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Summary of Ph.D. thesis

Drug permeation study through biological membrane barriers

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1. INTRODUCTION

Recently the topical drug administration routes have had an increasing role. They can have an edge over the oral route in the case of numerous drugs. 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 leads to reduced side effects. In addition, they have the advantages of continuous administration, predictable and extended duration of activity, applicability of drugs with a short biological half-life and enhanced patient compliance.

Non-Steroidal Anti-Inflammatory Drugs (NSAID) are often used for the treatment of chronic musculoskeletal injuries (e.g. rheumatoid arthritis, osteoarthritis). 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.

The main problem associated with the topical drug administration is that only a small number of APIs is suitable for overcoming the biological barriers of 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. The structure of this layer was pictorially described in the “brick and mortar” model, in which the embedded horny corneocytes without cell organelle represent the “bricks” and the intercellular lipids are the “mortar”. Due to its special and strictly ordered structure and its excellent diffusional resistance, it makes the transdermal delivery of APIs difficult or frequently impossible. Verifying the transdermal diffusion process is indispensable to the development of transdermal drug delivery systems.

Optimization of drug delivery through human skin is an important and innovative research area in the modern therapy. The feature 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 take into consideration.

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. The possibilities given by the alteration of the SC, were discussed in my thesis. It can be achieved in two ways. The first one is skin hydration, which may swell and open up the compact structure of the horny layer leading to an increase in penetration. The second one is the use of chemical penetration enhancers, which compromise the skin's barrier function reversible and consequently allow the entry of otherwise poorly penetrating molecules into the deeper layers.

Recently other application routes have been used besides the transdermal route, in order to increase the bioavailability of the API. A new and interesting area is the study of the human amniotic membrane's permeability. Amniotic membrane (AM) is the innermost layer of the placenta. AM transplantation has become frequently used in ocular surface surgery and has been found to be beneficial in a number of ocular surface diseases. 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. Its transplantation provides a new possibility in the treatment of corneal diseases, ulcers. In all cases topical treatment is essential after amniotic membrane transplantation. The pharmacokinetic impact of amniotic membrane, however, has not been exactly explored in the literature yet.

2. AIMS

The aim of my Ph.D. thesis was to investigate the drug permeation through various biological membranes.

1. In the first part of my Ph.D. work, the transdermal drug permeation 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 *in vivo*,
- to perform thermoanalytical measurements in order to find a connection between the hydrating effect and the water binding mechanism 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 *in vivo*.

2. The second part of my research work 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*.

3. MATERIALS AND METHODS

3.1. Materials

Various types of semisolid vehicles were developed and investigated, such as lamellar lyotropic liquid crystals (LLCs), polymeric emulsifier (PTR1) containing hydrogel and gel-emulsion, alginate (PA) containing hydrogel and oil dispersion, Carbopol based hydrogel. They were compared with two commonly used oil/water creams (Unguentum hydrophilicum anionicum and Unguentum hydrophilicum nonionicum, Ph.Hg.VII.) containing an anionic or nonionic emulsifier generally used in everyday dermatological practice.

A NSAID, the Ibuprofen (IBU) was chosen as API. IBU was incorporated into the Carbopol 971 based hydrogel (IBU gel) and two similar compositions were prepared, also containing penetration enhancer. The first one contained Transcutol (TR, Diethylene glycol monoethylether, Gattefossé and S&D Chemicals Ltd, Hungary) (IBU-TR gel) as penetration enhancer, the second one Sucrose laurate (D-1216, Mitsubishi-Kagaku Foods Co, Japan) (IBU-SE gel). This is a sucrose ester, representing the new generation of penetration enhancers.

3% commercially available Ofloxacin (OFL) eye drops (Floxal®; Dr. Mann-Pharma, Germany), a widely used topical antibiotic in ocular surface disease were used for studying the amniotic membranes' permeability.

3.2. Methods

In vivo tests

The semisolid vehicles were tested on healthy volunteers *in vivo*. The hydration state of the skin before and after treatment was recorded by using Corneometer® CM 825 (Courage and Khazaka Electronic GmbH, Cologne, Germany). The barrier function of the skin was detected by measuring TransEpidermal Water Loss (TEWL) with Tewameter® TM 300 (Courage and Khazaka Electronic GmbH, Cologne, Germany).

