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DEVELOPMENT OF AN *IN VITRO* MODEL TO STUDY COMPROMISED SKIN: PIGSKIN VERSUS THE PHOSPHOLIPID VESICLE-BASED PERMEATION ASSAY

BY

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Abstract

When the skin barrier is reduced, penetration of topical and transdermal drugs could potentially be enhanced and the risk of systemic effects is increased. The studies analysing penetration through intact or diseased skin are often limited by the variability in obtaining specimens of representative skin. The phospholipid vesicle-based permeation assay is an artificial barrier mimicking human *stratum corneum* and can be used to determine the permeability of drugs through the skin. The model is highly reproducible. In this study we aimed to further develop this model to represent the compromised human skin by inducing the changes in its preparation process.

To evaluate the applicability of the model, the results must be compared to penetration data found in *in vivo* or *ex vivo* studies. For this purpose, we used pig skin, both intact and with induced damage in the barrier properties. The study was performed on the Franz diffusion cell systems by following the amount of the penetrated drug. The comparison of the permeability through the artificial barriers and pig skin was evaluated by using the model drug, diclofenac sodium.

The results from the *in vitro* experiments showed that it is rather difficult to induce damage in the skin batter properties when using the pig skin. None of the treatments applied to damage the skin or enhance the penetration of drug as compared to intact skin, but an interesting finding was the extremely low penetration through the acid-damaged skin. However, we managed to increase the permeability of diclofenac sodium through the phospholipid vesicle-based permeation assay. The findings confirmed that, in order to follow the penetration of topically applied drugs through the damaged skin, a more reliable method to use is the non-animal skin-based models.

Key words: compromised skin; phospholipid vesicle-based permeation assay; pigskin

Sammendrag

Når hudbarrieren, eller nærmere sagt hornlaget, er redusert eller skadet vil dette potensielt kunne øke opptaket av påførte topikale eller transdermal legemidler. Dette kan også, uheldigvis, øke risikoen for systemiske effekter og bivirkninger. Få studier har per dags dato undersøkt gjennomtrenging av legemidler over svekket eller skadet hudbarriere, og disse begrenses dessuten av variasjon i biologisk materiale, samtidig som det er imot etiske prinsipper. Det er derfor svært ønskelig og heller kunne utføre slike studier på mer forutsigbare *in vitro* modeller som ikke er basert på dyre- eller menneskehud.

Et eksempel på en slik *in vitro* modell er den såkalte fosfolipid vesikkel-baserte permeabilitetsmodellen, PVPA. Den er laget av komponenter som gjenfinnes i human hud og kan dermed gi innsikt i hvordan gjennomtrengning av legemidler over hud foregår. En annen fordel med modellen er at den er lett å forandre på, men den gir likevel reproduserbare resultater. Gjennom denne studien forsøkte vi å modifisere denne modellen til å etterligne svekket eller skadet hud ved å gjøre endringer i produksjonsprosessen.

For å kunne vurdere om modellen er representabel som human hud, må resultatene sammenlignes med *in vivo* eller *ex vivo* studier. På bakgrunn av dette ble hud fra griseører valgt som materiale og behandlet med ulike metoder for å redusere barrieren. I disse metodene inngikk blant annet tape-stripping, behandling med syre eller base og direkte brenning av huden. Effekten av behandlingen ble evaluert ved å brukes Franz diffusjonsceller og ved å se på mengden legemiddel som trengte gjennom huden. Målinger fra friske griseører ble brukt som kontroll.

Resultatene fra *in vitro* studien visste at ingen av behandlingene førte til økt gjennomtrenging av diclofenac sodium, men en interessant måling var den reduserte mengden av legemiddel målt gjennom syre-skadet hud. Men resultater fra den fosfolipid vesikkel-baserte permeabilitetsmodellen ble mengden gjennomtrengt legemiddel økt betraktelig. På bakgrunn av våre funn, kan denne modellen også etterligne skadet hud og gi mer pålitelige resultater i studer i forhold til *in vitro* forsøk som bruker biologisk materiale.

List of Abbreviations

AD	Atopic dermatitis
CER	Ceramide
CHOL	Cholesterol
CHOLSUL	Cholesterol sulphate
DS	Diclofenac sodium
E-80	Egg phosphatidylcholine
FDC	Franz diffusion cell
FFA	Free fatty acid
GIT	Gastrointestinal tract
ICD	Irritant contact dermatitis
PB	Phosphate buffer
PC	Phosphatidylcholine
PCS	Photon correlation spectroscopy
PVPA	Phospholipid vesicle-based permeation assay
PAMPA	Parallel artificial membrane permeation assay
SC	Stratum corneum
SD	Standard deviation
SLS	Sodium lauryl sulphate

TEWL	Transepidermal waterloss

1. Introduction

1.1 Skin

The skin is the largest organ in the human body with 1.8 m^2 surface and it represents 16 % of the body's own weight (Sherwood, 2010). Historically, the skin has been used for topical delivery of drugs in many years, but since the 1970s the transdermal patches actually has been used for systemic delivery. This type of drug delivery is challenging because of restricted permeability across the skin barrier and it is consequently investigated (Prow et al., 2011). Transdermal development of drugs has grown in recent years, because it among other things reduces side effects and avoids first pass metabolism (Stahl et al., 2010).

1.1.1 Skin functions

The main function of the skin is to protect the body against external factors by providing:

- The physical barrier by the outer layer in the skin, *stratum corneum* (SC)
- The chemical and biochemical protection which involves skin lipids, acids, enzymes, microbiological peptides and macrophages
- The immunological protection composed of cellular and humoral compounds of the immune system (Baroni et al., 2012).

Others functions of the skin include preventing the loss of fluid, reducing the UV-penetration, the regulation of body temperature and the synthesis of vitamin D (Sherwood, 2010).

1.1.2 The skin structure

The skin consists of three main layers; the epidermis, dermis and subcutaneous layer, as shown in Figure 1. Each layer is further divided into more layers (Baroni et al., 2012), which makes it difficult for microbes to penetrate into the body and cause an infection (Sherwood,

2010). Epidermis is the outermost layer and it is about 0.8-1.5 mm thick and consists mostly of corneocytes (Gawkrodger, 2008). These are flat, non-nucleated (dead) cells containing water and keratin filaments (Baroni et al., 2012). The epidermis does not have any direct blood supply, but receives the nutrients through the diffusion from the underlying tissues (Sherwood, 2010).

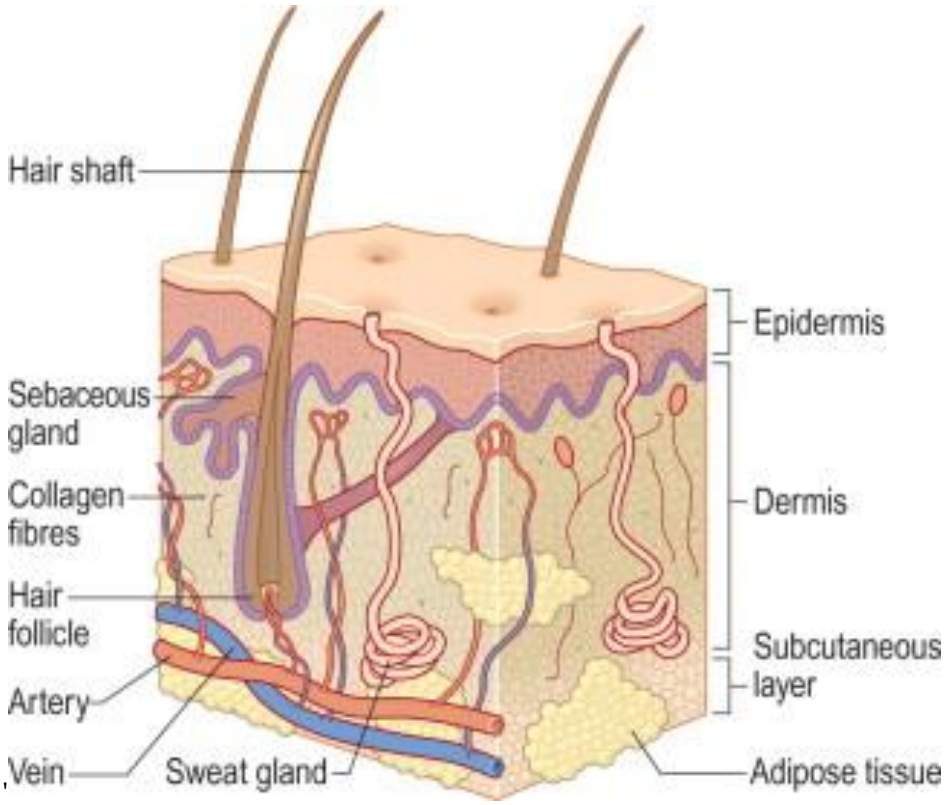

FIGURE 1: STRUCTURE OF THE SKIN (SPROAT ET AL., 2006)

The second layer is the dermis - a connective tissue with collagen fibres providing the strength, elastin fibres for the elasticity, the sweat glands for producing salts and the hair follicles for secretion of keratin/proteins to increase sensitivity (Sherwood, 2010). Dermis can vary in the thickness from 0.006 mm on the eyelids to 0.8 mm on the palms (Aulton, 2007). The dermis supplies blood vessels involved in temperature regulation and nerve endings able to detect pressure and pain. The subcutaneous layer is the underlying adipose tissue, attending for energy metabolism, storage and isolation of heat (Baroni et al., 2012; Sherwood, 2010).

