purified water (Ph.Eur.6.), the oil phase was isopropyl miristate (Ph.Eur.6.). In our experiment we developed lamellar LLCs because their structure

Table 1 Compositions of the studied preparations

Components/%	LLC1	LLC2	Oil-free PTR1 gel	PTR1 gel-emulsion	PA gel	PA oil dispersion
Purified water	30	10	99.7	69.7	95	85.5
Isopropyl miristate	10	60				
Miglyol 812			0	30	0	10
Cremophor RH40	60	30				
PTR1			0.2	0.2		
Trolamine			0.1	0.1		
PA					5	4.5

demonstrates the greatest similarity to the lipid bilayer of the stratum corneum (SC). LLCs are expected to be ideal dermal vehicles with the ability to integrate into the structure of the SC and restore it [11].

Gel-emulsions were prepared with the use of the polymeric emulsifier Pemulen™ TR1 (PTR1) (Noveon, USA), which served as an emulsifier and a gel-forming agent, too. This is a cross-linked block copolymer of poly(acrylic acid) and hydrophobic long-chain methacrylates. It is able to stabilize o/w emulsions because its short lipophilic part integrates into the oil droplets whilst its long hydrophilic part forms a micro-gel around the droplets arresting their fusion. The aqueous phase was purified water, the oil phase was neutral oil (Ph.Eur.6., Miglyol 812). The neutralizing agent was trolamine (Ph.Eur.6.).

For the formulation of hydrogels 1,2-propandiol-alginate (PA) and purified water were used. In order to ascertain whether oil as an occlusive ingredient has an influence on skin hydration, we investigated LLCs containing 10% and 60% of oil, we formulated an oil-free and an oil-containing system with PTR1, and as PA is ready to incorporate a small amount of oil without added surfactant, PA oil dispersion was also prepared.

Methods

In vivo skin tests

Fifteen healthy subjects (11 females and 4 males) of ages between 22 and 51 years without any known dermatological diseases or allergy participated in the experiment. Informed consent was obtained from all volunteers and the study was approved by the local ethics committee. During the test period, the subjects were not allowed to use any other skin care products on their hands. To prove the standard circumstances, all the skin tests were performed after the adaptation of the volunteers to room conditions (30 min at 23–25 °C and 40–50% RH). During the experiment, the samples were applied on the dorsal hand of all the subjects. Electrical capacitance, indicating the hydration level of the SC, was determined by Corneometer® CM 825. TEWL was evaluated as an indicator of skin barrier integrity using Tewameter® TM 300 (all devices by Courage and Khazaka Electronic GmbH, Cologne, Germany) [12–14]. The electrical capacitance of the SC and the TEWL value were determined before and after the sample application. The measured values were compared with the values detected on the non-treated skin, the changes in moisturizing level and in TEWL were expressed in percentage [15, 16].

Thermoanalytical measurements

The thermogravimetric analysis was carried out using a MOM Derivatograph-C (MOM GmbH, Hungary) instrument. Samples were weighed (40–50 mg) in platinum pans (No.4). The reference was an aluminium oxide containing pan. Two types of measurements were performed. At a slow heating rate the samples were heated from 25 to 120 °C at 1 °C min^{-1}, at a fast heating rate the systems were heated from 25 to 200 °C at 10 °C min^{-1}. TG (mass loss % versus temperature) and derivative TG (DTG) curves were plotted. Each study was repeated three times.

Results and discussion

During the investigation with derivatograph, the samples lost water due to heating, the TG curves show this weight loss. In the case of the fast heating rate they could be divided by the DTG curves into sections, which indicate different types of water spaces. More peaks could be distinguished in the DTG curves of the preparations with a complex structure (LLCs, PTR1 gel-emulsion, PA emulsion) (Fig. 1), where the water is supposed to be bound through various binding mechanisms. One peak may correspond to free water at about 100 °C, which is able to diffuse to the surface at a faster rate than the evaporation process itself, hence the process represents free surface evaporation. The other peak at about 140 °C may correspond to the residual water being strongly bound within the system [7]. Besides the free water, the LLCs also contain