Thermoanalytical investigations

The thermogravimetric analysis was carried out using a MOM Derivatograph-C (MOM GmbH, Hungary) instrument, which can give information about the water content of these preparations, and we could study the water binding mechanisms indirectly in them. 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}.

Rheological measurements

The viscosity and the flow properties of the preparations were investigated by 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. 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.

Drug diffusion and permeation investigations

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 synthetic membrane (Porafil membrane filter, cellulose acetate, pore diameter: 0.45 μm, Macherey-Nagel GmbH & Co. KG, Germany) (*in vitro*) and on heat-separated epidermis or amniotic membrane (*ex vivo*).

For the investigation of the reservoir function of the AM, pieces were soaked in 10 ml of 3% commercially available Floxal eye drops for 1 (Group 1), 2 (Group 2) and 3 (Group 3) hours.

The effective diffusion surface area was 1.767 cm^2. Phosphate buffer solution (PBS pH=7.4±0.15) thermostated at 37±0.5 °C was used as an acceptor phase to ensure sink conditions. 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.

Preparation of heat-separated epidermis

Excised human skin from patients who had undergone abdominal plastic surgery was used for the skin permeation studies. The epidermis was separated from the underlying dermis using the heat-separation technique. After excision, the subcutaneous fatty tissue was removed and individual portions were immersed in water bath at 60 °C for 90-120 s and the epidermis was gently removed from the underlying dermis using forceps.

Preparation of human amniotic membrane (AM)

Amniotic membrane (AM) is the innermost layer of the placenta, which was obtained by elective cesarean section. It was separated from the chorion as soon as possible, 1 hour after delivery at the latest, by blunt dissection and was rinsed with phosphate buffer (pH=7.4±0.15) until use or was stored cryopreserved.

ATR-FTIR spectroscopic investigations

The Fourier Transform Infrared (FTIR) spectroscopic measurement is a powerful tool for studying the structure of the SC at molecular level, characterizing its water, lipid and protein content, examining the drug penetration into the skin and verifying the acting mechanism of penetration enhancers. The sequential tape stripping technique makes it possible to get information also from the deeper regions of the SC. Samples from the SC layers can be collected with the use of adhesive tape (D-Squame® Skin sampling discs, CuDerm Corporation, Dallas, USA) and the composition of the SC can be determined on the ZnSe crystal of the equipment by infrared radiation.

All ATR-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 experiments were performed on 15-week-old male SKH-1 hairless mice, which were anesthetized. With the use of the adhesive tape, samples were collected from the uppermost layer of their dorsal skin 30 min after the application of the preparations. The stripping procedure was repeated for up to 18 strips recording an IR spectrum after each third tape strip.

4. RESULTS

4.1. Investigation of developed semisolid vehicles

In vivo skin tests and thermogravimetric prediction method for hydration effect

Based on the thermogravimetric measurements it could be established, that more peaks could be distinguished in the DTG curves of the preparations with a complex structure (LLCs, polymeric emulsifier containing gel-emulsion, PA oil dispersion, anionic and nonionic o/w creams,) where the water is supposed to be bound through various binding mechanisms. One peak may correspond to free water at about 100 °C, while the other peak at about 140 °C may correspond to the residual water being strongly bound within the system (*Figure 1*).