1.1.3 Lipid structure and cell differentiation in the skin

For skin to remain healthy and fresh, it needs to maintain the balance between proliferation and desquamation. As some cells divide, others need to die and be removed to provide space for the new cells. Normal skin in younger adults renews itself in approximately 20-28 days (Baroni et al., 2012; Grove and Kligman, 1983), but is lengthened by more than 10 days in adults. This is because the decline in the epidermal cell renewal is constant in the youngest, but drops dramatically after the age of 50 (Grove and Kligman, 1983). Unfortunately, a disturbance in the cell proliferation and desquamation-process can result in different skin disorders, such as psoriasis (Baroni et al., 2012). Cell-growth occurs in the lower epidermis and as the cells migrate through the skin-layers, they differentiate from being living keratinocytes to dead corneocytes (Bouwstra and Ponec, 2006).

During the migration in the layers, the cells are stimulated to produce keratin. Keratin accumulates in the keratinocytes in the basal layer, and throughout the different layers in the epidermis, the cells will interact with a protein called fillagrin. Because of these tight interactions and the exposed stress, the cells will collapse and become flat corneocytes. The corneocytes floats in the extracellular matrix and are surrounded by different proteins forming an envelope-like structure. The envelopes are held together by corneodesmosomes to provide a good cohesion within the skin (Bouwstra and Ponec, 2006; Baroni et al., 2012).

Free fatty acids (FFA's) are produced of skin-lipids by the keratinocytes and are packed in small organelles called lamellar bodies. The lamellar bodies fuse with the membrane of the cells and form a lipid envelope. The envelopes secrete lipids into the spaces between the cells and are arranged into intercellular lamellas, parallel to the cell surface. The lipid envelope will finally bind covalently to the envelope-like structure in the matrix, assuring a good chemical resistance (Baroni et al., 2012).

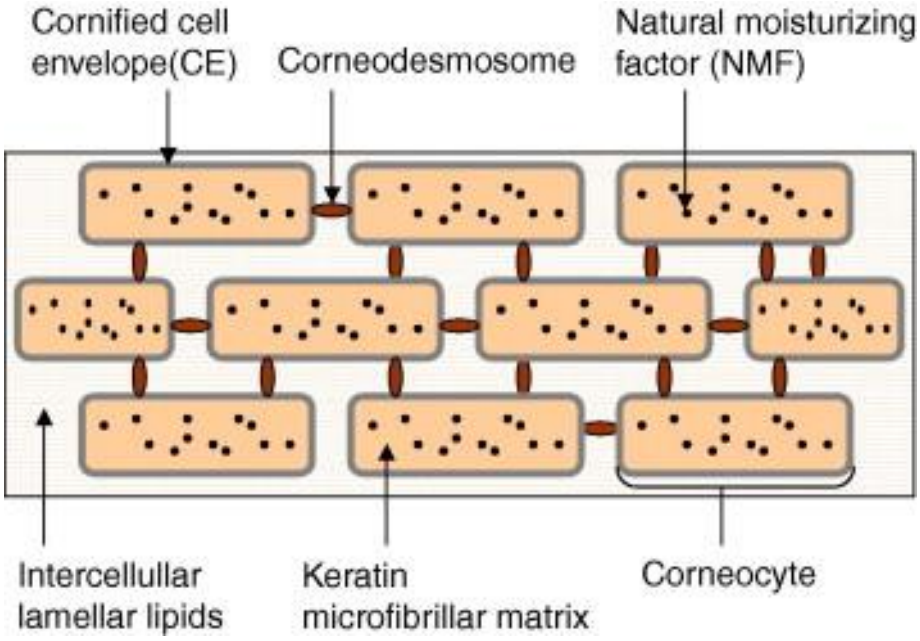

FIGURE 2: ILLUSTRATION OF THE "BRICK AND MORTAR"- ARRANGEMENT IN THE SKIN (WIEDERSBERG ET AL., 2008)

The envelope-structures are often described to be the "brick and mortar"-arrangement, as shown in Figure 2. The flattened corneocytes are referred to as the bricks and the mortars are the lipids arranged in the bilayers (Prow et al., 2011). These lipids can be FFA's, cholesterol (CHOL), cholesterol sulphate (CHOLSUL), ceramides (CER's), triglycerides or sterol-esters. Natural moisturizing factor is also present to maintain the suppleness (Benson, 2005).

The barrier function of the skin is dependent on protein structures and lipid organization present in the outermost layer, the SC (Bouwstra and Ponec, 2006).

1.1.4 Stratum corneum

The SC is a part of the epidermis and is about 0.4-10 µm thick. It is the most important layer in the skin regarding its permeability, because of the rate-limiting diffusion through the skin (Prow et al., 2011). The main building-components are FFA's, CER's, CHOL and a smaller amount of CHOLSUL. The most predominant cells present in the SC are the corneocytes (Prow et al., 2011). Although the cells are dead, the disulphide-bonding between the keratin assures the SC strength (Grawkrodger, 2008).

The SC can absorb impressively 3 times more liquid than its own weight (Grawkrodger, 2008). The water is an important molecule in respect to stabilization of the skin structure. Because most of the water is bound to proteins, it also provides mechanical properties (Konda et al., 2012). If the amount of water drops below 10 %, the skin will dry and its pliability fails (Gawkrodger, 2008). The SC also controls the diffusion of substances outside on the skin and the transport across this layer is mainly based on passive diffusion (Prow et al., 2011).

1.2 Transport across biological membranes/epithelial barriers through the skin

There are different approaches for drugs and metabolized substances to cross any biological membrane (Brandl et al., 2008). Some substances require a receptor to cross the barrier, which can be G-protein coupled- or inotropic receptors present in the skin (Baroni et al., 2012). Transport of different molecules can be classified as either active or passive transport. The differences between these are the consumption of energy during the process: the active transport requires energy to work and can therefore move against a concentration gradient (Brandl et al., 2008)

Passive transport is divided into three main groups where the simple diffusion is the most common. Simple diffusion is subdivided into the passive diffusion and facilitated transport. These two types are physiological processes which describe the net transport of the movement of a solute along a concentration gradient. The extent of diffusion of any molecule across membranes is affected by the physiological and chemical properties of the permeant, but also by the interaction between the molecule and the membrane itself (Brandl et al., 2008).

Simple diffusion is described by an equation called Fick's first law. The equation (1) identifies important parameters for diffusion across the skin. The steady-state flux (J) is dependent on:

- The net-amount of diffusion of drug in the SC dependent on time and surface unit, the diffusion coefficient (D)
- Membrane thickness/diffusional path length (h)
- The ratio of concentration of un-ionized drug between SC (lipophilic) and the vehicle , the partition coefficient (P)

- The concentration (C_o) of the applied drug (Benson, 2005)

$$J = \frac{DC\text{o}\text{P}}{\text{h}} \qquad \text{(EQUATION 1)}$$

Molecular sizes less or equally to 500-600 Da have shown to be optimal according to permeation, since it will make the diffusion coefficient high. The partition coefficient of a molecule should also be intermediate, because the compound then has adequate solubility within both lipophilic and hydrophilic regions in the epidermis and permeation will be easier When all parameters are ideal, transdermal delivery is feasible (Barry, 2001; Benson, 2005).

The facilitated transport occurs when molecules pass through membranes or barriers using special channels, but most intrinsic compounds and drugs use the passive diffusion to cross the membranes (Brandl et al., 2008).

Compounds that could cross the skin barrier, will pass through SC using two main routes called transcellular or intercellular, respectively. Transcellular path is described by the passage through the cell and intercellular as passing in between the cells, as shown in Figure 3 (Brandl et al., 2008). The intercellular route is favourable for most molecules, especially small and lipophilic solutes (Prow et al., 2011). The rate of diffusion is dependent on the lipophilicity and size of the molecule (Benson, 2005).