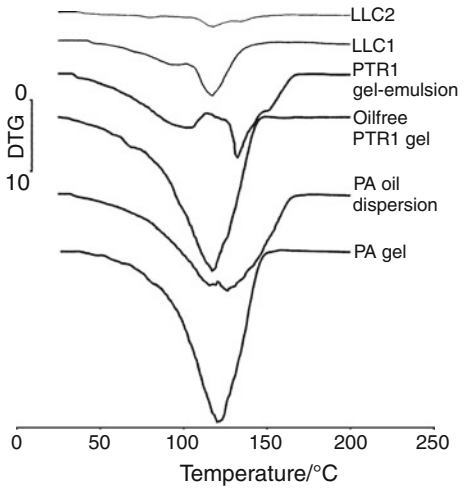

Fig. 1 The DTG curves of the investigated preparations (water distribution)

interlamellar water incorporated between the hydrophobic layers. The PTR1 gel-emulsion has a special structure, a part of the water is in a very strong bond around the oil droplet (micro-gel water), which is indicated by the second DTG peak, whilst in the PTR1 and PA gel the entire amount of water is bound but not so strongly as in the PTR1 gel-emulsion and PA oil dispersion, which is shown by the only DTG peak at 120 °C.

From the measured weight loss it was calculated how many percents of water evaporated from the preparations in the specified temperature ranges (Fig. 2a). It could be established clearly that the samples with more differentiated curves deliver the differently bound water in several steps, resulting in a lasting moisturizing effect.

It could be observed that the oil-containing PTR1 gel-emulsion, PA oil dispersion and the higher oil-containing LLCs hold approximately 5% water even over 150 °C. They have a significantly better water binding capacity than the oil-free samples. The reason for it could be that the oil arrests the evaporation. The occlusive effect of the oil was proved by corneometric measurements on the human skin, too, as the oil-containing PTR1 gel-emulsion and the PA oil dispersion showed a better moisturizing effect than the oil-free samples (Fig. 2b).

In order to examine the whole water content of the systems, a slow heating rate was used. A tangent was fitted to the TG curve in the peak of the DTG curve. From the slope of this tangent the maximum evaporation speed (MES) (mg/min) could be calculated. The steeper the curve is the faster the moisture evaporates from the preparation.

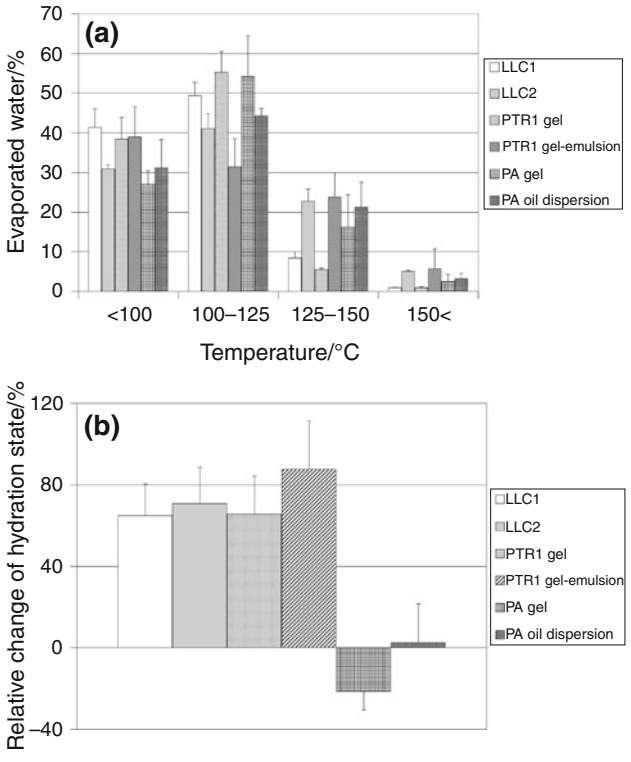

Fig. 2 **a** Percentage weight loss values over specified temperature ranges. **b** Mean values of the changes in the skin hydration of the volunteers