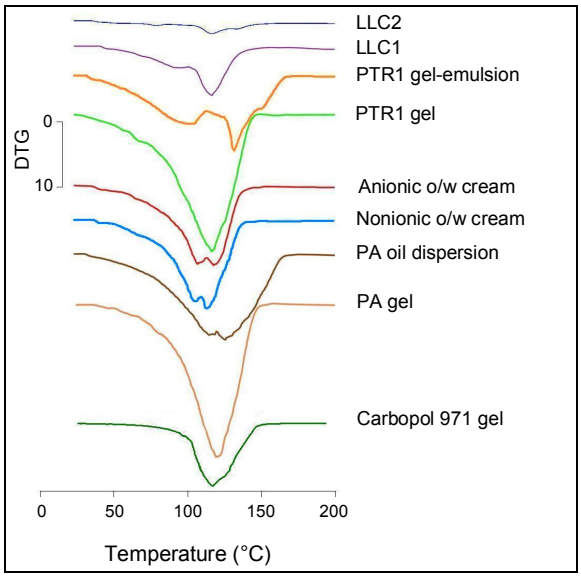
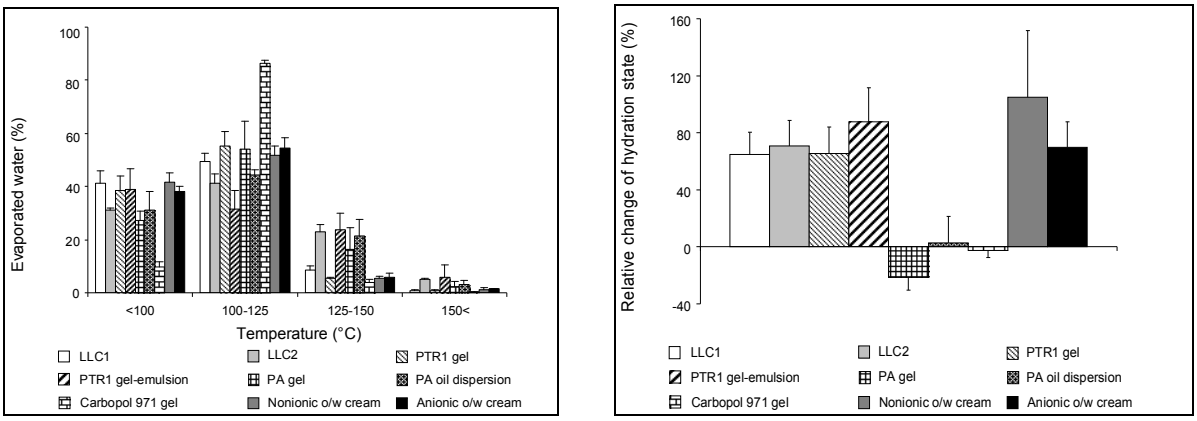


Figure 1 DTG curves of the investigated samples

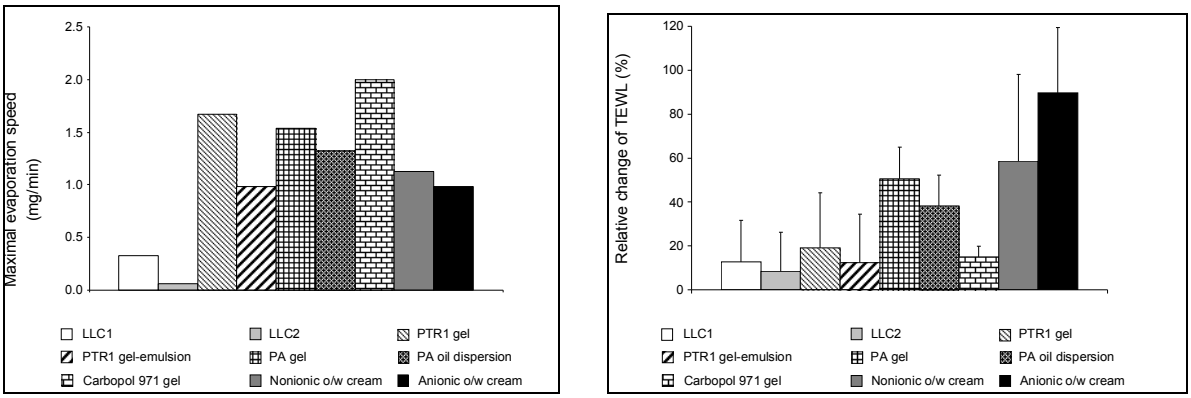
From the measured weight loss it was calculated how many percents of water evaporated from the preparations in the specified temperature ranges. It could be ascertained clearly that the samples with more differentiated curves deliver the differently bound water in several steps, resulting in a lasting moisturizing effect (*Figure 2a*). It could be observed, that the formulations with a complex structure and strong water binding capacity showed the best hydrating effect by the corneometric measurement, too (*Figure 2b*).



a) *b)*

Figure 2a) Percentage weight loss values over specified temperature ranges b) Mean values of the changes in the hydration level

The maximum evaporation speed (MES) (mg/min) could be calculated from the TG curves, which are in accordance with the results of the transepidermal waterloss measurements, too (*Figure 3*).



a) *b)*

Figure 3a) The maximum evaporation speed b) Mean values of the changes in the TEWL

Therefore, it could be stated that our thermogravimetric analysis seems to be a possible predicting method for the characterization of the hydrating effect and permanency of semisolid drug delivery systems.

