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LIMITATIONS OF PIG SKIN AS *IN VITRO* MODEL MIMICKING SKIN WITH DAMAGED BARRIER PROPERTIES

BY

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Abstract

Skin is an alternative route for the administration of drugs and has several advantages compared to the oral administration route. However, for a drug substance to act systemically after topical application it has to penetrate through the skin barrier (*stratum corneum*). Therefore it is important to investigate drug penetration through both healthy and damaged skin (reduced barrier properties).

In this study Franz diffusion cells was used to determine the extent to which chloramphenicol penetrated through intact and treated (different degree of induced damage) pig ear skin. The skin slices were treated with tape-stripping and treatment with strong alkali, heat, and burning of the skin, respectively. The results were not as expected; the cumulative penetration of chloramphenicol through the treated skin did not increased as compared to intact skin, except for the treatment with the strong alkali for five minutes. This treatment resulted in a modest enhanced penetration. However, the phospholipid vesicle-based permeation assay has been developed to mimic skin. This barrier is made on a filter support where small liposomes are fitted in the pores of the filter and large liposomes are deposited on the top.

The stability of the PVPA barrier was tested over a period of 4 week and the results indicated that the integrity of the barriers were not influenced after storage at – 75 ˚C for 21 days. In order to mimic the compromised skin, we attempted to induce leakiness of different degree to the PVPA barrier by changing the ethanol concentration in the liposome suspension. Although the results from the permeability experiments showed no significant differences between the barriers with various ethanol concentrations, it seems that the original PVPA model can be modified to mimic the compromised skin.

Comparing the *in vitro* model based on the damaged pig skin to the model based on the PVPA barrier, it seems that PVPA model provides more reliable and reproducible model.

Sammendrag

Huden er en alternativ administrasjonsmåte for topikale legemidler med mange fordeler sammenlignet med *per oral* administrasjon. Til tross for fordeler må en uansett overvinne hudbarrieren (*stratum corneum*) dersom en ønsker systemisk effekt. Dette gjør at det er viktig å utforske penetrasjonen av legemidler over både intakt og skadet hud (redusert hudbarriere).

I denne studien ble Franzdiffusjonsceller brukt for å undersøke i hvilken grad kloramfenikol penetrerer først intakt grisehud og deretter behandlet hud påført forskjellig type skader. Hudstykkene fra griseørene ble behandlet med tape-stripping, sterk base, varmt vann og åpen flamme. Resultatene fra forsøkene var ikke som forventet da den kumulative konsentrasjonen av kloramfenikol ikke økte for skadet hud sammenlignet med intakt hud, bortsett fra behandling med sterk base i fem minutter. Denne behandlingen resulterte i liten økning i penetrasjonen av kloramfenikol. Det har forøvrig blitt utviklet en fosfolipid vesikkel-basert permeabilitets modell (PVPA) for å etterligne huden. Denne modellen er laget på et filter hvor små liposomer setter seg i porene til filteret, mens de store liposomene legger seg på toppen av filteret.

Stabiliteten av denne fosfolipid vesikkel-baserte permeabilitetsmodellen ble undersøkt over en periode på fire uker og resultatene tyder på at integriteten av modellen ikke påvirkes etter å ha blitt oppbevart i – 75 ˚C i 21 dager. Etanol er en vesentlig komponent for å oppnå en tett barriere i PVPA modellen. For å etterligne hud med redusert hudbarriere, forsøkte vi å variere etanolkonsentrasjonen. Til tross for at resultatene fra permeabilitetsforsøkene ikke viste noen signifikant forskjell mellom barrierene med ulik etanolkonsentrasjon, kan det fremdeles tyde på at den originale permeabilitetsmodellen kan modifiseres for å etterligne hud med redusert barrierefunksjon.

Dersom en sammenligner *in vitro* modellen basert på skadet grisehud og PVPA modellen, kan det tyde på at PVPA modellen er en mer pålitelig og reproduserbar modell.

List of Abbreviations

ACD	Allergic contact dermatitis
AD	Atopic dermatitis
CD	Contact dermatitis
E-80	Egg phosphatedylcholine
ICD	Irritant contact dermatitis
PCS	Photon correlation spectroscopy
PAMPA	Parallel artificial membrane permeability assay
PVPA	Phospholipid vesicle-based barrier assay
SD	Standard deviation
TEWL	Transepidermal water loss
UV	Ultraviolet radiation

1. Introduction

1.1 Skin as site for drug delivery

The skin covers the entire body, serves as protective tissue during the contact between the body and the environment, and is used as a site for dermal and transdermal drug delivery. In dermal drug delivery the drug is applied topically in order to treat skin diseases. The advantages of delivering drug by this route are the ability to achieve a high concentration of the drug at the site of action (skin) and the potential for the reduction of the systemic drug concentration, hence the systemic side effects (Honeywell-Nguyen and Bouwstra, 2005).

The transdermal drug delivery route is an alternative route to oral administration in order to get the drugs into the systemic circulation. In this case, skin is the site of administration, not the targeted organ. The transdermal route offers several advantages compared to oral administration; the factors that can influence the gastro-intestinal absorption are circumvented, so is the hepatic metabolism. This makes the transdermal route suitable for drugs with low bioavailability. Other types of drugs that are suitable for transdermal delivery are drugs with a narrow therapeutic window. It is also possible to maintain a sustained drug permeation rate by this route (Honeywell-Nguyen and Bouwstra, 2005).

However, due to its strong barrier properties, skin allows also a very low permeation rate of drugs applied onto it (Honeywell-Nguyen and Bouwstra, 2005) and the drug characteristics such as lipophilicity, polarity, molecular weight, etc. will have a great impact on the success of the therapy (Benson, 2005).

However, some transdermal therapeutic systems have been successfully marketed for treatment of systemic disorders. Some examples where such systems are in currently in use are hormone replacement therapy, pain management, angina pectoris, smoking cessation and neurological disorders (Sinkò et al., 2012).

As the skin barrier is an obstacle for most drugs to permeate into/through (Sinico and Fadda, 2009), the skin structure is of a great interest to understand its barrier capacity.

1.2 Healthy skin

1.2.1 The anatomy and chemical composition of the skin

The skin, with its three layers; the epidermis, the dermis and the hypodermis, is the largest organ of the body. The three distinguished layers (Figure 1) vary in the thickness and composition (El Maghraby et al., 2008; Sherwood, 2010).

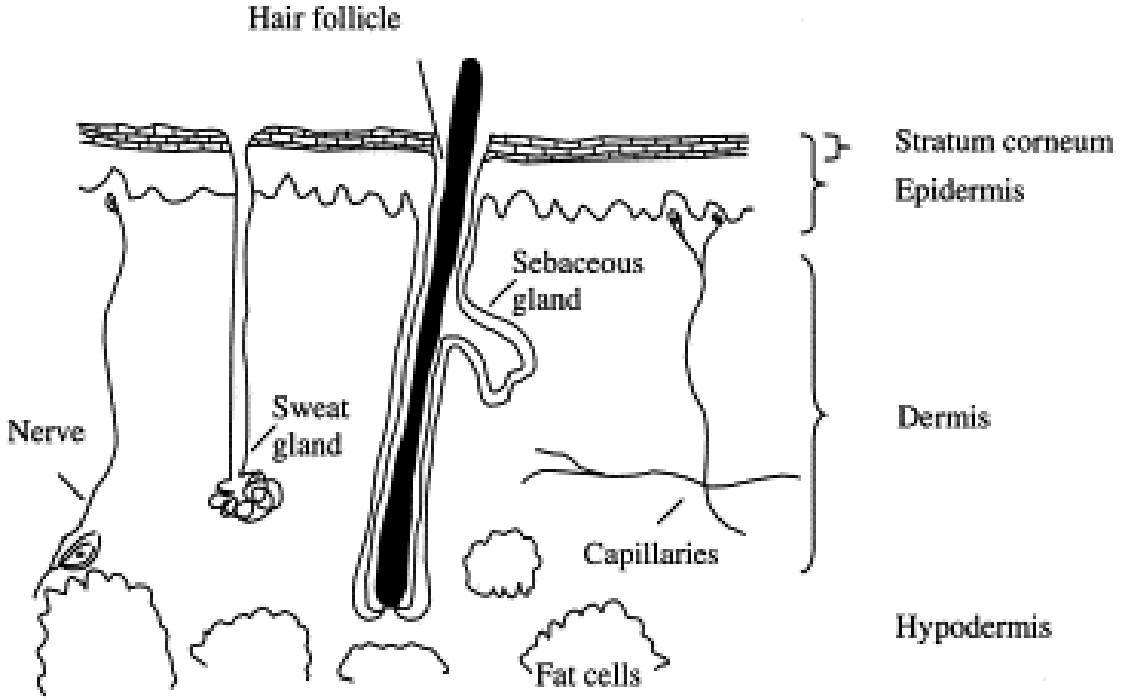

Figure 1: The skin structure (Moser et al., 2001).

The hypodermis contains most of the body fat and is located between the dermis and muscles or bones (Sherwood, 2010). The dermis is 3-5 µm thick (El Maghraby et al., 2008) and its composition includes elastin, collagen, blood and lymphatic, nerve endings, hair follicles and sweet glands (Sherwood, 2010). Epidermis can be divided in five separate layers, where *stratum corneum* is the outer most layer (El Maghraby et al., 2008). *Stratum corneum* consists of dead cells, corneocytes, while the other four layers consist of living cells, keratinocytes. Keratinocytes start to differentiate and migrates from the living layers up to *stratum corneum*. During migration they produce proteins and lipid and their structure and composition changes,

and in the end of the differentiation keratinocytes die and are transformed into corneocytes. Corneocytes are flat dead cells consisting of keratin, water and a cell envelop of proteins (Bowstra and Ponec, 2006).

1.2.2 The functions of the skin

The skin covers the body and has many functions. It takes part in the synthesis of vitamin D, it protects the body against foreign agents/bodies and ultraviolet (UV) radiation, it regulates the body temperature, is a part of the immune system, and it can sensor a stimuli through the numerous nerve endings (Lebonvallet et al., 2010). In respect to the barrier function, skin prevents transepidermal water loss (Abraham and Downing, 1989). Transepidermal water loss (TEWL) refers to the passive diffusion of water from the hydrated dermis to the *stratum corneum* (lower water content). In a healthy skin, the natural moisture factors manage to retain an amount of the water within *stratum corneum* while some of the water evaporates on the surface. TEWL is used as a way to measure the effect of external agents on the skin barrier and is very important for both dermal and transdermal delivery (Sotoodian and Maibach, 2012).

1.2.3 The barrier function of the skin

Although skin has many functions, the most important function is the protection of the inner organs from the outside environment (Baroni et al., 2012) and the prevention of water loss (Abraham and Downing, 1989; Prow et al., 2011). This function maintained through the multi-layered structure of epidermis, dermis and hypodermis (Wato et al., 2012). The efficacy of barrier function is mainly dependent on the *stratum corneum* (first-line barrier) (Baroni et al., 2012; Bouwstra and Ponec, 2006; Ochalek et al., 2012). A thin and irregular layer (0.4-10 μm) of dead skin cells, sebum, sweat and bacteria covers the corneocytes in *stratum corneum* and is not considered to enhance the barrier function of the skin (Prow et al., 2011).

The lower layers of epidermis contribute to the barrier functions as well. They represent a second-line barrier in which the cell-cell junctions in the granular layer (the layer below the lipid layer in *stratum corneum*) are adding to the skin barrier capacity (Baroni et al., 2012; Prow et al., 2011).

1.2.4. The structure of *stratum corneum*

The *stratum corneum* is the outermost layer of the epidermis and consists of 10-25 layers of corneocytes resulting in a 10-15 µm thick layer when in the dry state, which swells upon hydration (Ochalek et al., 2012). The corneocytes are surrounded firstly by the protein-rich envelope, which in turn is covered by a lipid envelope. In addition to these two envelopes, the corneocytes are finally embedded in the intercellular lipid matrix which is arranged in bilayers. This arrangement, protein-rich corneocytes embedded in the intercellular lipid matrix, is referred as "brick and mortar" model (Figure 2). Corneodesmosomes contribute to the barrier properties of the outer layer by associating the corneocytes to each other. The corneodesmosomes thus result in a regular packaging. The outer layers of *stratum corneum* has turnover of a 14 days period, in human, depending on the anatomically site and age. This turnover is a consequence of the desquamation of the outer layers of *stratum corneum* (Prow et al., 2011).