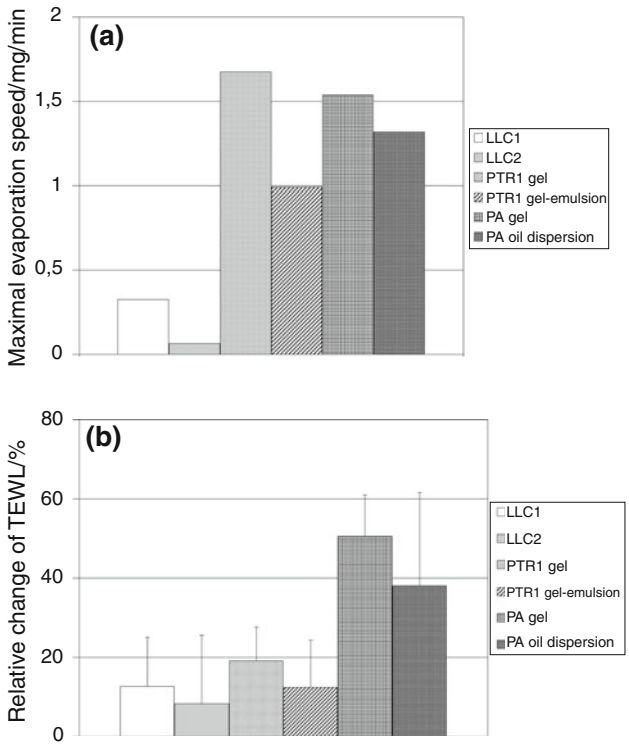

Fig. 3 **a** The MES of the investigated samples. **b** Mean values of the changes in the TEWL

The lowest MES shows the LLCs (Fig. 3a) and the least TEWL was also found in the case of the LLCs (Fig. 3b). The reason for it could be, on one hand, their lamellar structure with strong water bonds. Namely, they contain a major proportion of incorporated water concentrated in the layers between the hydrophilic domains. On the other hand, their lamellar structure is very similar to the human SC lipid bilayer, thus they are able to integrate into the structure of the skin and to restore its barrier function, arresting the TEWL [17].

A remarkable difference was found between the oil-containing and oil-free samples with respect to MES and TEWL changes. This could be explained with the more complex structure of the oil-containing PTR1 gel-emulsion and PA oil dispersion, which is made possible by the presence of water bound with different strengths in these preparations.

Conclusions

The ideal semisolid dermal drug delivery system ensures a good, lasting moisturizing effect and retards the evaporative water loss. It can be concluded from the results of our investigations that the oil-containing systems with a complex structure (LLC1, LLC2, PTR1 gel-emulsion) satisfy these requirements the most. The reason for their excellent moisturizing effect can be, on the one hand, that oil as an occlusive ingredient forms a protecting film on the skin surface, thereby arresting water loss. On the other hand, thanks to their complex structure (lamellar, micro-gel structure), they have a strong water binding capacity and they can deliver the differently bound water in several steps. These observations were also supported by thermogravimetric measurements as well as by corneometric and TEWL measurements on healthy volunteers. The results of the in vivo moisturizing tests are in accordance with the results of the thermoanalytical measurements. Thus, thermogravimetry can be used for characterizing the microstructure and water binding capacity of semisolid dermal vehicles, from which the rate of water release and the permanency of the skin hydrating effect can be concluded. Therefore, thermogravimetry seems to be a possible predicting method for the characterization of the hydrating effect of different semisolid preparations. It could be used for screening the potential DDSs cost and time effectively, reducing the number of in vivo tests.

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SHORT REPORT

Ibuprofen penetration enhance by sucrose ester examined by ATR-FTIR in vivo

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Abstract
The aim of this work was to investigate the skin penetration enhancer effect of a sucrose ester (SE) in an Ibuprofen (IBU) containing hydrogel and to examine its influence on the special lipid bilayer of the stratum corneum (SC). ATR-FTIR spectroscopic measurements were performed combined with tape stripping method on hairless mice in vivo. A SE containing gel was compared to another gel without SE. It was found that the preparations caused only minimal modifications in the lipid and the protein structure, promoting the skin hydration and therefore also the penetration of IBU. Although the degree of moisturization and penetration were more intense in the case of the SE containing gel treatment, it did not cause greater alterations in the SC structure than the gel without SE. It has been proven that SE acts as an effective and non-irritating hydration and penetration enhancer for IBU through skin.