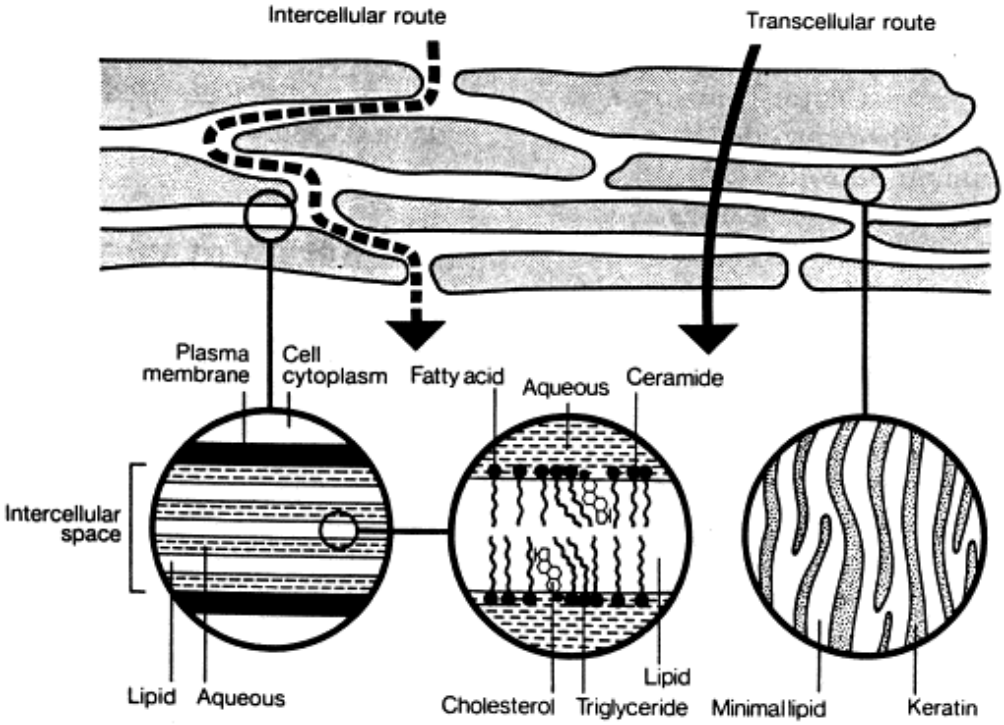

FIGURE 3: ILLUSTRATION SHOWING TWO MAJOR ROUTES FOR DRUG PENETRATION THROUGH THE SC (SUHONEN ET AL., 1999)

The transcelluar route is preferred for polar molecules or solutes (Prow et al., 2011), and is depending on the partitioning between the membrane and the aqueous compartment (Brandl et al., 2008). There are also existing other appendageal routes; through the sweat ducts and via the hair follicle or the sebaceous glands. These routes can be utilized when dealing with some of the polar and larger molecules or ions (Benson, 2005).

1.3 Compromised skin

When the skin is compromised by injuries or diseases, it will generate different degrees of inflammation (Aulton, 2007). Gattu and Maibach (2010) presented an overview over the few *in vitro* human studies performed to determine the absorption through diseased skin (not burned). Based on their findings, it can be concluded that it looks like the inflammations cause modest increased absorption, especially for hydrophilic compounds. This is why patients with skin diseases, such as psoriasis, always should be careful when administrating local therapy onto the exposed areas (Chiang et.al, 2012). If drugs are applied on the reduced

barrier, it can result in higher drug absorption and unwanted systemic effects (Mohammed et al., 2012).

The reasons for limited data regarding absorption through compromised skin are:

- Difficulty in obtaining specimens of diseased skin
- Varied reproducibility
- Variability of skin damage (Gattu and Maibach, 2010)

In recent years a novel parameter has been included in newer studies. The parameter examines the barrier properties in different skin disorders and is called transepidermal water loss (TEWL).

1.3.1 Transepidermal water loss

The transepidermal water loss presents the passive movement of water through the SC and gives an indication on how well this layer works. Studies have shown that compromised skin shows significantly increased water loss values (Noor and Hussein, 2013), because the skin is less efficient in maintaining the water when the barrier is reduced. Determination of TEWL is frequently used in cosmetic and pharmaceutical industry to examine the skin response to irritation (Machado et al., 2010; Sotoodian and Maibach, 2012). The cosmetic industry is mostly interested in hydration of their ingredients, while TEWL can be used, with limitations, to examine the efficacy of products for wound healing as well. Some ingredients, for example detergents, have been found to give fake TEWL data by providing hydration or even decreased barrier function. The main limitation of TEWL-investigations is that the parameter measures the water loss only. The method does not, unfortunately, give any information on how permeability will differ when administering drugs with different physiochemical properties (Sotoodian and Maibach, 2012).

1.3.2 Factors affecting the skin permeability

The pH of the skin varies in values from 4.0-6.0 (Zheng et al., 2012) and is important for cutaneous antimicrobial defence. It is also involved in the regulation of enzymatic activation

in the skin and skin renewal (Baroni et al., 2012). The pH can be affected in endogenous ways by moisture, sebum, sweat, anatomic site, age, genetic predisposition, SC thickness and skin-proteins (Baroni et al., 2012; Zheng et al., 2012). As most drugs are weak organic acids or bases, they predominate in aqueous solutions in either ionized or unionized form, dependent on the pH and pK_a of the drug. The unionized form of acid/base is often lipophilic and the ionized form is hydrophilic. When pH is lower than pK_a, the lipophilic property predominates and the penetration through the skin could increase. If the pH in the skin varies, this will give a possibility in fluctuations in drug available for percutaneous penetration (Konda et al., 2012).

The variability in both inter- and intra-structures of anatomical sites of the skin is reported. It is explained by the different degree of maturity of the corneocytes and surface area. Sites of the body with thinner skin (eyelids) have smaller cells with less surface area, fewer cell-layers and shorter permeation path length. This can cause higher drug absorption in specific areas of the body where skin is thinner (Mohammed et al., 2012).

The influence of ageing on the skin barrier and penetration is still controversial, while a study by Branchet et al. (1990) showed smaller decreasing values in epidermal thickness with age, but found no significant changes between men and women. Luebberding et al. (2013) showed that sebum production significantly decreases (between the ages 40-49) and surface pH increases significantly with ageing (between the ages 50-60). The same study also found that hydration of skin only showed a low significant difference in the elderly group, while TEWL only had minor variations. All these factors will, unfortunately, influence the barrier function and permeability properties (Luebberding et al., 2013).

In respect to the SC thickness, it is generally accepted that the thickness is maintained during ageing, but the differences in the underlying tissues are still discussed. S-Møller et al. (2003) especially reported differences in between body sites in relation to thickness, while Pellacani and Seidenari (1999) reported significantly increased thickness in the lower part of the facial skin in varying ages.

There are also external factors that can influence the skin barrier and the pH. Some of these factors are detergents, chemicals, cosmetics and topically administered drugs. Changes in the pH are also reported to be involved in pathogenesis of some diseases, such as different types of dermatitis (Baroni et al., 2012).

1.3.3 Contact dermatitis

This type of dermatitis includes a large spectrum of different inflammatory skin reactions after the exposure to external agents, where irritant contact dermatitis (ICD) is responsible for approximately 80 % of the dermatitis cases (Mark and Slavin, 2006). ICD is mainly caused by contact with the agents that induce irritation, such as chemicals or temperature extremes (Fonacier and Boguniewicz, 2010). It is non-immunological in nature and caused by the direct epidermal keratinocyte damage after stimulus from a releasing factor (Mark and Slavin, 2006). The damage can cause diminution of cells, disorganization of lipids in the epidermis resulting in increased TEWL-values.

A common category of ICD for infants between 9-12 months is the one caused by diapers; nappy rash. This is caused by occlusion and friction from diapers. The occlusion will cause maceration because of enhanced exposure to urine, water and faeces (Fonacier and Boguniewicz, 2010). The water will make the surface more fragile, more sensitive to friction and allow increased permeation of other irritant substances. The alkaline pH of the urine and the enzymes in the faeces will not make the skin resist further irritation (Atherton, 2001).

Allergenic dermatitis is another type of ICD and affects genetically susceptible individuals who have an already sensitized allergen exposure (Mark and Slavin, 2006). The most common allergen is nickel and exposure of this can results in a cell-mediated hypersensitivity reaction involving the immune system (Fonacier and Boguniewicz, 2010).

1.3.4 Atopic dermatitis

Atopic dermatitis (AD) is mainly caused by abnormalities in the CER's composition (Chiang et al., 2012), but whether the penetration is affected or not is still debated. A study from 2009 by Ortiz et al. (2009) showed no increased penetration in atopic skin. However, a study by Hata et al. (2002), on the other hand, reported that penetration of hydrophilic yellow dye was increased in proportion to the severity of disease. Another study by De Jongh et al. (2006) tested the diffusion of sodium lauryl sulphate (SLS) in atopic patients and found a 1.5-fold higher diffusion. An *in vivo* study by Jakasa et al. (2006) supported the finding of enhanced penetration in the atopic skin, by finding a 2-fold increased penetration of SLS. In all of the mentioned studies healthy subjects were used as a control.

1.3.5 Psoriasis

Psoriasis is a genetic disease characterized by a hyperproliferation of the keratinocytes in skin resulting in a dysregulated inflammation, with partly of completely damaged SC. The immune system is also involved by decreased lesinoal infiltration of cells. This damage may result in increased TEWL and skin penetration (Habif, 2010; Chiang et al., 2012). An older study by Schaefer et al. (1977) showed that human psoriatic skin exhibited 3- to 10-fold enhanced penetration of triamcinolone acetonide, compared to healthy skin. Another study by Colombo et al. (2003) recovered an increase in cumulative amount of 5-methoxypsoralen through *in vitro* human skin, which was intermediate to healthy and psoriatic human skin *in vivo.*

The common factor during the studies is the use of human skin. This is behind ethical principles, so the recent year's different *in vitro* models have been developed to reduce the excessive use of human and animal skin (Markovic et al., 2012).