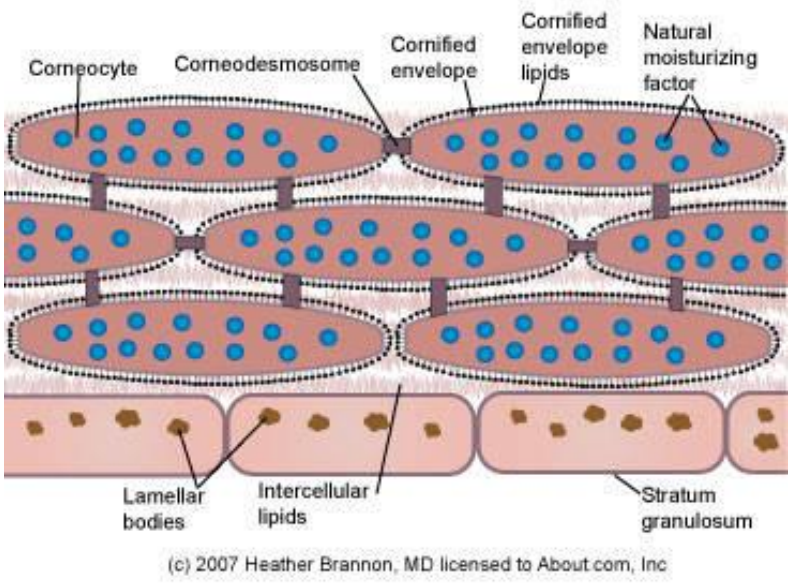

Figure 2: Illustrating the brick and mortar composition of stratum corneum. (http://dermatology.about.com/od/anatomy/ss/sc_anatomy.htm)

This lipid matrix contains the following four classes of lipids; ceramides, cholesterol, cholesteryl sulphate and free fatty acids, respectively. The ceramides represent the most abundant lipid (approx. 50 % of the lipid mass) while cholesterol and fatty acids represent 25

and 10 percent, respectively (Wertz, 2000). The ceramides are regarded as the crucial compounds in the barrier functioning. Ceramides with their polar head group play a crucial role in the formation of the membrane structure (Ochalek et al., 2012), while cholesterol is important for the balance between rigidity and fluidity of the epidermal barrier (Baroni et al., 2012).

1.2.5 Factors affecting the skin barrier

The main factors affecting the skin barrier are the pH (Ali and Yosipovitch, 2013), anatomic site (*stratum corneum* thickness) (Mohammed et al., 2012) and age (Konda et al., 2012).
The pH of the skin surface is normally 4-6, while the pH in epidermis and dermis is 7-9 (Ali and Yosipovitch, 2013). The skin pH is influenced by several factors such as hydration, presence of sweat and sebum, sex, anatomical site (Prow e al., 2011) and age (Ali and Yosipovitch, 2013).
The acidic pH has long been considered important for the barrier function of the skin and the prevention against invasion of microorganisms. This is due to the fact that pathogenic bacteria do not thrive in acidic environment. However, the pH also influences enzymes which are essential for the development and maintenance of the skin barrier (Ali and Yosipovitch, 2013). The age is another factor affecting the skin barrier. The aged skin exhibits changes such as increased dryness of *stratum corneum*, the decrease in skin surface lipids and the flattening of the epidermal-dermal junctions (Konda et al., 2012).

1.3 Transport through biological barriers

1.3.1 Mechanisms of transport across biological membranes

There are several mechanisms of transport across biological membranes; active transport by carriers, passive diffusion (simple diffusion and facilitated diffusion) and endocytosis/transcytosis. In active transport the drug is transported against the concentration gradient, because it uses a substrate-specific carrier and energy is required to transport the drug against the concentration gradient (Brandl et al., 2009).

Passive diffusion is a transport mechanism where no energy is needed, because it follows the concentration gradient, namely the transport from a place with high concentration to a place with lower concentration. The facilitated diffusion is quite similar to simple diffusion in a sense that it also follows the concentration gradient, and hence, no energy is required. However, the drug passes through the membrane by specific channels or is translocated in carrier proteins (Brandl et al., 2009).
Only simple passive diffusion will be discussed further, because the prepared *in vitro* barrier system does not mimic other transport mechanisms, thus only passive diffusion can be measured (Brandl et al., 2009).

Fick's first law of diffusion describes the permeation of a drug through *stratum corneum* (Benson, 2005).
The equation is given:

$$\frac{dm}{dt} = J = \frac{DC_0P}{h}$$

According to Fick's first law the rate of a steady state flux (J) is related to four parameters;
the diffusion coefficient (D) of the drug in *stratum corneum* ,the thickness of the membrane (h), the partition coefficient between *stratum corneum* and the vehicle (P) and the drug concentration in the donor (C_0)

1.3.2 Drug absorption through skin barrier

The *stratum corneum* is important for the skin's barrier function and makes intact skin impermeable for substances from the outside environment. The impermeability of the skin presents challenges both when the drug is delivered topically and transdermally. For topical application, only a small amount of the active drug reaches the target (Hadgraft, 2001).
As the barrier function of the skin is mainly due to *stratum corneum*, diffusion of a molecule through this layer will be the rate-limiting step (Prow et al., 2011). The transport of molecules through *stratum corneum* occurs by three routes; transcellular and intercellular penetration route (directly over *stratum corneum*) and transappendageal route through the sweat ducts and hair follicles (Figure 3) (Benson, 2005).

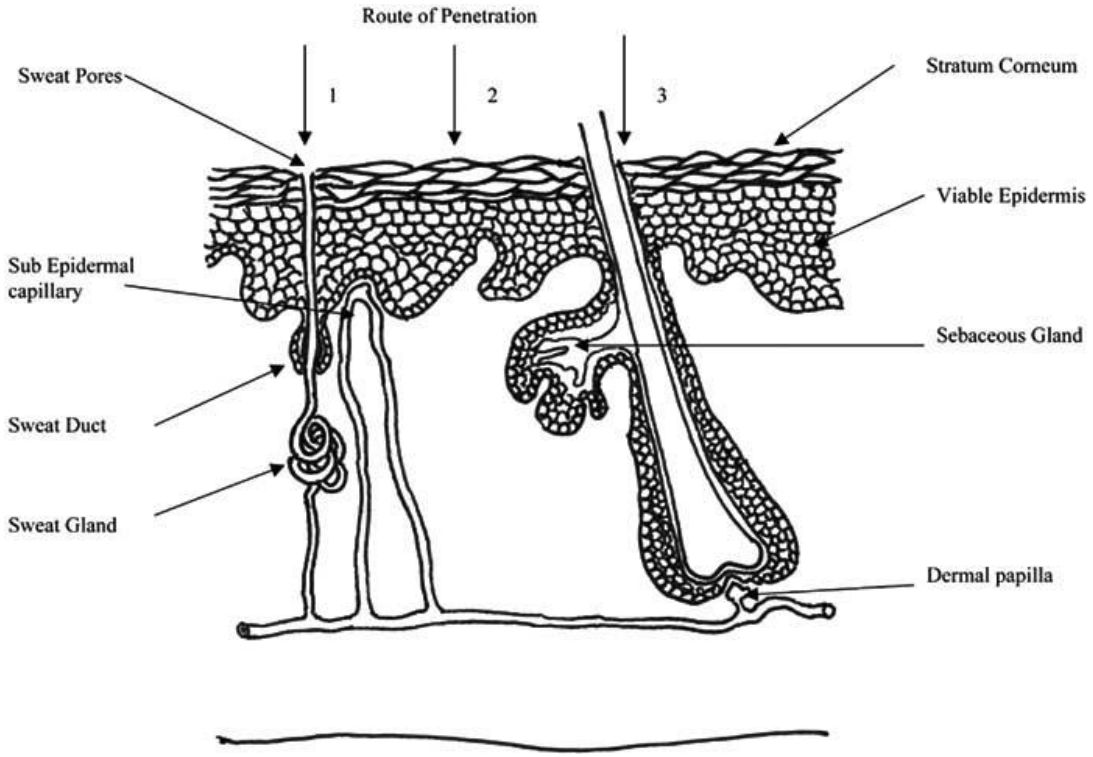

Figure 3: The penetration routes through skin (Benson, 2005).

The transappendageal route can be an alternative route for ions and large polar molecules which can hardly permeate directly through *stratum corneum* (Sinico and Fadda, 2009). Drug delivery across *stratum corneum* by this route is regarded realistic, and so far most of the work has been focused on hair follicles (Prow et al., 2011). The transepidermal pathway (Figure 4) is the main route for substances to permeate across the intact *stratum corneum* and can be achieved by two diffusional routes; the transcellular route and the intercellular route, respectively (El Maghraby et al. 2008; Sinico and Fadda, 2009).

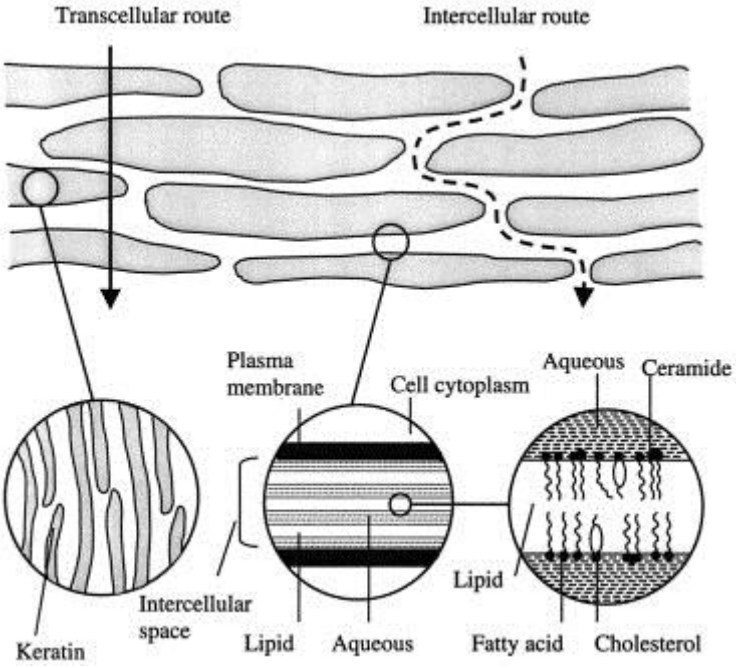

Figure 4: Permeation routes through the brick and mortar arrangement in the skin (Moser et al., 2001)

In the transcellular route, the molecule has to enter and pass through the corneocytes, and due to the "brick and mortar" arrangement of *stratum corneum* (El Maghraby et al., 2008; Prow et al., 2011) has to diffuse through the estimated 4-20 lipid lamellae (Benson, 2005) between the corneocytes in the intercellular lipid matrix before it can diffuse through the next corneocyte (Figure 4). A drug which uses the intercellular route to permeate through *stratum corneum* travels through the intercellular lipid-rich matrix between the corneocytes (Figure 4). The importance of intercellular lipid matrix is evident as both the intercellular and transcellular route require permeation into and through this intercellular matrix, making the lipids in this matrix crucial for the barrier function of the skin (El Maghraby et al., 2008).

The intercellular route is the main penetration route for most molecules through *stratum corneum* (Prow et al., 2011). The reason is the protein-rich envelope of corneocytes which limits the absorption of drugs into the corneocytes (Bouwstra and Honeywell-Nguyen, 2002). However, there are some optimal properties which the molecule should exhibit in order to be, at least theoretically, able to permeate through the skin (Barry, 2001). These properties are low molecular mass (less than 500 Da), sufficient solubility in both oil and water which

correlates to a partition coefficient (log P) between 1-3, low melting point (results in good solubility) and the ability to make hydrogen bonds (Benson, 2005; Prow et al., 2011). The lipophilic character of a molecule is crucial for the penetration rate through the intercellular lipid-rich matrix. Small molecules are able to penetrate this matrix easily, while the penetration of large molecules can be restricted (Prow et al., 2011).

1.3.3 Methods to promote drug penetration through the skin

However, *stratum corneum* can be modified under certain condition in order to promote drug penetration through the skin (Benson, 2005). These conditions include treatment with chemicals such as excessive water, surfactants, terpenes, alcohols, fatty acids, esters and azone (Barry, 2001; Benson, 2005). The hydrated *stratum corneum* has a more open structure compared to unhydrated skin, resulting in less compact *stratum corneum* when in hydrated state, providing a barrier which is more permeable to most substances, however not all. The use of transdermal patches, hydrophobic ointments and occlusive films will all increase the bioavailability of drug applied topically onto the skin since it prevent the water loss (Barry, 2001). This method could be applied to increase the penetration of both hydrophilic and lipophilic substances and is the safest to use among the penetration enhancers (Benson, 2005).