Keywords: FTIR, Ibuprofen, penetration enhance, stratum corneum, sucrose ester, tape stripping

Introduction

The principal function of the stratum corneum (SC) is to act as a barrier to the loss of tissue water due to its special lipid-protein biphasic structure. However, this excellent diffusional resistance of the SC makes the transdermal delivery of active pharmaceutical ingredients (API) difficult or frequently impossible.[1] Understanding the diffusion process across the skin is very important for the development of transdermal drug delivery.

Ibuprofen (IBU) is a non-steroidal anti-inflammatory drug widely used in the treatment of musculoskeletal injuries, but there is a great interest to develop the topical dosage forms of IBU to avoid its gastrointestinal side effects.[2,3]

The Fourier-Transform Infrared (FTIR) spectroscopic measurement by Attenuated Total Reflection (ATR) method is a powerful tool for studying the biophysical structure of the SC at the molecular level, characterizing its lipid, protein and water content[4–6] and for examining the penetration pathway, the biochemical modifications induced by the penetration and the influence of various penetration enhancers on the SC barrier function.[7–10] The big advantage is that this technique enables the elucidation of the extent and mechanism of percutaneous penetration enhancement in vivo.[11] However, the information obtained pertains only to the immediate layer in contact with the ATR crystal. Information from the deeper regions of the SC can only be obtained through sequential tape stripping.[12–15]

The aim of the present study was: (1) To evaluate the effect of a sucrose ester (SE), which is a member of the new generation of penetration enhancers, on the skin structure, and (2) to examine its influence on the penetration of IBU through SC in vivo by means of FT-IR spectroscopy combined with tape stripping method. Recently SEs have been in focus because they are biodegradable, do not remove the cutaneous fat film and would not denature the protein of the skin surface in contrast to the usual ethoxylated non-ionic surfactants, so they damage the barrier less than the other surfactants.[16–18]

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Materials and methods

Materials

5% IBU (Sigma, St Louis, MO, USA) containing gel (IBU gel) was prepared by the following procedure. IBU was dissolved in Polyethylene glycol 400 (20%) (Hungaropharma Ltd, Hungary) and this solution was added to Carbopol 971 (BF Goodrich Co., USA) hydrogel. The pH value was adjusted by adding Trolamine (7%) (Hungaropharma Ltd, Hungary). A similar composition (IBU-SE gel) was prepared by using SE (Sucrose laurate, D-1216, Mitsubishi-Kagaku Foods Co, Tokyo, Japan) containing Carbopol 971 gel.

Methods

The experiments were performed on 15-week-old male SKH-1 hairless mice (body weight: 35–41 g). The procedure and protocol applied were approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged in advance. Prior to the interventions the animals were anesthetized with a mixture of ketamine (90 mg/kg body weight) and xylazine (25 mg/kg body weight). The skin of hairless mice was treated with both of formulations. With the use of adhesive tape (D-Squames, CuDerm Corporation, Dallas, TX, USA) corneocytes were collected from the uppermost layer of their dorsal skin 30 min after the application of the preparations. Due to the possibility of surface contamination and to remove the excess of the preparation, the first tape was discarded. The stripping procedure was repeated for up to 18 strips recording an IR spectrum after each third tape strip. The spectrum of the non-treated skin was also recorded. In order to obtain a reference spectrum of the API, a KBr pellet containing 0.5 mg IBU was prepared and used. The spectra of the preparations were also recorded.

All FT-IR measurements were performed by an Avatar 330 FT-IR spectrometer (Thermo Nicolet, USA) equipped with a horizontal ATR crystal (ZnSe, 45°). Spectra were recorded between 4000 and 400 cm^{-1} at 4 cm^{-1} optical resolution, and 32 scans were co-added. The spectra of three parallel samples, gained from three different animals, were recorded and corrected with the spectrum of the tape. No ATR correction was performed.