4.2. Study of the Ibuprofen containing hydrogels

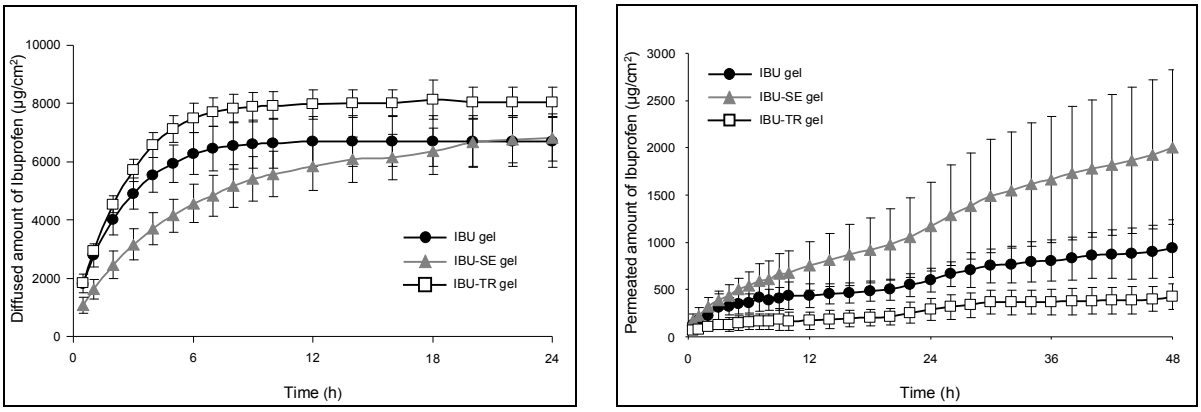
Henceforth Ibuprofen (IBU) containing Carbopol 971 hydrogels were investigated. The first one did not contain any penetration enhancer, in the second one Transcutol was used and in the third one sucrose ester was used as penetration enhancer.

Rheological characterization

Rheology is the study of how matters deform and flow under the influence of external forces. 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 relevantly.

Drug diffusion and permeation examinations

IBU diffusion from these preparations and skin penetration was examined by the vertical Franz diffusion cell system. However, the *in vitro* measurements through synthetic membrane give information only about the API liberation and diffusion. The skin penetration of the drug should be investigated *ex vivo* through human epidermis. Based on the membrane diffusion investigations Transcutol increased the diffusion of IBU efficiently. However, the SE hampered the drug diffusion process (*Figure 4a*).



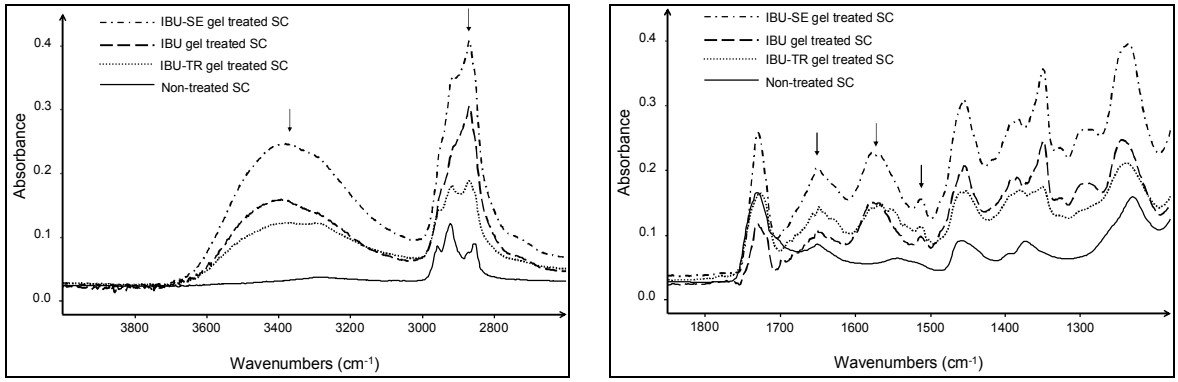
a) *b)*

Figure 4a) The diffused amount of Ibuprofen in vitro b) The permeated amount of Ibuprofen ex vivo

Different results were obtained with the *ex vivo* permeation experiments. The SE increased the API permeation through skin notably, while the Transcutol 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, while, the TR does not act by changing the skin structure, it increases the diffusion. The reason for the decreased *ex vivo* skin permeation could be the TR accumulation in the skin, which can cause API depot in the SC (*Figure 4b*).