1.4 Diseased skin and its barrier function

1.4.1 Skin diseases

Atopic dermatitis (AD, 6.9 %) and psoriasis (6.6 %) are two of the most common skin diseases in adults (Prow et al., 2011). The treatment of atopic dermatitis (Ong, 2009) and psoriasis (Chiang et al., 2012) are based on topical treatment. In order to optimize the treatment, it is important to understand the barrier properties of the diseased skin as they differ from those of the healthy, intact skin (Chiang et al., 2012).

1.4.1.1 Atopic dermatitis (AD)

AD is a chronic inflammatory skin disease and starts already in childhood for approximately 85 % of all patients. The symptoms are dryness, itch and *Staphylococcus aureus* colonization. The cause of this disease is not fully understood yet. It is debated whether the inflammation linked to this disease is caused by an immune defect or by a primary defect in the skin barrier. However, it is known that AD skin is deficient in various lipids and proteins that play a crucial role in the skin barrier and hydration. The patients with AD have a compromised barrier as compared to healthy persons (Ong, 2009). The barrier defect is connected with the reduced ceramide content in *stratum corneum* and shows increased TEWL (Pilgram et al., 2001).

Jakasa et al. (2007) measured a modest enhancement in the penetration of polyethylene glycols (lipophilic compounds) in AD patients as compared to controls, healthy individuals. The penetration of all glycols through AD skin was twice as high compared to the control subjects except for one patient who had only 60 % higher penetration. Yoshiike et al. (1993) measured the *in vitro* penetration of theophylline both for AD patients and healthy subjects. They reported increased theophylline penetration in AD patients both in involved and uninvolved atopic dermatitis skin compared to control subjects.

1.4.1.2 Contact dermatitis (CD)

Contact dermatitis is a widely used term and includes inflammatory skin reactions after the exposure to external agents. There are two types of CD; allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD). The external agents that can cause ICD are chemicals, detergents, solvents, alcohols, creams, lotions, ointments and powders. The environmental factors that also can stimulate ICD are wetting, drying, perspiration and extreme temperatures (Fonacier and Boguniewicz, 2010). ICD is more common and responsible for approximately 80 % of all cases. Both types of contact dermatitis include an inflammation. However, the inflammation in ICD is not dependent of the immunological memory (prior sensitization is not required); it rather results from the keratinocytes damage in epidermis after exposure to an external agent. ICD can affect anybody who has been exposed to the stimuli with sufficient concentration and duration. ACD, on the other side, affects only genetically predisposed

persons (Mark and Salvin, 2006). The ICD is characterized with the skin barrier which is compromised and exhibits increased TEWL (Fonacier and Boguniewicz, 2010).

Diaper dermatitis is one type of ICD. Diaper dermatitis is caused by several factors such as prolonged exposure to urine and faces, increased hydration and occlusion, altered microbial flora and altered pH. Altered skin pH is a result of the prolonged exposure to urine and feces which causes more alkaline environment due to the formation of ammonia. Ammonia will activate the fecal enzymes, resulting in breakdown of the skin barrier (Ali and Yosipovitch, 2013).

1.4.1.3 Psoriasis

Psoriasis is a genetically influenced disease (Gould et al., 2003) which is characterized by scaly lesions (Rang et al., 2007). The proliferation of keratinocytes is enhanced resulting in epidermis thickening, including *stratum corneum.* The lipid composition in psoriatic skin is altered resulting in the discontinued barrier with increased TEWL (Gould et al., 2003).

Several penetration studies have been performed through psoriatic skin. The reported data are rather conflicting. Shani et al. (1985) studied the penetration of electrolytes in both psoriatic patients and healthy volunteers who had bathed in Dead-Sea or in simulated bath-salt solution for 30 minutes over a period for four weeks. They found the significantly increased serum levels of Br, Rb, Ca, Zn only in the psoriatic group. Gout et al. (2003) also reported increased level of plasminogen activator inhibitor type 2 in psoriatic skin as compared to non-psoriatic skin. However, a study performed by Wester et al. (1983) showed no difference in the levels of hydrocortisone or dithranol between psoriatic and healthy skin. The results from these studies indicate the need for more penetration studies on psoriatic skin.

1.4.2 Altered barrier function of diseased skin

One of the first features of damaged skin barrier is significantly increased water loss. The correlation between TEWL and barrier disruption has been demonstrated in several studies (Sotoodian and Maibach, 2012). Increased TEWL will induce dryness of the skin, which in turn can lead to reduce mechanical flexibility of *stratum corneum*. Other properties that will

be influenced by increased TEWL are the skin's physical properties (viscoelastic characteristics) and functional properties (drug permeation and barrier function) (Sotoodian and Maibach, 2012).

1.5 *In vitro* permeation studies in screening of candidates for dermal and transdermal drug delivery

In order to find drugs which are potential candidates for dermal or transdermal application it is important to perform the permeation studies. The Franz diffusion cell system is one of the most common methods used to measure permeation across skin (Sinkò et al., 2012). These permeation studies should ideally be performed on human skin to evaluate its penetration capacity. This is problematic due to the ethical issues, limited availability and religious restrictions. Animal skin is an alternative to human skin, and porcine ear skin is a good candidate because it can be compared to human skin with respect to

i) *stratum corneum*,
ii) the epidermal thickness,
iii) follicular structure and
iv) the hair density (El Maghraby et al. 2008).

The use of pig skin for permeation studies is also ethically acceptable, as it is obtained as a waste from animal slaughtered for food (Barbero and Frasch, 2009). However, the thickness of the pig ear can vary according to the type of the animal, age and feeding habit, therefore artificial skin membrane models gained more popularity in the past several years.

1.5.1 Artificial skin membranes used for in vitro permeation studies

The Parallel Artificial Membrane Permeability Assay- PAMPA and Phospholipid Vesichle-Based Barrier Assay (PVPA) are two of the artificial membrane models used to predict drug permeability across *stratum corneum in vitro.* Other methods include immobilized artificial membrane chromatography (IAM) and immobilized liposome membrane chromatography (ILC) (Engesland et al., 2013).

1.5.1.1 Parallel Artificial Membrane Permeability Assay- PAMPA

The PAMPA model is used to mimic biological membranes and hence, makes it possible to identify drugs which can be absorbed or permeate (dependent of the membrane) over/ through biological membranes by passive transport. The membrane in PAMPA is prepared on a hydrophobic filter and the phospholipids are dissolved in the organic solvent on the filter (Markovic et al., 2012).

The permeability across *stratum corneum* can be tested by two types of PAMPA-based models. The first is rather fast and simple. Although the components of this PAMPA model include silicone and isopropyl myristate, which are not found in the skin, the prediction of permeability across human skin, even indirectly, is possible. The second PAMPA model includes ceramide analogues and, therefore, mimics the barrier function of *stratum corneum* to a greater extent (Markovic et al., 2012).

1.5.1.2 The Phospholipid Vesicle-Based Barrier Assay- PVPA

The PVPA membrane is another artificial membrane used *in vitro* to mimic biological membranes (Engesland et al., 2013). The PVPA barrier model is prepared on a filter support (Flaten et al., 2006a,b) by placing liposomes in the pores of the filter and on the surface of the filter in order to produce a tight barrier (Flaten et al., 2006b). The disposition of liposomes in the PVPA is shown in Figure 5.

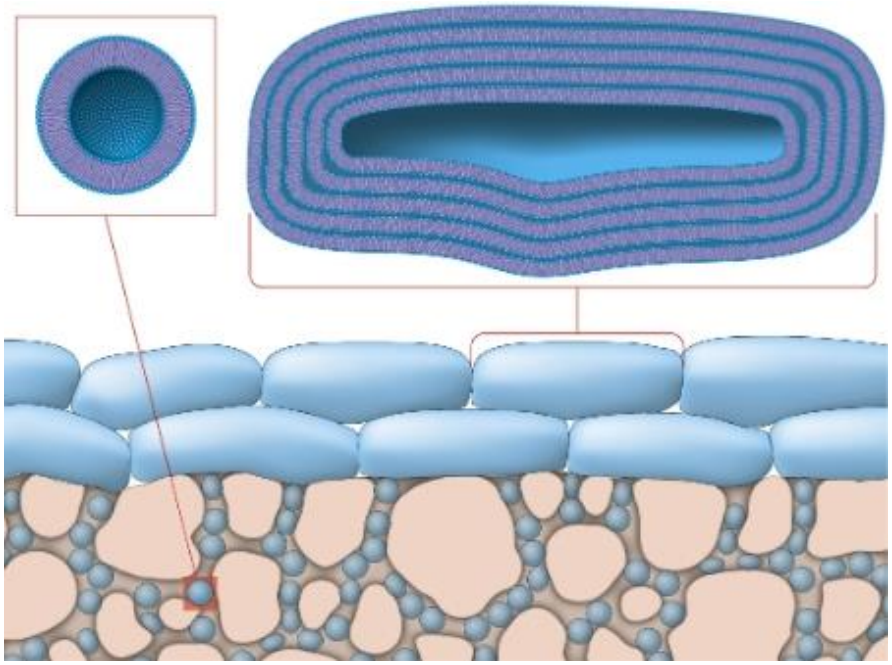

Figure 5: Schematic presentation of the liposome disposition in PVPA. (http://www.farmatid.no/id/605.0)

A liposome is a lipid vesicle that encloses an aqueous volume and consists of phospholipids (Elsayed et al., 2007). There are a number of techniques available to prepare liposomes. The different techniques to prepare liposomes and the type of lipids used can result in liposomes with different size either multilamellar (MLV) or unilamellar (SUV) in their structure. One of the simplest preparation techniques is the film hydration method. This method is based on preparing a thin lipid film on a glass surface. This lipid film is obtained by dissolving lipids in organic solvent followed by evaporation of the organic solvent. A lipid suspension is made by hydrating the lipid film with buffer and shaking the flask. The lipid suspension prepared by this method contains mulitilamellar vesicles. This suspension has to be treated further by high-pressure homogenization or filter extrusion to make liposomes of smaller size. Liposomes are also used as drug carriers and phosphatidylcholine is the primary lipid use to prepare these carriers (Brandl, 2001).

Liposomes are used to prepare skin model membranes because the intercellular lipids in *stratum corneum* form bilayers in a similar manner as liposomes (El Maghraby et al., 2008).

Due to this cell-like structure of liposomes, the membranes prepared from liposomes are capable to represent more *in vivo* like structure (Engesland et al., 2013).

The original PVPA was developed to predict passive drug permeability over/through the intestine, enabling rapid and reliable screening of novel drug candidates (Flaten et al, 2006a, b). This artificial membrane was prepared on a filter support, where liposomes made of E-80 is deposited, through centrifugation, both into the pores and on the surface of the filter (Flaten et al., 2006b).The barrier integrity can be tested by performing permeation studies with calcein and measuring the electrical resistance (Brandl et al., 2009).

This original PVPA have been used as the basis for the development for the PVPAs mimicking skin to a greater extend. The starting point was the ability of the original PVPA to distinguish between poorly and highly absorbable drugs. This was further extrapolated to the ability to distinguish between molecules which could be applied transdermally and those unable to penetrate the skin (Engesland et al., 2013).

The original PVPA was further developed into two new sub-models of PVPA; $PVPA_c$ and $PVPA_s$. $PVPA_c$ mimics the *in vivo* skin composition to a greater extent, due to the addition of cholesterol in the original composition. Liposomes in the $PVPA_c$ are made of E-80 (egg phosphatedylcholine) (77%, w/w) and cholesterol (23 %, w/w). Cholesterol contributes to the skin barrier properties and is important for the skin packaging. The inclusion of cholesterol thus makes the barrier composition of $PVPA_c$ more skin-like and provides a more robust system to stand the hasher procedure conditions (Engesland et al., 2013).

$PVPA_s$ consist of E-80 (50 %, w/w), ceramides (27.5 %, w/w), cholesterol (12.5 %, w/w), cholesteryl sulphate (2.5 %, w/w) and palmitic acid (7.5 %, w/w). The aim of choosing this lipid composition in liposomes was used to prepare a barrier which could mimic the healthy *stratum corneum* closely (Engesland et al., 2013).

These barrier mimics *stratum corneum* to a greater extent as compared to the barrier in PAMPA, because the barrier in this model consists of a layers organized in a similar manner as in skin (Engesland et al., 2013). The barrier in PAMPA models includes phospholipids dissolved in organic solvent. Another exciting side with the PVPA-based skin models is the ability to induce, in controlled manner, a leakage in a barrier, which could mimic the

permeation through compromised skin. This is a based on the correlation between the degree of membrane leakiness and the barrier properties in compromised skin (Engesland et al., 2013).

The PVPA model seems to be suitable for screening a larger number of drugs candidates in the earlier stage of drug development due to a less labor intensive techniques in the preparation procedure than comparable cell-based-methods (Brandl et al., 2009). The PVPA is also seen to be suitable for automation where a robotic system is connected to a plate reader (Engesland et al., 2013).