Results and discussion

It was examined whether the SE modifies the special lipid bilayer structure of the SC and the protein conformation in the corneocytes. The amount of the IBU in the upper layers of the skin was also studied.

Figure 1 shows the spectra of the pure IBU, pure SE and the applied preparations. The band of the C-C ring vibration could be seen at 1512 cm^{-1} in the spectrum of the IBU, which is considered as the most discriminative band and was used for the identification of the IBU.[19] The preparations have peaks at 1395 and 1562 cm^{-1}, which correspond to the symmetric and asymmetric

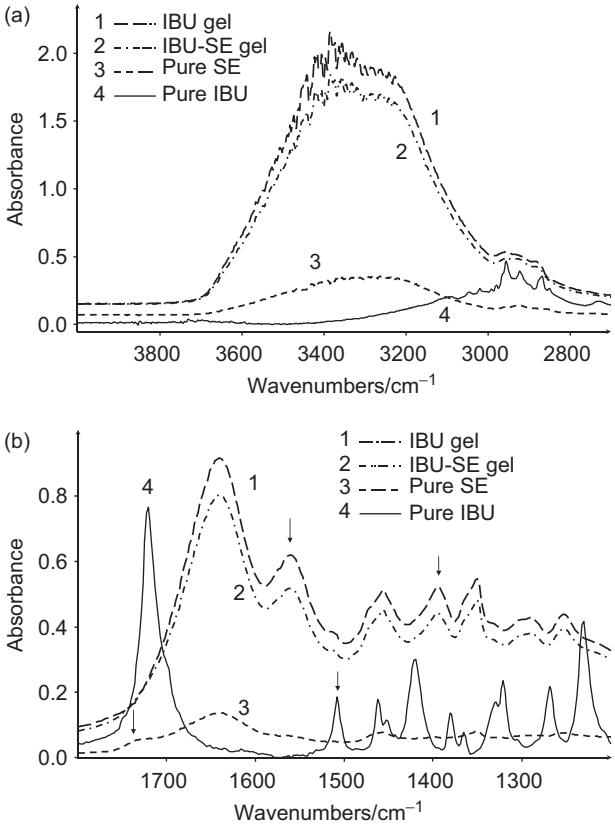

Figure 1. FT-IR spectra of the preparations and the pure IBU and SE.

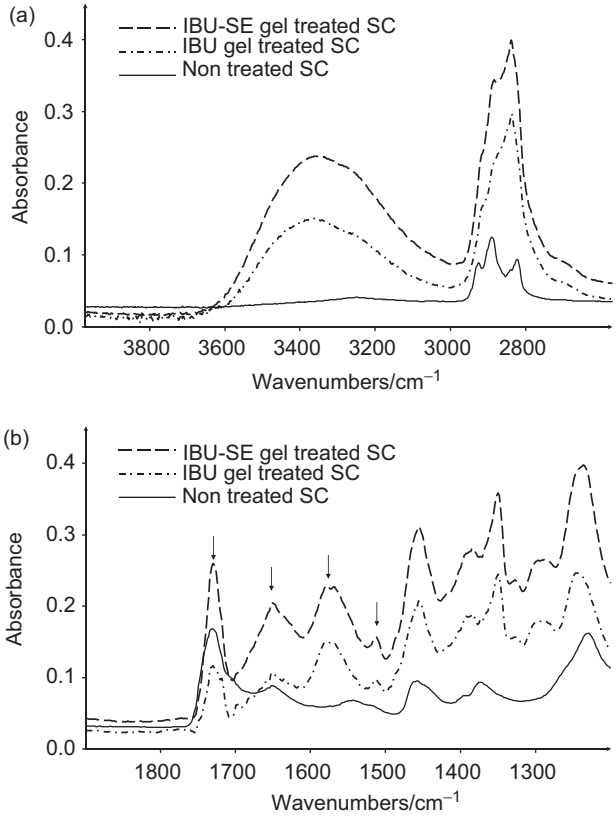

Figure 2. FT-IR spectra of the non-treated stratum corneum and treated by the IBU, and by the IBU-SE gel.