FTIR analysis

FTIR analysis was performed in order to confirm the assumptions about the permeation process. The continual line marks the spectrum of the non-treated skin. The band of the O-H stretching could be detected in the range of 3000-3500 cm^{-1}. If the spectra feature strong absorbance peaks in this region, it refers to skin hydration. The hydrocarbon chains of lipids give asymmetric and symmetric CH_2 stretching vibrations at 2920 and 2850 cm^{-1}. The alterations in the strictly ordered structure of the SC and lipid extraction induced by the applied materials can be observed in this region. The structure modifications of the lipid bilayer caused by penetration enhancers can be followed on the spectra. Any extraction of the lipids by enhancer results in a decrease of peak height and area. Some enhancers may fluidise the SC lipids, which can be noted from the shift of CH_2 stretching peaks to higher wavenumbers and a rise in peak width points to the fact that the rotational freedom of the lipid chains increased. 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.



a) *b)*

Figure 5a-b) FTIR spectra of the non-treated SC and treated SC by the Ibuprofen containing hydrogels

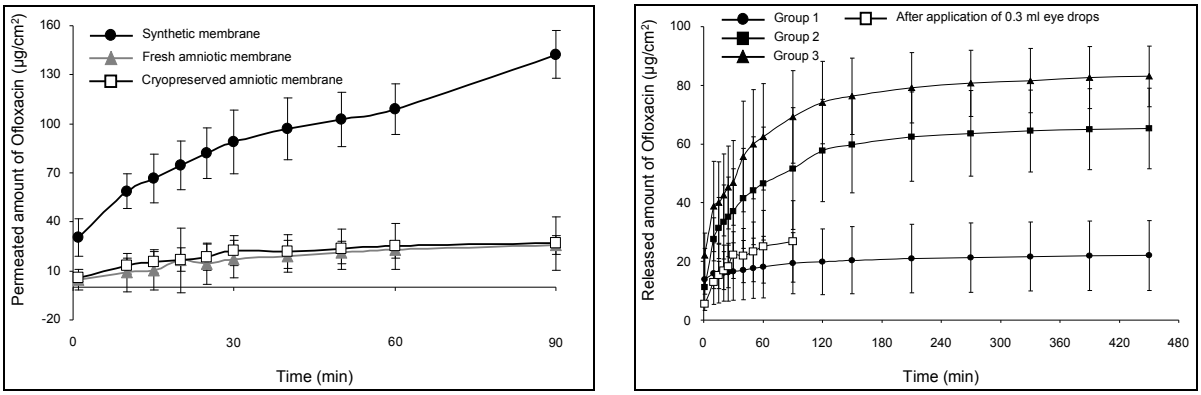
The results of the FTIR measurements showed that all the three hydrogels could increase the water content of the SC. The TR containing gel caused similar moisturization on the skin as the preparation without any penetration enhancer, but the SE containing gel had the most well-hydrating effect (*Figure 5*). We did not find any decrease in absorbance near 2850 cm^{-1}, however, we found that this absorbance peak increased in all cases, since the SC uptook the lipophilic hydrocarbon based components of the preparations. The most remarkable change was observed after the treatment with the SE containing sample. Minimal shifts were

observed in all cases. The amid I peak was found near 1650 cm^{-1}, which did not shift due to the treatment. 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 groups in the preparations. So the preparations also caused only minimal changes in the protein structure. The sign of the Ibuprofen could be successfully detected in the treated skin at 1512 cm^{-1} after all treatments. The SE could enhance the IBU penetration the most intensively. The study of the API distribution in the various SC layers revealed that SE containing 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. Extremely high absorbance was measured in the case of the IBU-TR gel application in the first layer. It can confirm our assumption about the TR and API accumulation in the SC. It has been proven that sucrose ester acts as an effective and non-irritating hydration and penetration enhancer for IBU through the skin.