1.6 In vitro permeation studies on the damaged skin

The numerous absorption studies were performed on the intact skin, however, the studies on chemical permeability through compromised skin are lacking. This is unfortunate as many topical drug products are used to treat skin diseases where the skin barrier is compromised, potentially resulting in increased penetration. The absorption of the drug could be altered significantly if the skin barrier is damaged as in diseased skin (Chiang et al., 2012).

1.6.1 Methods to induce skin barrier damage

There are several ways to induce the skin damage. The methods used can be classified as:
mechanical damage,
tape-stripping and abrasion (a brush brittle drawn across skin),
freezing, heating or branding, or
by using chemical irritants (Chiang et al., 2012).

1.6.1.1 Tape-stripping as a method to induce skin damage

Tape stripping is a common mechanical method used to damage the skin (Chiang et al., 2012). The skin (corneocytes) is removed by placing and pressing adhesive tape to the skin surface followed by removal of the tape (Lademann et al., 2009). This method leads to

increased TEWL (Chiang et al., 2012). There are several parameter which need to be taken into the consideration and these include the type of the tape used and the application method, inclusion of the tape-weighting, application of the pressure and the force used to remove the tape (Lademann et al., 2009).

Morgan et al. (2003) studied the absorption of penciclovir and aciclovir in time tape-stripped (0-40 times) human skin by the use of microdialysis. The results indicated the increased absorption of both drugs. The absorption of penciclovir was increased by 1300-fold and aciclovir by 440-fold. Morgan et al. (2003) also observed no more increase in TEWL after 40 strips, indicating the full disruption of *stratum corneum*. Tsai et al. (2003) performed an *in vitro* permeation study with a series of polyethylene glycols (molecular weight from near 300 to over 1000 Da) across tape-stripped murine skin. They demonstrated that molecules with greater molecular weight penetrated the tape-stripped skin (compromised skin) better than the normal skin.

1.6.1.2 Heating or branding to induce the skin damage

Moderate to severe burns results in completely destroyed barrier (Chiang et al., 2012). An *in vivo* study performed by Papp et al. (2009) showed the increased permeability of topically applied epinephrine in burned patients (serum levels of epinephrine increased) compared to the non-burned patients. This study confirmed that the burn skin has a damaged barrier property, as expected.

1.6.1.3 The use of chemical irritants to induce the skin damage

The chemicals applied to the skin have potential to alter the skin properties. However, both the concentration and the nature of irritant are of importance (Chiang et al., 2012). Malten and Thiele (1973) performed a study in humans to evaluate how much NaOH (pH 12) and Na_3PO_4 (pH 12) affected the TEWL. They found the TEWL to be increased to approximately 350 %. Since the increased TEWL is correlated to the reduced skin barrier, this method clearly resulted in the damaged barrier. In the studies performed by Petiot et al. (2007, 2010), who followed the penetration of uranyl nitrate through chemically (10 N HF, different concentrations of HNO_3, and 10 N NaOH) burned rat skin and intact skin, found no

significant difference in uranium concentration between 10 N NaOH burned rat skin and intact skin. However there was observed increased penetration of uranium after treatment with 10 N HF and various concentrations of HNO_3 and, due to increased disruption of *stratum corneum*.

1.7 Chloramphenicol as a model drug

We have selected chloramphenicol as a model drug with relevance for topical application onto the skin (Hurler and Skalko-Basnet, 2012). It is a broad spectrum antibiotic that is effective against both Gram-negative and Gram-positive organisms, *Rickettsiae* and *Chlamydia* (Heal et al., 2009). Its antibiotic effect is due to the inhibition of the protein synthesis in the microorganisms.

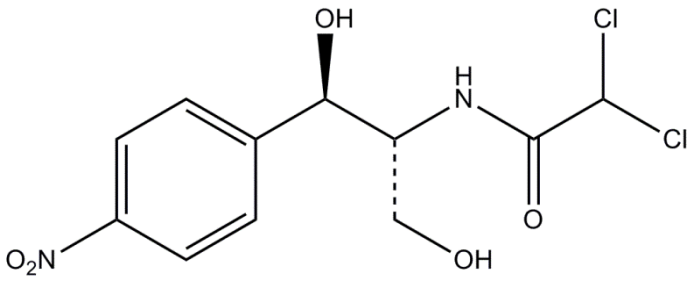

Figure 6: The structure of chloramphenicol

The oral use of chloramphenicol is restricted to severe infection due to its toxicity. It is widely used in bacterial conjunctivitis and the use is safe when given topically (Rang et al., 2007).

2. Aims of the study

Most of the current methods applied to study skin penetration include the use of skin samples obtained from animal, tested in the Franz diffusion cell system. However, it is a known fact that the animal origin and pretreatment of the skin samples affect the penetration studies. The main aim for this study was to develop a reliable and reproducible *in vitro* model, which would enable the evaluation of the skin barrier properties of the compromised skin. For this purpose, as the first step we induced the different degree of damage to the pig skin samples. The methods used to induce the damage in the skin barrier were:

- tape-stripping,
- treatment with strong alkali,
- treatment with heat and
- burning of the skin

The intact pig skin represented the control. Chloramphenicol was used as a model drug. The second step was to modify the already established PVPA barrier with different degree of leakiness to resemble compromised skin. The modification was based on the changes in the ethanol concentration in liposome suspension used to prepare large liposomes (1200 nm) that lie on the top of the filter support in this barrier. Finally, the stability of the modified PVPA barriers was tested to assure that the modified PVPA barrier can be scaled up.

3. Materials and Methods

3.1 Materials

3.1.1. Chemicals

Hydrochloric acid 37 % (w/w) (HCl), VWR International, BDH Prolab, Leuven, Belgium

Calcein, Sigma Aldrich Chemie GmbH, Steinheim, Germany

Chloramphenicol, Sigma Aldrich Chemie GmbH, Steinheim, Germany

Chloroform, Merck KGaA , Darmstadt, Germany

Cholesterol, Sigma Aldrich Chemie GmbH, Steinheim, Germany

Distilled water, Tromsø, Norway

Egg phosphatidylcholine (E-80), Lipoid, Ludwigshafen, Germany

Ethanol 96 % (v/v), Sigma Aldrich Chemie GmbH, Steinheim, Germany

Ethylenedinitrilotetra-acetic acid (Titriplex III), Sigma Aldrich Chemie GmbH, Steinheim, Germany

Methanol, Sigma Aldrich Chemie GmbH, Steinheim, Germany

Nitrogen (gas), AGA, Oslo, Norway

Potassium phosphate monobasic (KH_2PO_4), Sigma Aldrich Chemie GmbH, Steinheim, Germany

Sodium Chloride (NaCl), Sigma Aldrich Chemie GmbH, Steinheim, Germany

Sodium hydroxide (NaOH), Sigma Aldrich Chemie GmbH, Steinheim, Germany

Sodium phosphate dibasic (Na_2HPO_4) *12 H_2O, Merck KGaA, Darmstadt, Germany

TurboTorch Propan-gas (400 g), E-400, Primus AS, Oslo, Norway

3.1.2. Animal tissue

Pig ears, Nortura AS, Bardufoss, Norway

3.1.3. Equipments

Biocap BP-403, Erlab, Val de Reuil, France

Biofuge stratos, Heraeus instrument, Kendro laboratory products (GmbH), Osterode am Harz, Germany

Bransonic ultrasonic cleaner, 5510E-MT, Branson, Danbury, USA

Büchi rotavapor, R-124, Büchi vacuum, V500, Büchi vacuum controller, B-721, Büchi water bath, B-480, Büchi Labortechnik, Flawil, Switzerland

Costar assay plate 96 well black, Corning Inc., New York, USA

Costar UV-plate 96 well transparent, Corning Inc., New York, USA

Filter inserts transwell (d= 6.5 mm) and 24 transwell plates, Corning Inc., New York, USA

Forma Scientific freezer 923, Thermo Scientific, Marietta, USA

Franz diffusion cell 15 mm, 12 ml chamber, (#4G-01-00-15-12), Permegear Inc., Hellertown, USA

IBR Heat-Press, HP80-3500, IBR- Ingenierbüro, Waldkirch, Germany

REAX Top mini-shaker, Heidolph, Schwabach, Germany
Isopore membrane filters (1.2 µm), Merck Millipore, Billerica, USA

Julabo heating circulator, F12-ED, Julabo labortechnik GmbH, Seelbach, Germany

Custom made extruder Mgw Lauda RM-3 and thermostar, Dr. R. Wobser KG, Lauda-Königshofen, Germany

Millicell-ERS, Merck Millipore, Billerica, USA

NICOMP Submicron Particle Sizer, model 370, Particle sizing system (PSS), Santa Barbara, USA

Nitrocellulose mixed esters membrane (0.65 µm), Merck Millipore, Billerica, USA

Nuclepore membrane filters (0.4 µm), Whatman , Oslo, Norway

pH-meter, 744, Metrohm , Herisau, Switzerland

Polarstar Galaxy, BMG Labtech (GmbH), Ortenberg, Germany

Sartorius LP 4200S/ BP 211D/LP6 205/CP 225D/LP620S, Sartorius AG, Göttingen, Germany

Spectramax microplate reader, 190, Molecular devices, Sunnyvale, USA

Hamilton Syringe (250 µl), Hamilton, Reno, USA

Termaks incubator, KEBO AS, Bergen, Norway

Tesa SE 4124 adhesive tape (50 mm). Type of adhesive natural rubber. Total thickness 65 µm, Beiersdorf AG, Hamburg, Germany

TurboTorch Propan-gas (400 g), E-400, Primus AS, Oslo, Norway

Cellophane foil, Bringmann folia, Wendelstein, Germany

3.1.4 Computer programs

Fluorescence analysis: Fluostar galaxy (4.31.0), BMG Labtech (GmbH), Ortenberg, Germany

Particle size analysis: PCS CW388 (1.68 version 8.1). Nicomp Particle Sizing Systems (PSS), Santa Barbara, USA.

Microplate reader: SoftMax Pro (5.0), Molecular devices, Synnyvale, USA

3.2 Methods

3.2.1 Composition of phosphate buffer

A phosphate buffer with the following composition was used during the thesis:

Potassium phosphate monobasic (KH_2PO_4)	600 mg
Sodium phosphate dibasic dodecanhydrate (Na_2HPO_4 (*$12H_2O$))	6400 mg
Sodium Chloride (NaCl)	7240 mg
Ethylenedinitrilotetra-acetic acid Titriplex III (using only for calcein)	3720 mg
Distilled water	up to 1 l

The pH in the buffer was adjusted to 7.4 with 1 M NaOH solution/ 1 M HCl accordingly.

3.2.2 The Phospholipid Vesicle-Based Permeation Assay (PVPA)

3.2.2.1 Liposome preparation

The film hydration method followed by filter extrusion (Brandl, 2001) was used to prepare liposome dispersion. The liposome film was prepared by weighing egg phosphatedylcholine (E-80) (694 mg) and cholesterol (206 mg) into a round-bottom flask. Chloroform (15 ml) was added and the flask was shaken to dissolve the lipids. The chloroform was evaporated by the use of Büchi rotary evaporator at 47 ˚C (water bath), under vacuum and pressure (initially 400 mbar, but reduced slowly to 200 mbar) of 200 mbar. The round flask was maintained at 47 ˚C, under vacuum and a pressure of 200 mbar for the first two hours. Thereafter, pressure was reduced to 45 mbar under the vacuum and the flask was maintained at room temperature for another three hours to remove traces of chloroform. The lipid film was then hydrated with 13.5 ml phosphate buffer (pH 7.4), and the suspension was shaken by hand until all of the lipid film was dispersed in buffer. At last, ethanol (1.5 ml) was added. To prepare liposomes of two different sizes, the liposome suspension was further extruded in two portions, one portion through 0.4 µm Nucleopore filter and the other through 1.2 µm Isopore filter, respectively.

The filter extrusion was done as by Engesland et al. (2013), by use of a nitrogen driven-extruder, (Dr.R. Wobser KG), which was used at 10 bar after allowing the liposomal suspensions to heat in the extrusion chamber for 5 minutes at 40 ˚C. Each portion was extruded five times through the filter.

3.2.2.2 Characterization of liposomes

The size distribution of the liposomes dispersion was determined by photon correlation spectroscopy (PCS) on NICOMP Submicron Particle Sizer 370. The measurement was done right after extrusions, to ensure that the size reduction of liposomes was performed successfully. The preparation of sample and measuring conditions were done as described by Ingebrigtsen and Brandl (2002). The only difference was that the duration of each measuring cycle was 15 rather than 30 min as in the original method of Ingebrigtsen and Brandl, 2002.