1.4 Models for testing skin permeability

There are different model used to predict the profile of newly discovered drugs. Some important parameters to check are toxicity, absorption, metabolism and excretion. One of the models used for screening transdermal drugs is the phospholipid vesicle-based permeation assay (PVPA). It shows promising results for rapid screenings and provided the possibility of screening a huge number of drug candidates. The PVPA is made of liposomes based on phospholipids and provides a very cell-like condition (Engesland et al., 2013; Flaten et al., 2006a). Another technique for screening transdermal drugs is the parallel artificial membrane permeation assay (PAMPA). This is a simple barrier on a solvent-coated filter with phospholipids, which allows screening of permeability properties (Brandl et al., 2008).

1.4.1 Parallel artificial permeability assay, PAMPA

In general, PAMPA is a useful system for predicting any new drug's permeability because the high throughput capability (Markovic et al, 2012). The model is used for determination of transcellular *in vivo* absorption. The lipids are dissolved in an organic solvent, for example n-

hexadecane, and placed on a filter support. Compounds are screened by coupling a spectrophotometer to the plate, which makes it a rapid and automated analysis (Brandl et al., 2008). There are also developed different types of PAMPA-models containing artificial compounds as isopropyl myristate and silicone or bio-mimicking barrier with CER analogues (Markovic et al., 2012). Also models containing sink-conditions is developed and used (Brandl et al., 2008).

Since PAMPA is prepared by using an organic solvent, it may not mimic an optimal *in vivo* situation, but the PVPA has its advantageousness to mimic this situation more closely. This is because it is composed of unique liposomes (Flaten et al., 2006a; Engesland et al., 2013).

1.4.2 Liposomes

Liposomes can be made of different types of phospholipids, among others phosphatidylcholine (PC). It is endogenous biomaterial and is considered to be non-toxic. PC is a quaternary ammonium choline with a hydrophilic head-group linked to a glycerol backbone and an ester. The organization of the compound is the hydrophilic part of the lipid towards the water-phase, and the alkyl-chains towards the middle of the membrane, as illustrated in Figure 4. Phospholipids are rarely used alone to build liposomes, but other lipids could be added to improve *in vitro* and *in vivo* stability. Liposomes can be prepared by several techniques, but the general principles of preparation are hydration of lipids, formation of liposomes and adaptation of size. Depending on the technique and lipid composition, the number of lamellae could vary (Brandl, 2001). The vesicles can;

- Vary from nanometre to micrometre in size
- Be either mono-disperse (same size) or poly-disperse (different sizes)
- Be uni-lamellar (one layer around the core) or multi-lamellar (more layers around the core)

These factors are important to consider when preparing the liposomes and further use (Brandl, 2001).

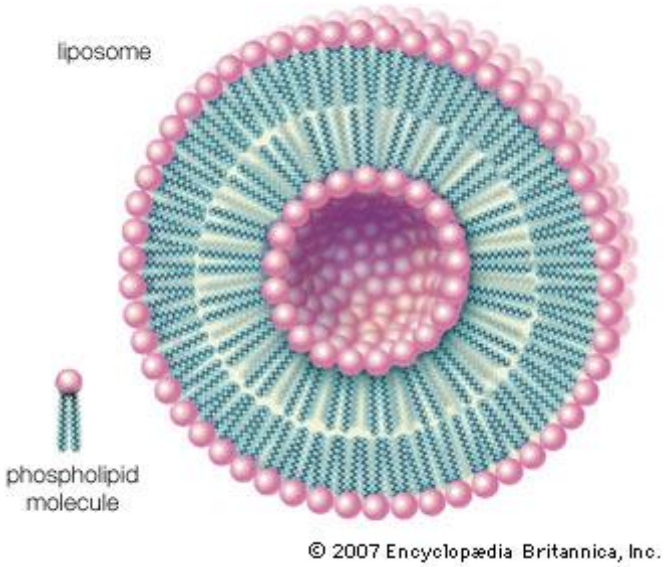

FIGURE 4: A TYPICAL STRUCTURE OF A UNI-LAMELLAR LIPOSOME (HTTP://WWW.BRITANNICA.COM/)

Liposomes have great potential as drug delivery systems. The vesicles can be used for:

- Diagnostic imaging
- Delivery of antibiotics
- Anticancer therapy
- Pharmaceutical application in general, but especially as drug carriers (Brandl, 2001)
- Mimicking biological barriers (Flaten et al., 2006a)

When liposomes are used as drug carriers, they can even solubilize water-insoluble drugs, provide a controlled release, be designed to target a specific site, overcome biological barrier and enhance immune response in vaccines (Brandl. 2001).

1.4.3 The phospholipid vesicle-based permeation assay, PVPA

The PVPA is an artificial barrier composed by liposomes, which originally was prepared from lipids resembling human intestinal epithelia (Flaten et al., 2006a). Because of improved importance and awareness of new drug's safety-profile in early drug development, it was necessary to develop a screening model for quantifying permeability of drugs. Many of the previous models for screening of intestinal drug permeation were too complicated, time-

consuming and did not model intercellular or active transport pathway. With this background, the PVPA was created for measuring the passive drugs permeability for oral drugs, and has been characterized, evaluated and tested with a range of drugs (Flaten et al., 2006a; Flaten et al., 2006b; Flaten et al., 2007; Flaten et al., 2008). The PVPA with the same component has been used for lead compound selection and formulation optimisation (Fischer et al., 2011; Hansen et al., 2011) and it can even measure very rapid permeation processes (Brandl et al., 2008).

The liposomes in the barrier are oligo- and/or multilamellar structured vesicles and mimic an *in vivo* condition (Flaten et al., 2007; Engesland et al., 2013). During studies, the PVPA with E-80 has shown to be better in predicting passive diffusion compared to bio-mimicking PAMPA and equally as good as PAMPA containing sink conditions (Flaten et al., 2006a).

Another relevant factor is the influence of agitation. The PVPA with E-80 is not affected by shaking/stirring, and studies have shown that it can withstand 200 rpm without any disturbance in terms of resistance. This is important because of a possibility in giving limited diffusion of lipophilic drugs by underestimating drug absorption (Flaten et al., 2007). Kansy et al. (2004) showed that highly lipophilic drugs, such as testosterone, exhibit a tendency to be affected by the stirring conditions in *in vitro* permeability assays. The reason why the PVPA is not affected by agitation is because of the characteristic morphology of the barriers, involving mostly aqueous compartments immobilized in a lipid-matrix (Engesland et al., 2013).

The model has recently also been developed to further mimic skin and has been suggested to be used as model for skin permeability for both healthy and compromised skin. The lipid organization in the human SC differs greatly from other barriers in the body, but by doing small changes in the lipid composition; the PVPA can easily mimic the SC. Since the model is a structured permeability model with the ability to manipulate the properties of the barriers, it opens the possibility of correlating the degree of leakiness with the degree of damage in compromised skin. The barriers can be developed to an *in vitro* model with SC properties mimicking both healthy and diseased skin, which would reduce the excessive use of animal and human studies for topical products in early drug development (Engesland et al., 2013).

1.4.3.1 Description and preparation of the PVPA

The barriers are made of tightly packed liposomes in different sizes. The liposomes can be extruded into desired size distributions and the preparation involves placing them onto a filter and further centrifugation. During the centrifugation, the smallest liposomes will be deposited in the filter-pores and the larger will lie on the top, as shown in Figure 5. By confocal laser scanning microscopy, it was proven that the filter pores were filled with liposomes and water channels were not present through the barriers (Flaten et al., 2006b). The barriers also need to undergo a freeze-thaw cycle to promote liposome fusion and to make it tight barriers. The final stage is to check the permeability of the barriers using calcein and to measure the electrical resistance over each barrier (Brandl et.al, 2008). Normal electrical resistance values for an intact barrier with E-80 are above 1000 Ω (Flaten et al., 2008). This value may vary depending on the lipid composition.

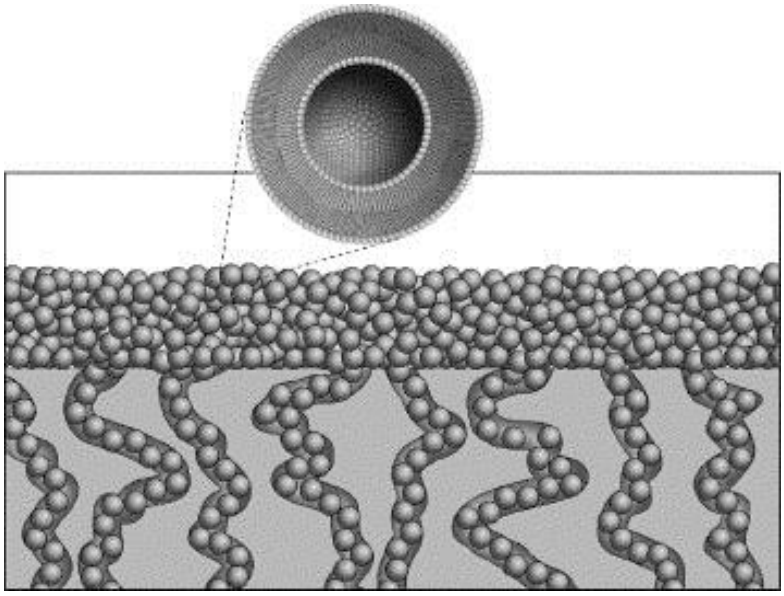

FIGURE 5: A HYPOTHESIZED ARRANGEMENT OF THE PVPA (FLATEN ET AL., 2006B)

Barriers containing only E-80 are tested to be stable in (80^{o}C) for 2 weeks (Flaten et al., 2006b), which makes it possible to produce bigger batches and the laboratory work more efficient. The same barriers have shown to be produced by robotic systems connected to a plate reader and this makes it useful for industrial screening (Flaten et al., 2009).