4.3. The permeability of human amniotic membrane

The ocular tolerance of artificial substances such as hydrogels or therapeutic contact lenses can be problematic, however, amniotic membrane is well tolerated and absorbed or integrated by the corneal tissues. Its transplantation provides an alternative possibility in the treatment of corneal diseases and ulcers. However, its barrier capacity and pharmacokinetic properties have not been described in the literature yet. Therefore, a drug permeation study was also performed through amniotic membrane after the application of Ofloxacin containing antibiotic eye drops.

First, the measurements were executed through fresh membrane and the results were compared with the data of the drug diffusion through synthetic membrane. It was found that the AM acts as a barrier, but the API could permeate through it. In addition, the permeability was also examined through cryopreserved AM, in order to study the influence of the freezing on the membrane. Cryopreservation is practical because in this way transplantation need not be connected to another operation. The results did not show any significant difference between the permeability of the fresh and the cryopreserved ones (*Figure 6a*).



a) *b)*

Figure 6a) Ofloxacin permeation through AM b) Ofloxacin release from AM after soaking in Floxal

Reservoir function of the AM was also supposed. It would be advantageous, because a pre-treated membrane could act as a drug release system after the transplantation. Then drug release would begin faster and would be more continuous, than by dropping. Our experiments demonstrated that the reservoir capacity is dependent on the duration of soaking. It could be shown that amniotic membrane can store more Ofloxacin 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. The results after 1 hour soaking were equivalent with the results after the application of the eye drops (*Figure 6b*). The clinical relevance of our findings might be, that the individual pre-treatment of AM 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.

5. SUMMARY

The aim of my work was to investigate the drug permeation through various biological membranes. My experimental results can be summarized as follows:

- 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.
- It could be assessed, that the API incorporation into the vehicle did not influence the consistency of the drug delivery system notably.
- It could be established that TR is an effective diffusion increaser for Ibuprofen, but it could not enhance its skin penetration.
- On the basis of our investigations, sucrose ester is better enhancer for Ibuprofen than TR. SE has promoted the skin penetration of IBU relevantly.
- From our study, it seems that the *in vitro* measurements give information only about the drug release and diffusion. It is also indispensable to examine permeation through the skin 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.
- The ATR-FTIR spectroscopy 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 Transcutol causes Ibuprofen accumulation in the SC, hereby ensuring a sustained effect.
- It has been proven that sucrose ester acts as an effective and non-irritating hydration and penetration enhancer for Ibuprofen without any harmful effect on the skin.
- 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.

ARTICLES RELATED TO THE PH.D. THESIS

I. M. Resch, B. Resch, **E. Csizmazia**, L. Imre, J. Németh, P. Révész, E. Csányi: *Permeability of human amniotic membrane to ofloxacin in vitro.*
Invest. Ophth. Vis. Sci. (2010) 51, 1024-1027 *IF: 3.431*

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ABSTRACTS

I. M.D. Resch, B.E. Resch, **E. Csizmazia**, L. Imre, J. Nemeth, P. Revesz, E. Csanyi: *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 E.**: *Félszilárd készítmények stratum corneumra gyakorolt hatásának vizsgálata,* Magyar Tudomány Ünnepe, Szegedi Akadémiai Bizottság Székháza, 2009. november 10. Szeged

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VII. Berkó Sz., Erős G., **Csizmazia E.**, Csányi E.: *Hatóanyagok bőrön keresztüli penetrációjának vizsgálati lehetőségei II., In vivo egér model,* „XVI. Országos Gyógyszertechnológiai Konferencia" és „VIII. Gyógyszer az Ezredfordulón Konferencia" 2010. október 20-22. Siófok (EA-06)

IX. **Csizmazia E.**, Erős G., Berkesi O., Berkó Sz., Csányi E.: *Hatóanyagok bőrön keresztüli penetrációjának vizsgálati lehetőségei III., FT-IR alkalmazás,* „XVI. Országos Gyógyszertechnológiai Konferencia" és „VIII. Gyógyszer az Ezredfordulón Konferencia" 2010. október 20-22. Siófok (EA-07)

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