3.2.2.3 Preparation of the phospholipid vesicle-based permeation assay (PVPA)

The phospholipid vesicle-based barriers were prepared as described by Engesland et al. (2013). Nitrocellulose filter (0.65µm pore size) were cut and fused onto transwell inserts (d=6.5 mm) at 150 ˚C for 30 seconds by using IBR heat press.

Figure 7: Showing the 24 transwell plate and inserts carrying the nitrocellulose filter.

The filter inserts carrying the 0.65 µm nitrocellulose filter (Figure 7) were transferred to 24-well transwell plates and 100 µl of liposome suspension (extruded through 400 nm filter) added to each insert, and centrifuged at 950 *g* (2500 rpm) in 15 minutes. This procedure was repeated once. Upon centrifugation, the inserts were placed on a paper (to dry out the traces of liposomes which had passed through), then transferred to new plates, and further placed in a heating cabinet (Termaks incubator) from KEBO, at 50 ˚C for 45 minutes. The plates were left at room temperature for 10 minutes, before 100 µl of liposomes (extruded through 1200 nm filter) were added. The plates were centrifuged at 1030 *g* (2600 rpm) for 60 minutes. After the centrifugation, an access of supernatant on the top of the filter inserts was usually visible. To remove this access of supernatant, the plates were centrifuged in upside down position at 25 *g* (300 rpm) for five minutes. The plates were stored in the freezer at -75 ˚C until further use. Before the permeability testing, the membrane inserts were taken out of freezer and transferred to new 24-transwells plate, which was then placed in the incubator at 30 ˚C for two hours to thaw.

3.2.2.4 The standard curves for chloramphenicol, calcein and ibuprofen

The standard curve of chloramphenicol was done with a stock solution of chloramphenicol (0.00548 g in 10 ml of phosphate buffer (pH 7.4)). The dilution series used to prepare standard curve for chloramphenicol; 1:400, 1:200, 1:100,1:50, 1:33.3, 1:25, 1:16.6, 1:14.3, 1:12.5 and 1:11.1. The absorbance of the solutions was measured spectrophotometrically. The standard curve was gained with a wavelength of 285 nm and R^2-value of 1. This standard curve was used to calculate the concentration of chloramphenicol in the samples after experiments. The calibration curve for calcein was done as Flaten et al. (2006a) and the ibuprofen standard curve was done as Engesland et al. (2013).

3.2.2.5 Integrity/ permeability testing of PVPA barriers using calcein, chloramphenicol and ibuprofen

The permeability studies were performed as described by Flaten et al. (2006a). These studies were performed in 24-transwell plates where all wells contained 600 µl of phosphate buffer (pH 7.4) as acceptor phase maintaining sink conditions. The inserts were placed in different wells and 100 µl of 5 mM calcein added to each insert to test the integrity of membrane

inserts. The same procedure was used for permeability testing, in which chloramphenicol solution (0.539 mg/ml) and ibuprofen solution (5 mM) was added in the inserts. The permeability/integrity experiments were run for five hours at room temperature. For the first three hours, the inserts were moved to new wells every hour and for the next two hours, every 0.5 hour. After five hours, 200 µl from each well (acceptor phase) and 2 µl from the donor phases (chloramphenicol and ibuprofen) were diluted with 198 µl of phosphate buffer and transferred to 96 well UV transparent plates (chloramphenicol and ibuprofen) and the 96-well black plates (calcein), respectively. The absorbance of chloramphenicol was measured spectrophotometrically at 285 nm, while fluorescence of calcein was measured with excitation filter at 485 nm and emission filter at 520 nm as done by Flaten et al (2006a). The resistance over the PVPA membrane was measured directly in the inserts immediately after experiments by Millicell-ERS. The permeability experiments were performed with 12 parallels (n= 2) for chloramphenicol and with 15 parallels (n= 2) for ibuprofen. The integrity experiments with calcein were performed with 18 parallels (n= 3).

The calculation of the apparent permeability coefficient (P_{app}) for chloramphenicol, calcein and ibuprofen over the barriers was calculated as by Flaten et al. (2006a) with the following equation;

$$P_{app} = \frac{J}{AC_d}$$

where, J is the flux rate (nmol/s), A is the surface area of the insert (cm^2) and C_d is the concentration in donor (nmol/mL).

3.2.2.6 Storage stability of the PVPA barriers

Flaten et al. (2006b) reported the simpler PVPA barriers made for intestinal permeability studies were stable up to two weeks. With the new lipid composition the aim was to investigate how long the membrane inserts can be stored at -75 ˚C. Inserts (a batch of 24) were prepared as described earlier and stored for different periods of time at -75 ˚C, from one day to four weeks. The permeability studies were performed as described earlier in 3.2.2.5 and the electrical resistance over the membrane was measured immediately after experiments by Millicell-ERS. The first permeation studies was performed with four membrane inserts that

were stored in the freezer (- 75 ˚C) only for one day and then thawed for two hours. After the thawing process the membrane inserts were left in room temperature overnight. The rest of the inserts were taken out of the freezer on the same day when the permeation study was performed. All the studies were performed once with four parallels that were stored in the freezer (- 75 ˚C) for the same time.

3.2.3 *In vitro* penetration studies using Franz diffusion cell

3.2.3.1 Penetration studies with chloramphenicol solution (3 mg/ml)

The Franz diffusion cell system was used to test the penetration through pig skin as the Franz diffusion cell system method is the most commonly used in *in vitro* permeation studies (Sinkó et al. 2012). The pig skin was used as skin model for the penetration studies because it resembles the human skin (Barbero and Frasch, 2009). Each cell in this system consists of an acceptor chamber (12 ml) and a donor chamber with a diameter of 15 mm. A membrane is then placed between these two chambers as shown in Figure 8.

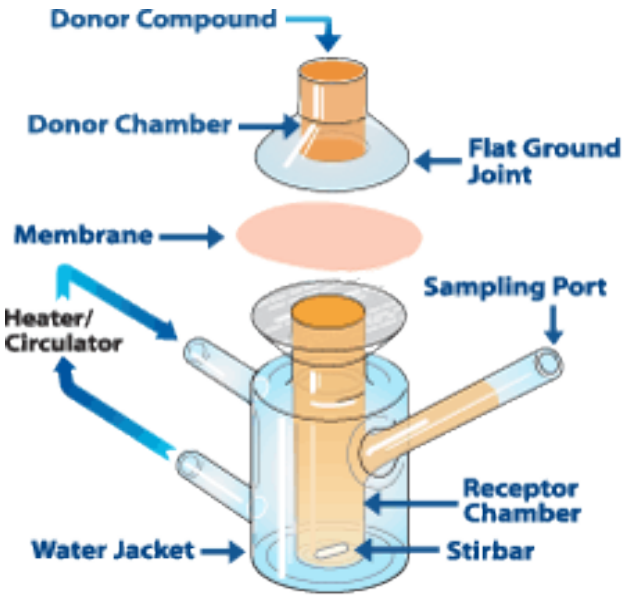

Figure 8: Franz diffusion cell systems. (http://www.permegear.com/)

This system was washed prior to use with methanol, deionized water and distilled water under the stirring, respectively; 30 minutes with each solvent. The membranes/skin slices used during penetration testing were either cellophane membrane (used during establishing the penetration testing method) or the pig ear skin slices. These membranes were placed between two O-rings in order to prevent leakage. The acceptor chambers were filled with phosphate buffer (pH 7.4) and a stir-bar was used to ensure uniform stirring (Figure 9).

Figure 9: The Franz diffusion cell systems (http://www.permegear.com/)

The temperature during penetration studies were maintained at 32 ˚C by the help of heating circulator. The donor chamber was placed on top and fitted with a bracket, and 300 µl of chloramphenicol solution was added. The donor chamber and the port of the acceptor chamber were covered with a double layer of parafilm to avoid evaporation. The samples (200 µl) were taken with a Hamilton syringe (250 µl) after the first 10 minutes and then every 0.5 hours for five hours. The acceptor chamber was refilled with buffer. After the completion of the experiment, the rest volume in the donor chamber were measured and a volume of 2 µl from all donor chambers was collected and diluted with 198 µl of phosphate buffer. All the samples were transferred into the 96- well transparent plate and measured at the wavelength of 285 nm as previously described.

When cellophane was used as a membrane, the samples were taken every hour for three hours and then every 0.5 hour for two hours. The chloramphenicol solution used was 0.539 mg/ml. The preliminary penetration studies with cellophane membrane were performed twice with 4 parallels in each study. While the penetration studies with intact skin were performed twice with 4 and 3 parallels in the respective studies.

3.2.3.2 The preparation of pig ear slices

The pig ears were purchased from a slaughterhouse (Nortura AS, Norway) and prepared on the day they arrived. The cartilage, excess fat and blood vessels were removed from the skin by using a scalpel. The skin slices were wetted with phosphate buffer and immediately packed in a plastic wrap and stored frozen (-20 ˚C). The slices were taken out of the freezer and placed in a fridge one day before the experiments were performed.

3.2.3.3 The penetration studies using chloramphenicol solution (3 mg/ml) and skin with damaged barrier properties (tape stripping)

The outer side of the pig ears was cleaned with ethanol 96% to remove fat from the skin surface. The tape stripping was performed on the outer side to induce damage to the *stratum corneum*. The tape used was Tesa SE 4124 (adhesive tape; 50 mm with total thickness of 0.65 µm). The tape stripping was performed 21 and 41 times respectively. The penetration studies with skin sample tape stripped 21 times were done once with 4 parallels, while the penetration experiment with 41 times tape stripped skin was performed twice with 3 parallels in each experiment. The skin sample used for penetration testing was 15 mm in diameter, as the outer diameter of the acceptor chamber. The penetration experiments were performed as described in 3.2.3.1.
The tape stripping was used to damage the skin as it has been proven to be suitable to damage the skin barrier (Morgan et al., 2003).

3.2.3.4 The penetration studies using chloramphenicol solution (3 mg/ml) and skin with damaged barrier properties (strong alkali)

The pig ear was treated with 3 M NaOH for either 5 or 15 minutes. This was done by placing the skin in a small white plastic box (d= 7cm) which was covered with aluminum foil. The skin was placed in a way that only the outer part (*stratum corneum*) was exposed to the strong alkali. A glass pipette was used to apply 3 M NaOH. The skin associated to the wall, but a glass pipette was used to spread the base over the skin during the whole treatment. The skin was placed in fresh phosphate buffer (pH 7.4) immediately after the treatment. The skin sample used for penetration testing was of 15 mm in diameter, as the outer diameter of the acceptor chamber. The penetration testing was performed as described in 3.2.3.1. The penetration studies with skin treated for 5 minutes with strong alkali were performed twice with 4 and 3 parallels respectively. While 3 parallels were performed in each penetration experiment with skin treated with strong alkali for 15 minutes. Malten and Thiele (1973) originally reported that strong alkali (pH 12) affected the barrier properties of the skin.

3.2.3.5 The penetration studies using chloramphenicol solution (3 mg/ml) and skin with damaged barrier properties (heat-induced damage)

The skin slices were placed on the bottom of a bottle with the outer side of the *stratum corneum* pointing out. The slices were taped around the bottle to assure that the inner side of the skin did not get into the contact with hot water (89-91˚C) during the exposure (temperature was measured during the exposure to hot water). The bottle with attached skin was held in water bath for 15 seconds and immediately placed in the phosphate buffer (pH 7.4). The skin sample used for penetration testing was 15 mm in diameter, as the outer diameter of the acceptor chamber. The penetration was performed as described in 3.2.3.1. The penetration studies with heat treated skin were performed twice with 3 parallels in each experiment.

3.2.3.6 The penetration studies using chloramphenicol solution (3 mg/ml) and skin with damaged barrier properties (burned skin)

The skin slices were placed in a big steal bowl with the outside of the skin (*stratum corneum*) pointing out. A propane burner (TurboTorch® Propane, Primus AS, Oslo) was used to obtain an open flame. The flame size was approximately 2 cm in length and was held near the skin to ensure burning. The skin was exposed to the flame for total 25 seconds. It was ensured that the whole skin was burned by moving the flame up and down covering the whole area of the skin. The skin sample used for the penetration testing was 15 mm in diameter, as the outer diameter of the acceptor chamber. The penetration was performed as described in 3.2.3.1. The penetration studies with burned skin were performed twice with 3 parallels in each experiment.
The burned skin is expected to exhibit high damage to its barrier properties (Papp et al., 2009).