1.4.3.2 PVPA intestine with E-80

As mentioned earlier, this barrier is a model for medium-throughput screening permeability of drugs in the intestine. The major phospholipid in the barrier is PC from egg, E-80 (Flaten et al, 2006a).

The pH of the gastrointestinal tract (GIT) in humans is different in fasted and fed conditions, but it also varies in location. Between proximal jejunum and distal ileum is can vary in values from 6.0-8.0. It is therefore very advantageous that the PVPA is set to be stable under various pH-conditions (range of 2.0-8.0). This can give a more realistic insight in drug absorption in the GIT (Flaten et al., 2006b; Flaten et al., 2008).

1.4.3.3 PVPA with E-80/CHOL

This model is used to mimic the human SC and can be used to screen permeation of transdermal drugs. The only difference from PVPA originally developed with E-80 is the addition of CHOL (Engesland et al., 2013), which is one of the major lipids present in human skin. CHOL is a common lipid and introduces rigidity to the barriers and makes it more compact. The addition will influence fluidity and pliability, but the degree of influence is dependent on the composition of lipids (Wertz, 2000). CHOL is also making the barriers more robust to withstand harsher procedure conditions (Engesland et al., 2013).

1.4.3.4 PVPA with E-80/ CER/ CHOL/ CHOLSUL/ palmitic acid

This artificial barrier is made for mimicking the lipid composition in healthy human SC with all major lipid classes in the skin for screening permeation of transdermal drugs (Engesland et al., 2013). The preparation of the PVPA barriers with E-80/ CER/ CHOL/ CHOLSUL/ palmitic acid is slightly more complicated and complex as compared to PVPA with E-80/ CHOL, and the cost is higher.

1.5 Other in vitro skin models

Porcine skin is often used as an *in vitro* model for human skin in studies testing percutaneous absorption (Jacobi et al., 2007). Pig and human skin are more similar than first expected, where both have a compact layer with epithelium. The porcine skin shows a line of basal cells in epidermis at the border of the dermis. There are also sweat glands, hair follicles and fat cells present in the underlying tissues, likely to substitute human skin (Jacobi et al., 2007). The lipid organization of the pig SC is set to be almost similar to human SC, although the CER's isolated from pig SC are slightly different (Bouwstra and Ponec, 2006; Cilurzo et al., 2007).

Jacobi et al. (2007) measured the thickness of porcine epidermis on the ear to be 72 µm, whereas the human epidermis on the shoulder was 70 µm. New-born pig skin is said to be thinner and were representative than older pigs, but has a higher amount of hair follicles present, but has been investigated for being an alternative for human epidermis (Cilurzo et al., 2007).

The study by Jacobi et al. (2007) also observed an average of 20 hairs at 1 cm^2 at the porcine ear and a number of 14-32 hairs in human skin. They reported that porcine ear skin is the best suitable model of mimicking human skin, although the diameter of hair and infundibular orifices were different. Klang et al. (2012) also reported that pig skin shows essential permeation characteristics *in vitro* with human skin and said that porcine skin is a highly suitable model for *in vitro* tape stripping.

1.6 Inducing the damage to SC

1.6.1 Different ways to damage SC

There are many ways to induce disturbances to the SC, which either can be by tape-stripping, abrasion, heating, freezing or applying different chemicals (giving irritation or delipidisation). This can theoretically result in enhanced percutaneous drug absorption and increased TEWL values (Chiang et al., 2012).

Freezing is mostly used for conserving skin, but the effects it has on the skin composition are not known yet. A study of Sintov and Botner (2006) used diclofenac in vehicles on skin from rats, guinea pig and pigs, and showed decreased penetration when skin was frozen (-20 °C), compared to fresh (4 °C) skin. The explanation of this could be an effect in the structured lipid bilayer for lipophilic solutions, but more studies are needed to ensure the cause (Chiang et al., 2012).

However, the different methods used in studies of inducing damage to the SC can vary and give varying results (Chiang et al., 2012). This was demonstrated by Akomeah et al. (2008) in a study showing that destruction of SC with a rotating brush had higher drug permeation, while delipidisation and tape-stripping were second and third. But it is also important to remember that each study use different types of materials from different species, anatomical sites and thereby kinetics. Because of this, it is necessary to provide a well-characterized method to investigate absorption through compromised skin, especially during tape-stripping (Lademann et al., 2009).

1.6.2 Mechanically disrupted skin barrier

Tape stripping is a well-established method used to damage SC (Klang et al., 2012), and gives the possibility to study penetration and reservoir behaviour of topical drugs, physiology of SC, epidermal wound healing and excretion of endogenous compounds (Lademann et al, 2009). Such damage to SC would, in theory, increase percutaneous drug absorption and TEWL, especially for water-soluble drugs (Chiang et al., 2012). This was shown in a study by Akomeah et al. (2008), by increased permeation of angiotensin II when damaging the skin with tape stripping. The study also reported that hydrophilic components were more affected than lipophilic. One major advantage with this method is the way of controlling the damage by complete or partial removal of the SC, which can facilitate solute permeation by reducing resistance (Akomeah et al., 2008).

A study by Löffler et al. (2004) showed that in order to produce reliable and reproducible results when using tape-stripping, the procedure must be standardized. All parameters that can influence the result must be eliminated or controlled as far as possible (Lademann et al.,

2009). Such parameter could be anatomical site, skin-type, thickness of SC, pH, type of adhesive tape, duration of pressure onto the skin and removal of the tape (Löffler et al., 2004).

1.6.3 Chemically disrupted skin barrier

Most chemicals may penetrate the skin if they are administered topically, but the nature and concentration of chemicals are crucial. When applying chemicals on the skin, it can cause delipidisation by extraction of fatty materials. Current and previous used chemicals to influence skin barrier function are acetone, alcohol or chloroform. Acetone is said to increase penetration of hydrophilic compounds, but more data is needed to know the effects on lipophilic compounds (Chiang et al., 2012). Ethanol will cause delipidisation and gaps between the adherent cornified cells or even reduced cohesion between the keratinocytes. This will increase the volume available for the drug to cross the SC (Akomeah et al., 2008). SLS, a surfactant, is also a known skin irritant and is often used as a model for inducing dermatitis (Benfeldt et al., 1999). The explanation is said to be by modifying the structure of keratin in the skin, which makes the SC swell and hydrate (Chiang et.al, 2012).

An *in vivo* human experiment performed by Malton and Thiele (1973) showed that exposure to NaOH and Na_3PO_4 resulted in a 350 % increase in water loss. They explained this by collapse of skin proteins and increased porosity. Petitot et al. (2007) did a rat experiment with NaOH. These experiments did not show any change in uranium percutaneous penetration when comparing intact and chemically disrupted skin.

1.6.4 Burned skin

Skin-damage caused by burnings can be either moderate or severe conditions, resulting in bullas or complete destruction of the skin barrier. Experimentally, burning does not show any significant effect in temperatures under 70 ºC; the higher the temperature, the higher permeation-rate through skin (Boosalis et al., 1987). An *in vivo* study by Papp et al. (2009) measured serum concentration of epinephrine in burned patients when it was topically applied, and showed an increased concentration compared to intact skin.

1.7 Diclofenac Sodium as model drug

Diclofenac sodium (DS) is a non-steroidal anti-inflammatory drug of the group phenylacetate (Rang et al., 2008). Its structure is shown in Figure 6. The use of DS is widely known and there are registered different formulations (capsules, gel etc.) on the Norwegian market (the list can be found on the website of the Norwegian Medicines Agency).