Table 1. Intensities in the different layers of the treated stratum corneum.

	Treated with IBU gel		Treated with IBU-SE gel	
	Absorbance at		Absorbance at	
The number of tape strips	3392 $cm^{-1}\nu_{O-H}$	1512 cm^{-1} IBU	3392 $cm^{-1}\nu_{O-H}$	1512 cm^{-1} IBU
Tape 3	0.1968	0.1403	0.2305	0.1495
Tape 6	0.1507	0.0970	0.2374	0.1568
Tape 9	0.1119	0.0847	0.1808	0.1351
Tape 12	0.0849	0.0706	0.1509	0.1143
Tape 15	0.0720	0.0540	0.0931	0.0812
Tape 18	0.0673	0.0699	0.0695	0.0742

carboxylate vibrations. The most characteristic bond of the surfactant assigned to the ester group can be seen at 1728 cm^{-1} in spectrum 3.

Figure 2 shows the IR spectra of the non-treated and the IBU gel and IBU-SE gel treated SC after the 6th tape stripping. The spectra feature strong absorbance peaks in the region of 3000-3500 cm^{-1} after the treatment with the preparations, which come from water, because the band of the O-H stretchings of the SE are too weak and IBU has no absorbance peak in this range (Figure 1a). So both hydrogels can hydrate the SC well, especially the IBU-SE gel.

The hydrocarbon chains of lipids give asymmetric and symmetric CH_2 stretching vibrations at 2920 and 2850 cm^{-1}, respectively. Any extraction of the lipids by enhancer results in a decrease of peak height and area. We found that the SC took up the lipophilic hydrocarbon based components of the preparations, so the absorbance peak near 2850 cm^{-1} increased significantly. Some enhancers may fluidise the SC lipids, which can be noted from the shift of CH_2 stretching peaks to higher wavenumbers (trans to gauche conformation) and an increase in peak width. Minimal shifts were observed in both cases.

The absorption bands at around 1650 cm^{-1} (amid I) and 1550 cm^{-1} (amid II) are typical protein bands which arise mainly from C=O stretching and N-H bending vibrations. These frequencies are sensitive to the conformation of proteins present in the SC. We found the amid I peak at 1670 cm^{-1}, which did not shift due to the treatment, so the preparations caused only minimal changes in the protein structure.[20] But the amid II peak range showed strong overlapping bands around 1570 cm^{-1} in the case of the treated SC, which can be assigned to the carboxilate modes of Carbopol and the IBU itself produced by the neutralization of the preparations by Trolamine.

The sign of the IBU could be detected in the treated skin at 1512 cm^{-1}. Various alterations can be seen near 1728 cm^{-1} compared to the non-treated skin. The peak is less intense in the spectrum of the IBU gel-treated sample than in the non-treated one, while a higher peak was found in the IBU-SE gel-treated case. It is caused by the strong C=O band of the SE.

Table 1 lists the relative absorbance values at 3392 cm^{-1}, which are proportional to the water content of the SC, and at 1512 cm^{-1}, which correspond to the amount of IBU penetrated to the SC layers. It was found that the upper layers of the SC were the most hydrated, and the water content decreases with the number of the tape strips. The amount of IBU also decreases in deeper layers. Furthermore, it could be assessed that the IBU-SE gel treatment achieved higher water and IBU content in each layer compared to the IBU gel treatment, in spite of the same API content in both preparations.

Conclusion

Thus it can be ascertained that both preparations caused only minimal modification in the lipid and the protein structure, promoting the skin hydration and therefore also the penetration of IBU. Although the degree of the moisturization and penetration were more intense in the case of the IBU-SE gel treatment, it did not cause greater alterations in the SC structure than the IBU gel. It has been proven that SE acts as an effective and non-irritating hydration and penetration enhancer for IBU through skin.

Declaration of interest

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