3.2.4 Compromised PVPA membrane

3.2.4.1 Varying the content of ethanol (96 %, v/v) in the PVPA barriers to mimic compromised *stratum corneum*

Engesland et al. (2013) experienced that varying ethanol concentration in the PVPA preparation presented different degree of leakiness, thus introducing a tool to alter barrier leakiness. Liposome suspension for the layer of large liposomes (1200 nm extrusion) remaining on the top of the filter support of the PVPA barriers was made with varying concentration of ethanol. The small liposomes (400 nm extrusion) going into the filter support were made with 10 % ethanol as described in 3.2.2.1. The PVPA barriers were prepared as described in 3.2.2.3. The final concentrations of ethanol in the large liposomes were 0, 5, 10, 15 and 20 % (v/v), respectively. The permeability studies were performed as described earlier in 3.2.2.5, except that the concentration of chloramphenicol solution was 3 mg/ml. The permeability experiments with PVPA barriers prepared with large liposomes containing 0 and 5 % ethanol in large liposomes were done twice. While the experiments with PVPA barriers prepared with large liposomes containing 10, 15 and 20 % ethanol were done three times. The

permeability experiments were performed with 14 parallels for 0 and 5 % ethanol, 18 parallels with 10 % ethanol and 22 parallels for both 15 and 20 % ethanol.

4. Results and Discussion

4.1 The Phospholipid Vesicle-Based Permeation Assay (PVPA)

4.1.1 Liposome characterization

One of the characteristics of the PVPA is that the barriers are made of liposomes with specific size distributions able to build layers to resemble cell-like structure. The liposome suspension was extruded through two filter sizes, namely 400 and 1200 nm, which is required to prepare the PVPA barrier mimicking *stratum corneum*. The liposomes extruded through 400 nm, fit into the pores of the filter support (650 nm), while the 1200 nm extruded liposomes stay on the top of the filter support (Engesland et al., 2013). Liposomes extruded through 400 nm filters exhibited a size distribution of 278 ± 110 nm and the size distribution of liposomes extruded through 1200 nm was 906 ± 82 nm.

4.1.2 Integrity testing of PVPA with calcein and permeability testing with chloramphenicol and ibuprofen

Calcein with relatively low molecular weight (622.55) and polar groups is not expected to readily cross biological barriers. The fluorescent property makes it ideal as a marker to examine batch-to-batch variations and as integrity control, supported by the resistance measurements (Flaten et al., 2006a). Chloramphenicol was used as model drug. The permeability studies with chloramphenicol solution (0.539 mg/ml) on PVPA barriers were performed to examine the ability of PVPA to mimic the skin barrier. The permeability of chloramphenicol was measured to be $2.046 \times 10^{-6} \pm 0.660 \times 10^{-6}$ cm/s with 12 parallels (n= 2) indicating that this method is reproducible. The permeability of calcein in the integrity testing of PVPA was measured to be $0.061 \times 10^{-6} \pm 0.053 \times 10^{-6}$ cm/s with 18 parallels (n= 3) and the resistance was measured to be 2072 ± 891 Ω. Engesland et al. (2013) determined the permeability of calcein to be $0.081 \times 10^{-6} \pm 0.041 \times 10^{-6}$ cm/s, demonstrating that the method was reproducible. Results from the permeability trials performed initially with ibuprofen demonstrated also the reproducibility of the PVPA barriers as the permeability of ibuprofen was measured to be $2.715 \times 10^{-6} \pm 0.822 \times 10^{-6}$ cm/s with 15 parallels (n= 2), which were close to the results reported by Engesland et al. (2013). They determined the permeability of ibuprofen to be $2.80 \times 10^{-6} \pm 0.60 \times 10^{-6}$ cm/s. Another artificial membrane which mimics the

skin is the parallel artificial membrane permeability assay (PAMPA). This membrane is based on a filter support where ceramides are dissolved in organic solvent. This arrangement of ceramides in organic solvent is different from the skin. Thus, the PVPA barriers mimic the *stratum corneum* to a greater extent (Engesland et al., 2013).

4.1.3 Stability of the PVPA barriers upon storage

The aim of performing the stability studies was to see whether the integrity of the PVPA barriers was affected during the storage at -75 ˚C. The stability studies of the PVPA barriers were performed by testing the permeability and resistance after 1, 7, 13, 21 and 28 days using 5 mM calcein that was freshly prepared.

The results, shown in Figure 10, indicated that the PVPA barriers could be stored at -75 ˚C for 21 days. Further tests with a higher number of barriers would be necessary before drawing a final conclusion.

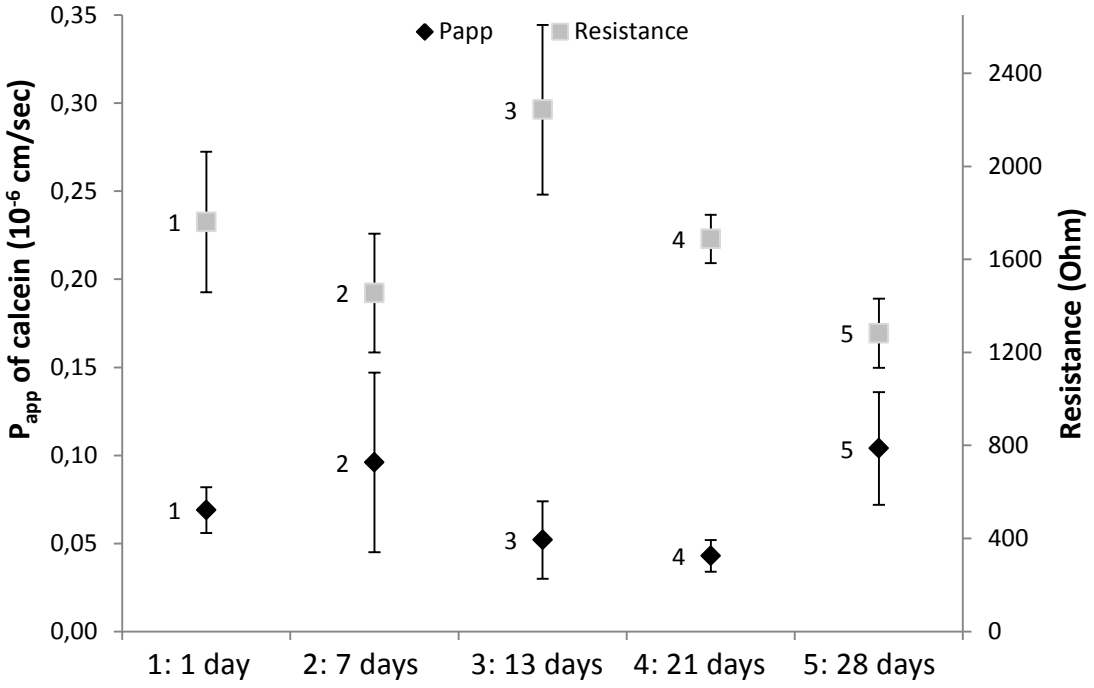

Figure 10: The results from permeability testing with calcein after storage of the PVPA barriers in the freezer. (n=1) with four parallels.

4.2 *In vitro* penetration studies using the Franz diffusion cell system

4.2.1 The preliminary penetration studies

The cellophane was used as a membrane to establish and optimize a method to perform penetration studies on the Franz diffusion cells. A chloramphenicol solution (0.539 mg/ml) was used during the optimization of the method. In order to assure that no degradation of chloramphenicol occurred upon storage, we prepared the fresh solution every week and measured the absorbance on every day the solution was used. A plug was initially used to cover the donor chamber, however, placing a plug on the donor chambers applied pressure to the cellophane membrane (Figure 8). As a consequence, we decided to use double layer of parafilm to prevent evaporation. Bubbles were observed when the penetration studies with cellophane were performed. We expected that bubbles between the acceptor and barrier could influence the penetration of the drug due to reduce contact between the acceptor and the barrier. Therefore, (when sample of pig skin was used as the barrier), prior to the start of each experiment we assured that no bubbles were present.

The result from the preliminary penetration studies showed a cumulative penetration of chloramphenicol to be 58.13 ± 8.44 % with 8 parallels. The results are shown in Figure 11.

4.2.2.1 The penetration studies using chloramphenicol solution (3 mg/ml) and intact pig skin

When the method for testing penetration on Franz diffusion cell was established, *in vitro* penetration studies using both the intact and treated (to induce damage in the barrier properties) pig skin were performed. The pig skin was used as skin model in this study for its ability to resemble human skin (Barbero and Frasch, 2009).

All the *in vitro* studies were performed with chloramphenicol solution of 3 mg/ml. The penetration studies using the intact pig skin were performed to determine the extent to which chloramphenicol penetrates through the skin with normal barrier properties. The results from these experiments with intact skin were hence used to compare cumulative chloramphenicol penetration through treated skin.

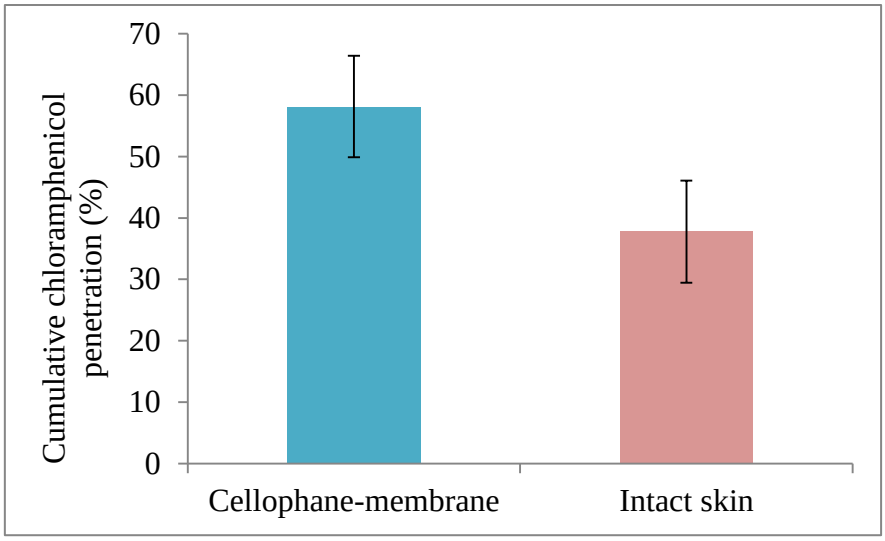

Figure 11: Cumulative chloramphenicol penetration through the cellophane membrane and intact pig skin after 5 hours. (n = 2)

The cumulative penetration of chloramphenicol was measured to be 37.76 ± 8.32 % with 7 parallels. The results are shown in Figure 11. However, the pig ears used in the penetration experiments were stored at -20 ˚C. Freezing of the skin can alter the permeability through it (Chiang et al., 2012). In an *in vitro* study performed by Brain et al. (2005) it was observed that penetration of diethanolamine applied as an simple aqueous solution increased in frozen human skin (-20 ˚C, stored for at least 1 week) while no difference in penetration of leave – on formulations of diethanolamine was observed in fresh skin. They also observed that the penetration of the leave-on formulations were low both in the frozen and fresh human skin (Brain et al., 2005). Increased penetration of diclofenac sodium was also shown for frozen pig skin as compared to fresh pig skin in a study performed by Sintov and Botner (2006). In all penetration experiments we used a chloramphenicol solution. The penetration of chloramphenicol may be increased in a frozen skin compared to fresh skin. Therefore, the results from our experiment indicate a higher penetration of chloramphenicol than what may have been the case if the fresh pig ear were used. Ideally fresh skin should be used. However, access to fresh pig skin is limited.

4.2.2.2 The penetration studies using chloramphenicol solution (3 mg/ml) and skin with damaged barrier properties (tape-stripping)

The *in vitro* penetration studies were performed on the tape-stripped pig skin. Tape-stripping is a commonly used method to damage the skin (Chiang et al., 2012). This method was used to evaluate the extent to which chloramphenicol penetrates through damaged skin. The damage to skin was induced by performing tape-stripping 21 and 41 times, respectively, to remove layers of corneocytes of *stratum corneum* in order to induce different degree of damage. The whole outside surface of the skin sample was covered with the adhesive tape. The first tape did not adhere to the skin; hence ethanol was used to clean fat from the surface. The second tape (applied after the removal of fat from the skin surface) adhered to the skin. The experiment showed that the cumulative chloramphenicol penetration through the skin tape-stripped 21 times was 18.41 ± 5.79 % with 4 parallels. For 41 times tape-stripped skin the cumulative penetration of chloramphenicol was 23.19 ± 9.17 % with 6 parallels. Results are presented in Figure 12.