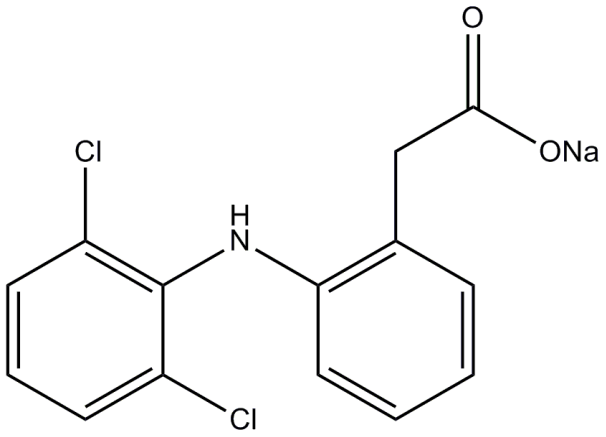

FIGURE 6: THE STRUCTURE OF DICLOFENAC SODIUM

The mechanisms for the major effects are mainly described by inhibition of the cyclooxygenase enzyme, which changes aracidonic acid to prostaglandins. This will result in decreased amount of prostaglandins in the body that leads to:

- Anti-inflammatory effects by reduced vasodilatation and indirectly less oedema
- Analgesic effects because of less sensitisation nociceptive nerve-endings and decreased vasodilation
- Antipyretic effects by preventing cytokines to elevate the set point of temperature control in the hypothalamus (Rang et al., 2008)

2. Aims of the study

Currently, there are very few studies dealing with penetration of drugs through diseased skin. Since most of the skin diseases are treated with topically administered drugs, the risks of having increased penetration and adverse effects are clearly present. The PVPA has recently been developed to mimic human skin and is suggested to be used as a model to determine permeability of topically or transdermally applied drugs. Our main aim in this study was to further develop this model into a suitable model mimicking the compromised skin. This could be done by making the changes in the preparation process, which would lead to the increased permeability of our model drug, DS.

In addition, the stability of such a model should be evaluated and we followed the integrity of the model during the long-term storage in the freezer (uptil 9 months).

To evaluate the applicability of the model, the permeability from the PVPA must be compared with the skin penetration data found through *in vivo* or *ex vivo* studies. By inducing the different levels of damage, we could gain insight on the extent of the skin barrier damage by following the penetration of DS. The damage of the skin barrier was induced by the following means:

- Tape-stripping
- Chemical treatment with an acid and alkaline solution
- Burning

The intact pig skin served as a control.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and drugs

Calcein, Sigma Aldrich Chemie GmbH, Steinheim, Germany

Chloroform, Merck KGaA, Darmstadt, Germany

Cholesterol, Sigma Aldrich Chemie GmbH, Steinheim, Germany

Diclofenac Sodium (DS), AWD Pharma GmbH, Radebeul, Germany

Distilled water, Tromsø, Norway

Egg phosphatidylcholine, Lipoid, Ludwigshafen, Germany

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

Ethylenedinitrilotetra-acetic acid (Titriplex III), Merck KGaA, Darmstadt, Germany

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

Methanol, Sigma Aldrich Chemie GmbH, Steinheim, Germany

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 dodecahydrate), Merck KGaA, Darmstadt, Germany

3.1.2 Equipment and devices

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

Biofuge stratos centrifuge, Heraeus instrument, Kendro laboratory products GmbH, Osterode, Germany

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

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

Cellophane foil, Bringmann folia, Wendelstein, Germany

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

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

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

Filter inserts (d= 6.5 mm) and 24-transwell plate, 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

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

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

Isopore membrane filters (1.2 µm), Merck Millipore, Billerica, USA

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

Millicell-ERS, Merck Millipore, Billerica, USA

NICOMP Photon correlation spectroscopy (PCS), model 370, NICOMP Particle sizing system (PSS), Santa Barbara, USA

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

Nitrogen gas, compressed (5.0), AGA, Oslo, Norway

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

PH-meter, 744, Metrohm, Herisau, Switzerland

Polarstar Galaxy, BMG Labtech GmbH, Ortenberg, Germany

REAX Top mini-shaker, Heidolph, Schwabach, Germany

Sartorius weight LP 4200S/ BP 211D/ LP 6205/ CP 225D/ LP 6205, Sartorius AG, Göttingen, Germany

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

Termaks incubator, KEBO AS, Bergen, Norway

Tesa SE 4124 adhesive tape (width of 50 mm), Beiersdorf AG, Hamburg, Germany

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

3.1.3 Animal tissue

Pig ears, Nortura AS, Bardufoss, Norway

3.1.4. Computer programs

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

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

Particle size analysis: PCS CW 388 (1.68, version 8.1), NICOMP Particle Sizing Systems (PSS), Santa Barbara, USA

3.2 Methods

3.2.1 Preparation of phosphate buffer, PB

1 litre PB was composed with:

- 600 mg KH_2PO_4
- 3720 mg Titriplex III (only if using calcein)
- 6400 mg Na_2HPO_4 dodecahydrate
- 7240 mg NaCl
- Distilled water up to 1000 ml

The pH was adjusted with 1 M HCl- or 1 M NaOH-solution to pH reached 7.4. The PB was used during all experiments in the thesis.

3.2.2 The Phospholipid Vesicle-based Permeation Assay, PVPA

3.2.2.1 Preparation of liposomes

Egg phosphatidylcholine, E-80 (694 mg) and cholesterol (206 mg) were dissolved in chloroform (15 ml) and hand-shaken until clear solution was observed, as described by Engesland et al. (2013). The organic solvent was evaporated using Büchi rotavapor. The temperature during evaporation was set to 47 ^{o}C and the pressure to 400 mBar and the pressure were lowered slowly down to 200 mBar to avoid boiling the solution. After the initial 2 hours, the pressure was set to 45 mBar for additional 3 hours at room temperature. PB (13.5 ml) and 96 % (v/v) ethanol (1.5 ml) were added to the dried film until everything hydrated. The dispersion was extruded at 10 bars in the Custom made extruder using compressed nitrogen gas at 40 ^{o}C. When the dispersion was filled in the chamber of the extruder the first time, it had to reach the actual temperature before extrusion (5 minutes). The

liposomes were extruded to obtain two different sizes through the 1.2 µm and 0.4 µm membrane-filters, 5 times each, respectively.

3.2.2.2 Particle size analysis

The size distribution (mean value) of the liposome-dispersion after extrusion was measured with NICOMP PCS. The sample preparation and measuring were done according to the method described by Ingebrigtsen and Brandl (2002). In brief, all glassware was sonicated with distilled water in Branson sonicator. Preparation of the samples was done in a laminar airflow bench to prevent contamination. The samples with vesicle dispersion were diluted with filtrated PB to reach count rate of 250-350 kHz in the PCS. The samples were placed in the machine for 5 minutes to eliminate the temperature differences (24 ^{o} C ± 1 ^{o}C). The analysis cycle was 15 minutes, whereas the original method was based on 30 minutes cycles.

3.2.2.3 Preparation of the PVPA

Each filter insert (d= 6.5 mm) was fused with 0.65 µm nitrocellulose-membrane, as described by Engesland et al. (2013) at 150 ºC on the IBR Heat-Press for 30 seconds. 100 µl of liposomes extruded through 400 nm were added to each insert in a 24-transwell plate, as shown in Figure 7. The plate was centrifuged at 950 *g* (2500 rpm) in Biofuge Stratos centrifuge. The sequence was repeated by adding another volume (100 µl) liposomes extruded through 400 nm on top and centrifuged with the same settings and time interval as previous mentioned. During this process, the small liposomes were filled into the filter pores preventing leakiness by water channels (Flaten et al., 2006b).

FIGURE 7: A 24-TRANSWELL PLATE WITH INSERTS

After placing the inserts on a slice of paper to remove liquid, they were placed in a 50 ^{o}C incubator for 45 minutes to evaporate the solvent. After 10 minutes at room temperature, 100 µl of liposomes extruded through 1200 nm was added and the plates was further centrifuged at 1030 *g* (2600 rpm) for 60 minutes. During this centrifugation, the bigger liposomes were placed on the top of the filter making it a tight barrier (Flaten et al., 2006b).

During the centrifugation, liquid is concentrated on top of the filters. This was removed by turning the inserts upside-down with a paper in the lid and centrifuged at 25 *g* (300 rpm) for 5 minutes. At least, the inserts were stored in freezer (-75 ^{o}C). Prior to the permeability experiments, the inserts were thawed at 30 ^{o}C in an incubator for 120 minutes. If the barriers were partly wet after the initial time, the process was lengthened until complete dryness was observed. The process with solvent evaporation and freeze-thaw cycle is used to promote tighter fusion of the liposomes in the barriers (Flaten et al., 2006a)

3.2.2.4 Permeation and integrity experiment of PVPA

The PB (600 µl) was added to each slot as acceptor medium (maintaining sink-conditions) in a 24-transwell plate. One insert was placed in each slot. The 5 mM calcein solution was used as a marker and added (100µl) to each insert when the analysis started. This solution was only

used to test the integrity of the barriers. Each sample from the acceptor medium in each slot (200 µl) was transferred to a 96-well black plate. Another sample (2 µl) from the supernatant on the top of each insert was taken after the analysis and diluted (1:100) with PB. The dilutions were transferred to the same plate as well. The standard curve of calcein was received from the Drug Delivery Transport and Delivery Research Group, Department of Pharmacy, University of Tromsø. The integrity was measured by using Polarstar Galaxy with excitation filter at 485 nm and emission filter at 520 nm.

For the permeability-testing, PB (600 µl) was added to each slot and a solution drug (100 µl) was added to the inserts. In the preliminary experiments an ibuprofen-solution (5 mM) was used, which was further replaced by DS (9.4 mM) as a model drug. Prior to the integrity-testing, each sample (200 µl) from the acceptor medium in each slot was transferred to a 96-well transparent plate. A sample (2 µl) from the supernatant on the top of each insert was taken after the analysis and diluted (1:100) with PB. The dilutions were transferred to the same 96-well transparent plate. The concentration of the samples were measured by using Spectramax 190 plate reader, which was programmed to the optimal wave length for DS; 280 nm. This was decided by making a standard-curve as described in 3.2.3.

The apparent permeability coefficient (P_{app}) was calculated by the equation:

$$P_{app}(cm = s) = \frac{J}{AC_d} \qquad \text{(EQUATION 2)}$$

J represents the observed flux rate (nmol/s), A is the surface area of the insert (cm^2) and C_d is the concentration of the donor (nmol/ml) (Engesland et al., 2013).

The experiments testing integrity and permeability lasted for a total of 5 hours in room temperature, where each insert was moved one slot the first 3 hours, and then every 30 minutes for 2 hours.

3.2.2.5 Electrical resistance measuring

Immediately after every permeation and integrity experiment, the electrical resistance over each barrier was measured. The resistance is measured to have a pointer in how compact the

barriers were, and is only a supporting measurement in according to P_{app}. Normal values for an intact barrier made of only E-80 lipids are reported to be above 1000 Ω (Flaten et al., 2008).

The measuring was done by using Millicell-ERS and the tip of the device was cleaned in fresh PB and placed into the supernatant of every insert until a stable value was displayed.

3.2.3 Preparing standard-curve for Diclofenac Sodium, DS

A standard-curve is needed for estimating the concentration of the drug (for DS in the thesis) in each sample. The preparation was done by making DS-dilutions from 1:5 to 1:10.000. A sample (200 µl) from every dilution, 12 copies of each, was transferred to a 96-well transparent plate and measured using Spectramax 190 plate reader. By doing literature search, the wavelength to screen the samples was stated to either 273 or 275 nm (http://www.medicinescomplete.com/). This was provided as a good starting point on which wavelength was relevant to use. The screening was done by varying the wavelengths from 220-280 nm. The results were recalculated and the optimal curve showing the best correlation (R^2-value= 0.9996) was chosen. The best wavelength in our experiment was decided to be 280 nm.

3.2.4 In vitro penetration on the Franz diffusion cell system, FDC

3.2.4.1 In vitro penetration experiment using FDC

When testing *in-vitro* penetration of drugs over the skin, a system called FDC is used. The system consists of a donor chamber (d= 15 mm), a membrane and a receptor chamber (12 ml volume), as shown in Figure 8. During the experiment, the present drug will diffuse from the donor chamber, through the membrane and down to the receptor chamber, where samples are taken.

Before doing permeation analysis, the FDC was cleaned with methanol, ionized water and finally distilled water. Each cleaning process lasted for about 30 minutes.

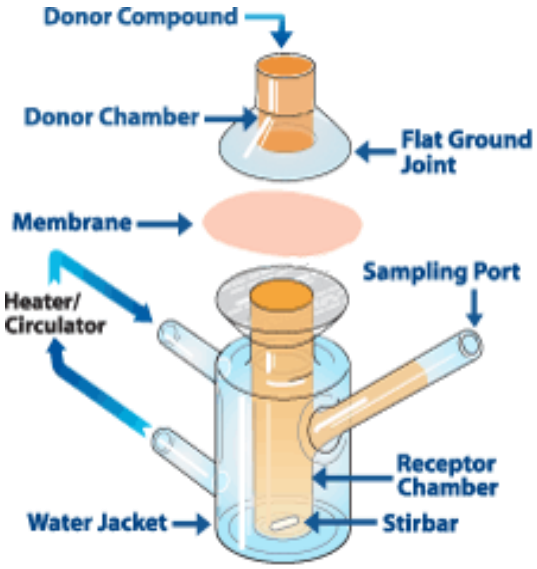

FIGURE 8: AN ILLUSTRATION OF THE FDC SYSTEM (HTTP://WWW.PERMEGEAR.COM/)

An O-ring was placed both over and under the membrane to prevent leakage. The membranes used during the experiments were made of cellophane (Bringmann folia) or the slices of pig ear skin; either in the intact form or with compromised barrier properties (tape stripped/chemically damaged/burned). The cellophane membrane was only used to establish an optimal method.

The typical set up of the experiments was as follows:

The receptor chamber was filled with PB (12 ml) and the stirrer inserted to ensure uniform stirring, as shown in Figure 9. The temperature was set to 32 ^{o}C by the help of a heating circulator. The membrane was placed on the receptor chamber and the donor chamber was placed on the top. They were both fastened with a pinch and filled with the sample (0.3 ml) containing DS solution (3 mM for cellophane membrane and 9.4 mM for pig skin). The chamber and the ports had to be covered with double layer of parafilm to prevent evaporation of the solution. The samples from the receptor chamber were taken continuously using a Hamilton syringe (250 µl).

FIGURE 9: PERMEGEAR V6A STIRRER (HTTP://PERMEGEAR.COM/)

The experiment lasted for a total of 5 hours. When using the cellophane membrane, the samples (200 µl) were taken with the same intervals as for experiments with PVPA. When using pig skin, a sample was taken after 10 minutes and later every 30 minutes. After each sample collection, the volume of the receptor chamber had to be replaced by PB to maintain the same volume. All the samples (200 µl) from each acceptor chamber during the experiment were collected directly in a 96-well transparent plate, covered with parafilm to prevent evaporation. Also samples (2 µl) from each donor chamber, diluted with PB (1:100) were transferred to the same plate as well. The remaining volume in the donor chamber was measured to do further calculations involving drug distribution in the system. The concentration of the samples was measured by using Spectramax 190 plate reader at 280 nm.

3.2.4.2 Preparation of skin slices

Pig ears are, by Jacobi et al. (2007), reported to be a suitable *in vitro* model for mimicking human skin when testing percutaneous absorption.

The ears were purchased from a slaughterhouse, Nortura AS, Norway. The pig ears were prepared by using a scalpel to remove blood vessels, cartilage and excess fat. Immediately after the preparation, the skin slices were rinsed in PB and wrapped in plastic (cling film). The

slices were stored in the freezer until the day before use. The defrosted, untreated skin was cut into slices matching the diameter of FDC (15 mm) and the penetration experiments were performed according to the procedure in 3.2.4.1.

3.2.4.3 Inducing SC-damage by using adhesive tape

The use of adhesive tapes to induce damage to the SC is a well-established method to investigate skin penetration of topically applied substances (Klang et al., 2012), and Morgan et al. (2003) showed in their study that it is an effective treatment to reduce the human SC.

Our procedure included cleaning the ears with 96 % (v/v) ethanol to remove surface fat. The skin was stripped with Tesa SE 4124 adhesive tape (width of 50 mm; Beiersdorf AG, Hamburg, Germany). The stripping was done only on the outer layer where SC is present. The number of stripping varied from 4 to 10, whereas one stripping (the first) was not counted because of lack in adherence. The stripped skin was cut into slices matching the diameter of FDC (15 mm) and the penetration experiments performed as described in 3.2.4.1.

3.2.4.4 Inducing SC-damage with HCl or NaOH

The use of different chemicals on the skin is used in studies to reduce the skin barrier function. Previous studies by Petitot et al. (2007) have used both HNO_3, HF and NaOH to check how the skin barrier was affected.

A small box (d= 7.0) was covered with aluminium-foil and the ear was placed into the box with the outer side up. The process involved pouring a 1 or 3 M HCl- and NaOH-solution on the skin. When the whole surface was covered, time of treatment was taken. For HCl, the treatment lasted 39 seconds, and for NaOH the time was 39 seconds and 5 minutes, respectively. During the treatment, excess solution was squirted around the whole pig slice to make sure the whole surface was treated. The ears were washed in PB immediately after the treatment to remove the chemical. The chemically disrupted skin was cut in slices matching the diameter of FDC (15 mm) and the penetration analysis was done equally as described in 3.2.4.1.

3.2.4.5 Inducing SC-damage by burning the skin

Burnings can result in complete destruction of the skin barrier, and Papp et al. (2009) showed that burned skin caused higher serum concentration of epinephrine in the experience of reduced barrier function.

The pig skin was placed with the outer side up in a big metal bowl. The damage was done by using a flame made by propane-gas, TurboTorch E-400. The flame was held close to the skin in order to induce severe burning to the SC, for a total of 25 seconds. The burned skin was cut in slices matching the diameter of FDC (15 mm) and the penetration analysis was done equally as described in 3.2.4.1.

3.2.5 Compromised PVPA with different volume of large liposomes on the top layer

The PVPA can easily be adapted to be used as model for testing permeability through compromised skin (Engesland et al., 2013). The upper layer in the PVPA with liposomes extruded 1200 nm is mimicking the human SC. Our hypothesis was when the volume of liposomes extruded through 1200 nm added on top was reduced, it would introduce different degree of leakiness to the barriers.