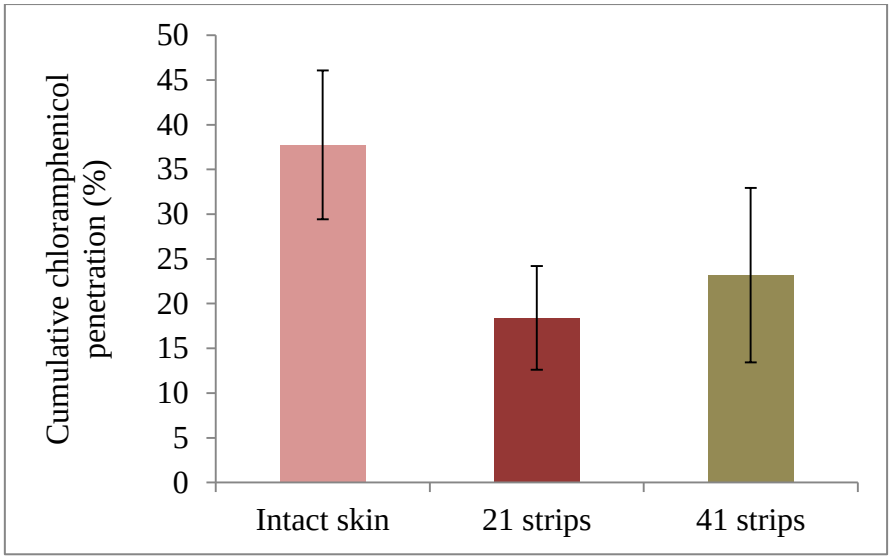

Figure 12: Cumulative chloramphenicol penetration through the intact (n= 2), 21 times tape-stripped (n= 1) and 41 times tape-stripped (n=2) pig skin after 5 hours.

These findings indicate that the tape-stripping did not result in damaged skin. However, there are studies that have reported increased penetration of substances through the tape-stripped skin. Morgan et al. (2003) observed increased absorption of penciclovir (1300-fold) and

aciclovir (440-fold) in the tape-stripped human volunteers as compared to intact skin. However, these drugs are hydrophilic while chloramphenicol is neither lipophilic nor hydrophilic drug with a log P value of 1.1 (http://www.medicinescomplete.com/about/index.htm). It seems that chloramphenicol penetrates less through the damaged skin.

However, there are some factors, which are important to be taken into the consideration when tape-stripping is used to induce damage to the skin, in respect to achieving homogenized and reproducible removal of corneocytes. These factors are the type of tape used; it should have an adhesive layer that is uniform both in the composition and distribution. The whole skin sample should be covered and the application pressure and duration should be the same for every tape application and the removal velocity and direction should be the same for all tape strips. The tape strips can be weighted before and after use to determine the mass of removed *stratum corneum* or the protein content on the tape can be used to determine the amount of removed *stratum corneum* (Lademann et al., 2009). When we applied the tapes the whole *stratum corneum* were covered and we tried to have the same pressure every time the tapes were applied, we also tried to keep the duration constant and to remove the tapes with the same velocity every time.

However, based on the discussion proposed by Landemann et al. 2009, a more reproducible method to perform tape-stripping should have been used. A more reproducible method to perform tape-stripping can be gained by fixed weighting, fixed duration of applied tape on skin, a roller used to adhere the tape can be used to adhere the tape uniformly and by removing the tape with the same velocity every time.

We also observed the greasy layer over the pig ear skin, which seemed to protect the actual removal of corneocytes. The skin from the bigger pigs was thicker which will affect the penetration of chloramphenicol with limited lipophilicity.

The tape-stripping procedure was stopped after 41 tapes because Klang et al. (2012) observed a peak transepidermal water loss value after the removal of 40 tapes and further removal of strips resulted only in the minor increase in transepidermal water loss. In addition, chloramphenicol, being neither highly hydrophilic nor lipophilic, might behave differently than some of the more hydrophilic drugs.

4.2.2.3 The penetration studies using chloramphenicol solution (3 mg/ml) and the skin with damaged barrier properties (strong alkali)

The aim of treating the pig skin with strong alkali, 3 M NaOH, was to see if this could yield increased damage to the skin barrier. The exposure times of 5 and 15 minutes were used, with the aim of complete barrier disruption. The treatment was done only to the outside of skin (*stratum corneum* site) and if the alkali came into the contact with the inside side of the skin sample, the skin was discarded. When the exposure time was 5 minutes, we observed a change in the color of the skin from pink to slightly yellow. The skin was also sticky. When the skin was treated with 3 M NaOH for 15 minutes, the skin became clearly yellow and sticky. Interestingly, the experiments showed that the cumulative chloramphenicol penetration through the skin treated with 3 M NaOH for 5 minutes was 49.79 ± 9.74 % with 7 parallels. Whereas the cumulative chloramphenicol penetration for 15 minutes exposure was 31.28 % ± 2.39 % with 6 parallels (Figure 13). This would indicate that shorter exposure to strong alkali leads to increased penetration of chloramphenicol.

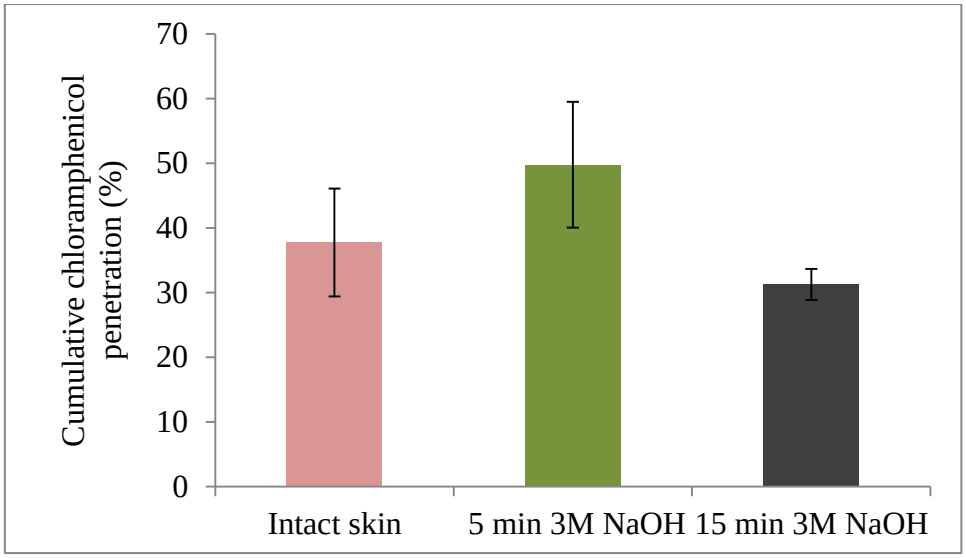

Figure 13: Cumulative chloramphenicol penetration through the intact and skin treated with 3 M NaOH for 5 and 15 minutes. The data are presented after 5 hours experiments. (n= 2)

One possible explanation on why shorter exposure leads to increased penetration could be the swelling of the skin and a more loose structure of *stratum corneum* is gained, as immediate reaction to the contact with strong alkali. After longer time, delipidization occurs in a way

which makes the skin contract and reduced penetration of chloramphenicol is observed. This phenomena needs to be further evaluated and may be of particular interests in respect to the development of *in vitro* models of compromised skin. However, the untreated skin showed a lower penetration of chloramphenicol. Petiot et al. (2007; 2010) reported no significant difference in penetration of uranyl nitrate through the rat skin treated with 10 N NaOH with different duration of exposure. The duration of exposure in their experiments was 30 minutes, 2 hours and 6 hours.

4.2.2.4 The penetration studies using chloramphenicol solution (3 mg/ml) and the skin with damaged barrier properties (heat-induced damage)

After failing to prove significant damage to the skin barrier by the strong alkali, we used the heat as a means to damage the skin. We prepared the experimental set up in a way to ensure that only the outside of the skin was exposed to hot water. The ear was held in a hot water for 15 seconds and the temperature during the exposure was maintained at 89-91 ˚C. The damage was visible as the skin, which was exposed to the warmed water, visually swelled. This type of the damage, by hot water, included two known factors influencing the penetration through *stratum corneum*; namely hydration and heat. The cumulative penetration of chloramphenicol through the skin held in warm water was 23.87 ± 7.07 % with 6 parallels (Figure 14).

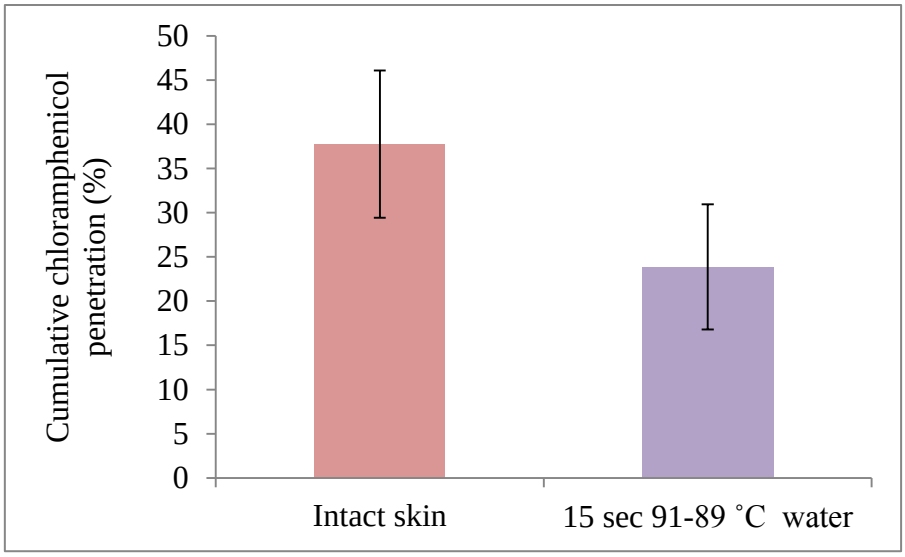

Figure 14: Cumulative chloramphenicol penetration through the intact and skin treated with hot water after 5 hours. (n= 2)

Although the hydration of *stratum corneum* is expected to result in a less compact structure of this layer, which in turn results in an increased penetration of most substances (Barry, 2001), we did not observe the same trend. However, the mechanism of how exactly the hydration increases the transdermal delivery of substances are not fully understood (William and Barry, 2004). The temperature of the water used to damage the skin should have been effective in inducing damage, as it has been reported that to damage the skin barrier by heating/branding, the burn temperatures should at least reach 70 ˚C (Behl et al., 1980; Flynn et al., 1981). Flynn et al. (1981) investigated how scald burns caused at 60-98 ˚C (for 60 seconds) influenced the penetration of mouse skin and observed that the penetration increased with increasing temperature. The experiments showed that penetration increased mostly in the temperature range from 70 to 80 ˚C. Another interesting finding was that the compounds that had more hydrophilic characters showed increased penetration through burned rat skin (Flynn et al., 1981).

However, the results from our experiments showed not increased penetration of chloramphenicol, which might by due to the fact that chloramphenicol is not highly hydrophilic and the duration of the exposure (15 seconds) was not enough to open up the

structure of the *stratum corneum*. However, swelling of the skin sample was observed which may be due to the hydration of the skin.

4.2.2.5 The penetration studies using chloramphenicol solution (3 mg/ml) and the skin with damaged barrier properties (burned skin)

In order to completely damage the barrier properties of the skin, we burned the skin with an open flame (propane burner) from approximately 2 centimetres distance that was moved in cycles in order to treat the whole skin sample. The burning resulted in a clear change of the skin colour, from pinkish into brown after the burning. The penetration of chloramphenicol through the burned skin was 20.68 ± 5.06 % with 6 parallels, surprisingly less that the penetration through the intact skin (Figure 15).

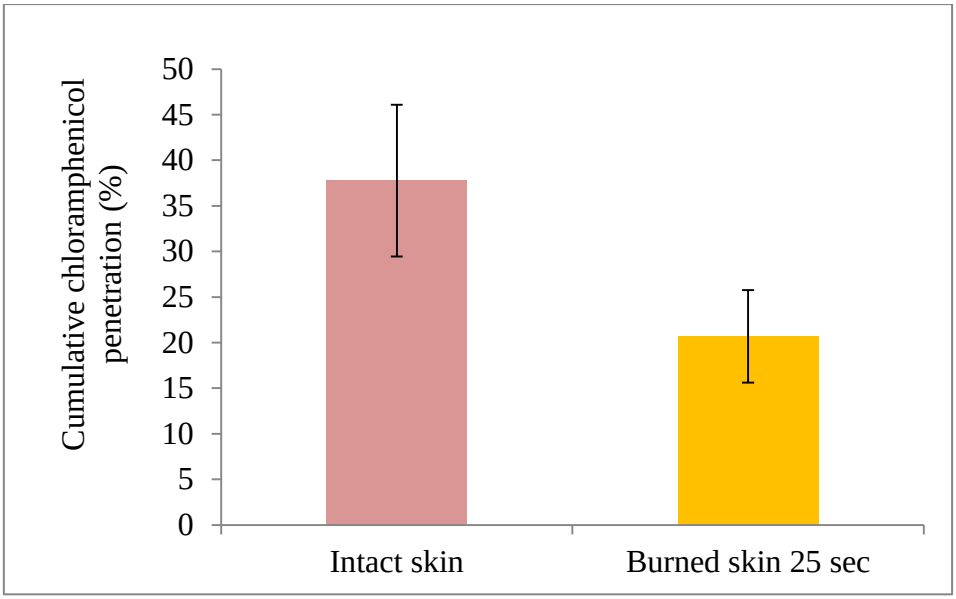

Figure 15: Cumulative chloramphenicol penetration through the intact and burned skin after 5 hours. (n= 2)

Behl et al. (1980) and Flynn et al. (1981) reported that a temperature used for burning the skin should be above 70 ˚C. The temperature of the flame obtained from propane burner was not measured. However, the damage was visible as the skin was brown after the burning. If the skin were burned further (more than 25 seconds) the skin would be black which in turn would

colour the acceptor phase in the Franz diffusion cell and influence the absorbance measurements performed after the penetration experiments. However, the penetration of epinephrine through burned skin was reported, by Papp et al. (2009), to increase compared to non-burned skin. The results from our experiments did not showed increased penetration which can be due to the method used for burning of the skin. We tried to hold the flame near the skin during the exposure, but the distance was not fixed and although the flame was moved around the whole skin it might be that the parts were not exposed equally to the flame. We tried to assure a constant flame distance (2 centimetres) every time, however, the flame size was difficult to control.

4.2.2.6 Summary of the penetration study with chloramphenicol solution (3 mg/ml) through the intact and compromised skin

The results from all experiment with the intact and treated skin are presented in Figure 16. None of the results from the penetration study indicates that the treatments used to induce the damage to skin in this study increased the penetration of chloramphenicol as compared to the penetration through the intact skin.

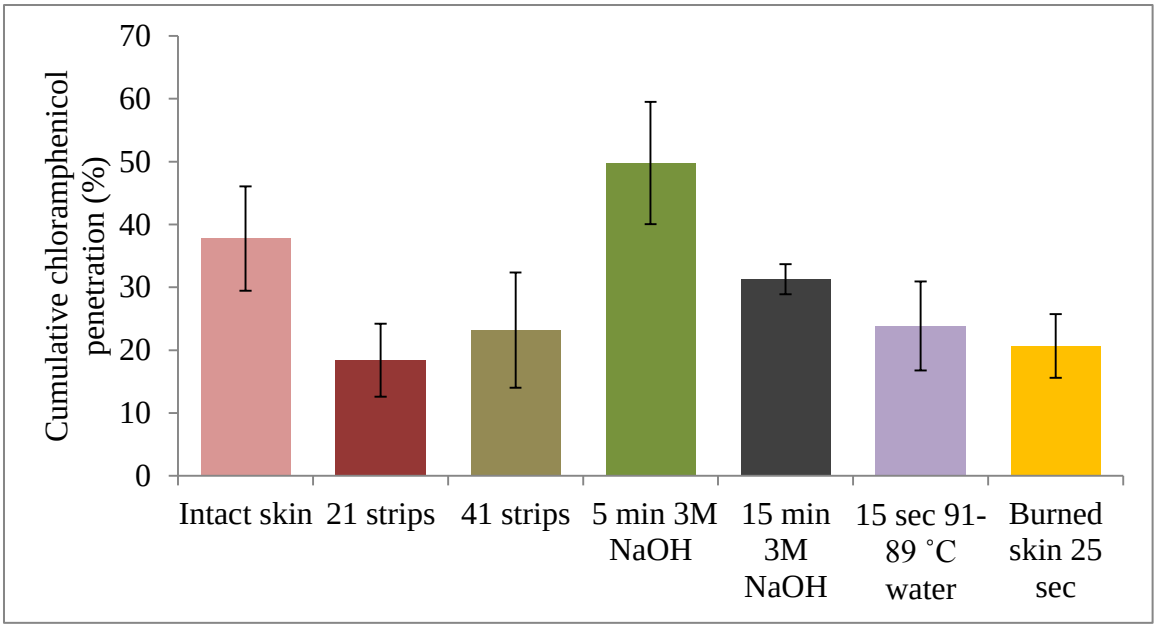

Figure 16: Summary of the cumulative chloramphenicol penetration through the intact and compromised skin after 5 hours. (n=2) for all experiments, except for 21 times tape-stripped skin (n=1)

Although it may appear that the methods used to induce the damage in skin barrier were not successful, one should also consider that the pig ears (biological material) were collected from different pigs (age). We observed the differences in the skin during the performance of the experiments. Some of the skin slices were greasier that influenced the tape stripping of the skin. This can be observed in Figure 16 where the standard deviation of the 41 times tape-stripped skin is large. However, the tape-stripping was not performed with an optimized method. The thickness of the skin slice varied (1.20-1.99 mm) as some of the pig ears slaughtered were clearly bigger.

However, another important consideration is that only two experiments were performed with intact and compromised skin, but one with 21 times tape-stripped skin. The numbers of parallels might be too low to draw a conclusion. However, the findings also indicate that the use of animal skin as an *in vitro* model for skin with damaged barrier properties might not be the best choice. The PVPA method has shown to be reproducible when permeability studies were performed with calcein and ibuprofen, as the permeability values achieved by us was

reproducible compared to Engesland et al. (2013). The permeability experiments with chloramphenicol performed by us showed also that the PVPA method is reproducible.

4.3 The PVPA barriers and the effect of ethanol concentration

The PVPA models for skin permeability can be tuned to express desired level of leakiness mimicking compromised skin (Engesland et al., 2013). Ethanol is used to prepare the PVPA barriers as it induces higher degree of fusion in the freeze-thaw process during the preparation of PVPA barriers (Flaten et al., 2006a).

Liposome suspensions of large liposomes (extruded through 1200 filter nm), remaining on top of the filter support of the PVPA barriers, were prepared with varying concentration of ethanol. The concentrations of ethanol in the large liposomes were 0, 5, 10, 15 and 20 % (v/v), respectively. The small liposomes (extruded through 400 nm filter) going into the filter support were prepared with 10 % ethanol. The aim of preparing liposomes (extruded through 1200 nm filter) with varying concentration of ethanol was to prepare the PVPA barriers with different degree of leakiness as it was recently reported by Engesland et al. (2013) that varying the ethanol concentration in the PVPA preparation resulted in the different degree of leakiness. However, Engesland et al. (2013) changed the ethanol concentration in the liposome suspension used in the preparation of both 400 and 1200 nm liposomes used to make PVPA barriers. In this study the difference in the ethanol concentration was only made in the preparation process for large liposomes. The permeability studies were performed with chloramphenicol solution (3 mg/ml). The results from this study are presented in Figure 17.

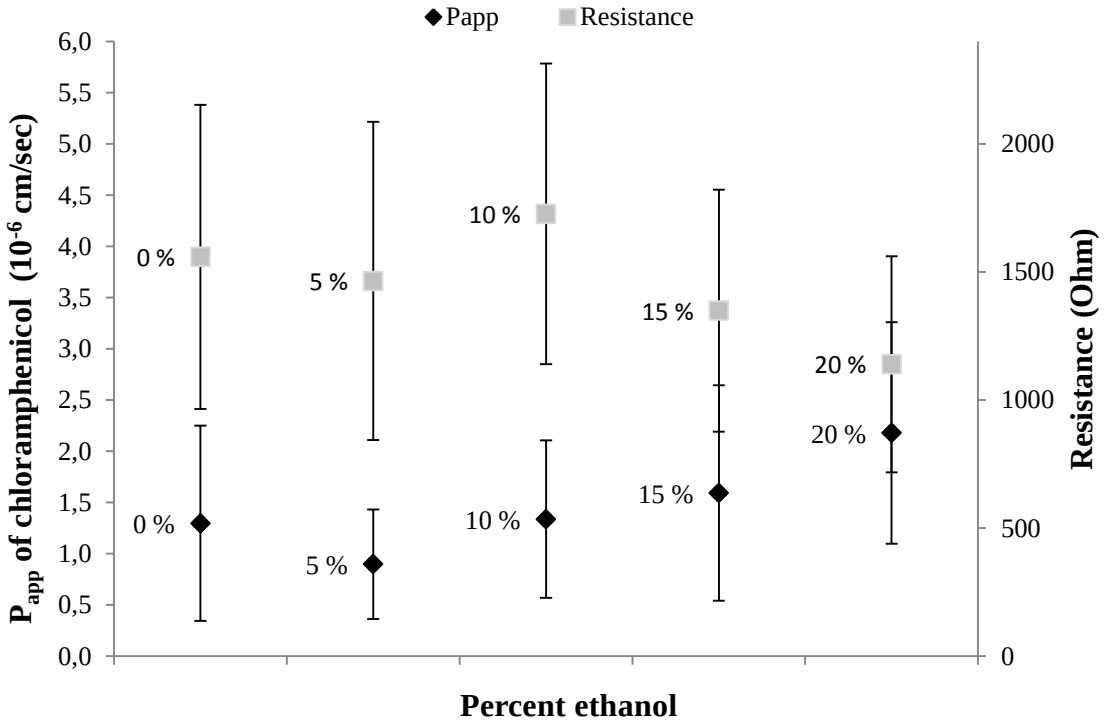

Figure 17: The effect of increasing ethanol concentration in the liposome suspension on the permeability values (P_{app}) of chloramphenicol and barrier resistance (ohm). (n= 2) for 0 and 5 % ethanol. (n= 3) for 10, 15 and 20 % ethanol.

The results from the permeability experiments with PVPA barriers prepared with various concentration of 96 % (v/v) ethanol in the liposome suspension showed different results than expected. Our results showed that changing ethanol concentration in the preparation of 1200 nm liposomes did not result in increased permeability. The permeability experiments were performed with 14 parallels with 0 and 5 % ethanol, 18 parallels with 10 % ethanol and 22 parallels for both 15 and 20 % ethanol in the PVPA barriers. The results presented in Figure 17 (both permeability value and resistance) indicate no differences in the integrity of PVPA barriers with the various ethanol concentration which is different from the results obtained by Engesland et al. (2013). They observed that the PVPA barriers prepared with 10 % ethanol concentration were tightest with the most reproducible results. While the barriers with the ethanol concentrations below and above 10 % (v/v) were more permeable to calcein. However, it is difficult to conclude that changing the ethanol concentration in the liposome suspension does not make a barrier with different degree of leakiness based on the data presented in Figure 17, as the number of parallels included in the permeability studies was too

small. If the permeability studies were performed with a larger batch with each concentration, we may have obtained different results. Another factor affecting the permeability studies is that chloramphenicol exhibits low absorbance and the measurement of absorbance in the sample from the acceptor chamber (after the experiment) is performed spectrophotometrically. To avoid the problem with chloramphenicol having low absorbance the HPLC method can be used to measure the permeability of chloramphenicol through the PVPA barriers.

5. Conclusions

Compromised skin and the penetration of drugs applied to compromised skin represent a challenge in development of topical and transdermal formulations. Our results from the penetration experiments with chloramphenicol as model drug showed that the conventional methods used to induce damage often fail to increase the penetration as compared to intact skin. Only the short time exposure to strong alkali damaged the barrier properties and resulted in increased penetration. We showed that the use of animal skin (biological material) is limited by the variations (thickness, age and greasiness) that are difficult to control.

Therefore, it is important to develop a model which is reproducible and herewith we are proposing the modified PVPA barrier. The permeability of both calcein and ibuprofen were measured with PVPA barrier assay that mimics skin and the results were found to be close to those earlier reported by our group. The PVPA barriers can be used to model the compromised skin and by the targeted manipulation of the preparation procedure, it is possible to mimic the different degrees of barrier damage. Moreover, the preliminary stability testing of the barriers indicates their stability, a very important parameter in respect to potential scale up.

6. Perspectives

Development of the phospholipid vesicle-based permeation assay (PVPA) as a model of compromised skin

- In the further development of the PVPA barriers, the experiments with larger number of parallel are required as the number of parallels in the experiments in this study was too small
- Development of a HPLC rather than spectrocopical method can be necessary follows the low concentrations of the drug. Another drug with different lipophilicity could be applied, as well as highly hydrophilic drug

Stability testing of PVPA barriers

- More experiments during longer time frame could be performed.

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