A batch of 16 inserts was prepared following the protocol of 3.2.2.1 and 3.2.2.3. The only difference was the volume of liposomes extruded through 1200 nm added on top. Briefly, the preparation was done by adding 100 µl liposomes extruded through 400 nm on the filter and centrifugation at 950 *g* for 15 minutes. Another volume of 100 µl liposomes extruded 400 nm was added and centrifuged with the same interval as previously described. The inserts were placed in a 50 ^{o}C incubator for 45 minutes for solvent evaporation. The volume of liposomes extruded through 1200 nm on top was: 6 inserts with 25 µl, 6 with 50 µl and 4 with the original volume (100 µl) made for comparison. The permeability was tested by using a 9.4 mM DS-solution as described in 3.2.2.4. Resistance was measured as described in 3.2.2.5.

To have reliable results, the previous experiment was reproduced. Another batch of 48 inserts was made; the concentration and resistance were measured as described in the previous

paragraph. The volume of liposomes extruded through 1200 nm in each insert was; 21 with 25 µl, 18 with 50 µl and 9 with original volume (100 µl) added.

3.2.6 Stability analysis of the barriers

The stability of the inserts was tested to determine how long the inserts could be stored in the freezer without affecting the integrity of the barriers. This can also favour production of industrial batches by making it less labour intensive (Engesland et al. 2013). Previous studies have shown that the barriers made by E-80 could be stored at -80^{o}C for up to 2 weeks without any changes in the calcein permeability (Flaten et al., 2006b). It was therefore favourable to check long-term stability with the barriers containing both E-80 and CHOL.

A set of 17 inserts from different batches were obtained:

- 5 inserts from 5th of May 2012
- 5 inserts from 27th of November 2012
- 6 inserts from 13th of December 2012

The experiment was carried out 22th February 2013, checking stability on the inserts after approximately 9 months, 3 months and 2 months storing in the freezer.

The inserts were made as described in 3.2.2.1 and 3.2.2.3, by adding liposomes extruded through 400 nm and 1200 nm, solvent evaporating, centrifuging and finally by freeze-thaw cycle. The permeability was analysed by using a marker-solution of calcein (5 mM), according to Flaten et al. (2006b). The solution was freshly made one day before the experiment to eliminate stability-issues of the solution. Permeability analysis was done as described in 3.2.2.4. The resistance was also measured as described in 3.2.2.5. by using Millicell-ERS.

4. Results and Discussion

4.1 Particle size analysis

The liposomes were extruded to obtain desired size distributions, so the smallest liposomes (400 nm) could fit into the pores of the filter and prevent leakage through the barriers (Flaten et al., 2006b). The liposomes extruded through filters with a pore size of 400 nm had a mean diameter of 360 ± 42 nm. For the liposomes extruded through 1200 nm filters, the mean diameter was 912 ± 80 nm. These liposomes, due to their size will not pass through the filter, but lay on the top contributing to a tighter barrier (Engesland et al., 2013).

4.2 Permeability and integrity analyses of the PVPA with E-80 and CHOL

Permeability and integrity analyses were performed as described in the section 3.2.2.4. The permeability studies are important as a validation of the transport through the biological barriers (Flaten et al., 2006a). The first experiments used a common drug, ibuprofen (5 mM), to test the permeability through the barriers. This has also been performed by previous studies (Engesland et al., 2013). Later in the experiments a DS-solution (9.4 mM) or a marker-solution (5 mM calcein) was used to determine permeability and integrity. The experiments lasted for a total of 5 hours. The concentration and electrical resistance were measured and P_{app} was calculated by using Equation 2. The results are presented in Table 1 with calculated standard deviation (SD), where N represents the number of experiments.

TABLE 1: CALCULATED P_{APP} (CM/S) AND RESISTANCE (Ω) MEASUREMENTS ON THE PVPA, USING CALCEIN (N=3), DS (N=2) AND IBUPROFEN (N=2) AFTER 5 HOURS

Permeation of drug through the PVPA					
	Parallels	**P_{app} (10^{-6} cm/s)**	**P_{app} SD**	**Resistance (Ω)**	**Resistance SD**
Calcein	18	0.061	0.053	2072,778	891,125
DS	18	1.125	0.759	545.00	96.969
Ibuprofen	15	2.707	0.892	1446.667	347.145

Calcein is a hydrophilic marker and was expected to have a limited permeability through the barriers because of the molecular character. The results showed a P_{app} of 0.061 ± 0.053 10^{-6} cm/s for calcein, which is lower as compared to the values for the DS of 1.125 0.759 10^{-6} cm/s. For ibuprofen, the P_{app} was 2.707 ± 0.892. The mentioned resistance values are only reflecting the permeation values; when the resistance is high, the P_{app} is low. Previous studies, with the same lipid composition, have shown a mean calcein permeability of 0.081 ± 0.041 x 10^{-6} cm/s and a mean P_{app} for ibuprofen at 2.8 ± 0.6 10^{-6} cm/s (Engesland et al., 2013). These results, although not identical, are close to our findings and indicate that the barriers are reproducible.

The PVPA was suggested as a permeability model able to mimic human SC by Engesland et al. (2013). To evaluate the applicability of the model, the permeability from the PVPA must be compared with the skin penetration data found through *in vivo* or *ex vivo* studies using skin samples in the FDC. The aim of this study was to examine data from the penetration through the intact pig skin, and further induce different levels of damage in the barrier properties, in order to evaluate to which extent the compromised SC was affected.

4.3 In vitro penetration experiments in FDC using the cellophane membrane and damaged pig skin

All the experiments were performed according to the section 3.2.4.1. The preliminary experiments were conducted on the cellophane membrane, followed by the experiments on intact or damaged pig skin. The DS-solutions used throughout the experiments was either 3 mM (cellophane membrane) or 9.4 mM for damaged skin slices. The penetration of the drug was calculated by using Equation 2. The results are presented in figures or tables, where N expresses the number of experiments with each membrane/skin slice.

4.3.1 Penetration through the cellophane membrane

The use of cellophane membrane and FDC is a commonly used method to investigate penetration of drugs. Cellophane membranes are semi-permeable, where molecules penetrate through the pores by the same principle as given in Equation 1 (Loftsson et al., 1994; 2002). We used cellophane membrane in the beginning of the study to validate the method prior to

the skin FDC experiments. The total number of parallels was 8 and the amount of penetrated drug through the cellophane membrane was 78 ± 25 %, as shown in Figure 10.

During the validation of the FDC tests using the cellophane membrane, bubbles between the membrane and the acceptor medium were observed. This could influence the contact between the acceptor medium and the membrane, providing less area for the drug to penetrate through the membrane. In later experiments, the receptor chamber was overfilled with PB and the skin slices were carefully placed on the top avoiding the formation of bubbles. This was done for all samples before the start of the experiments. Another change during the validation was the covering of the donor chamber. The chamber was originally covered with plugs, but closing by plugs caused unnecessary exposure of the membranes to pressure. We decided to replace the plugs with double layers of parafilm in all experiments in the FDC system.

4.3.2 In vitro penetration study on intact pig skin

The aim of these experiments was to find normal penetration values of drug through intact pig skin, making it possible to compare the values with damaged skin later in the thesis. The total number of total parallels performed was 12.

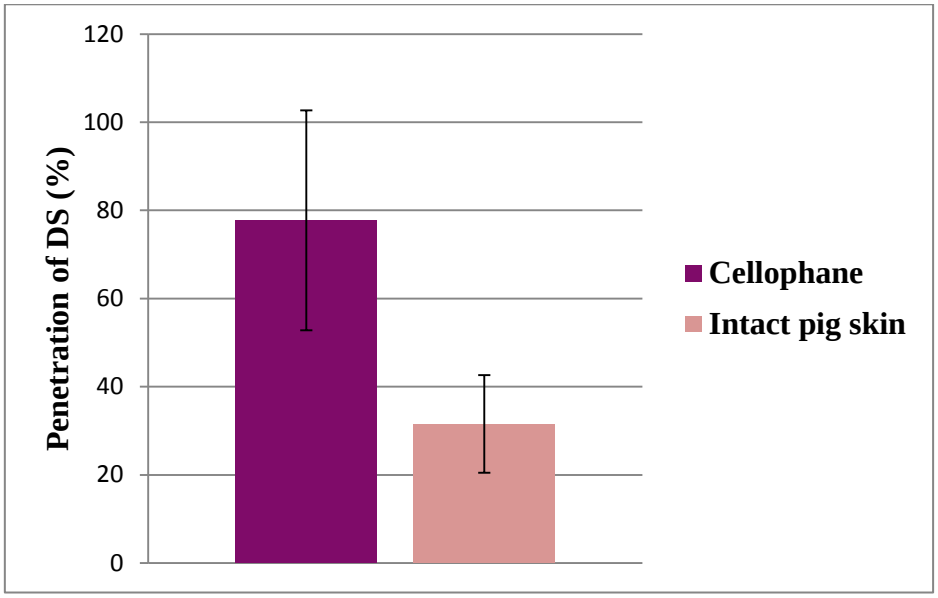

FIGURE 10: PENETRATION (%) OF DRUG THROUGH THE CELLOPHANE MEMBRANE (N= 2) AND FOR INTACT SKIN (N=3) AFTER 5 HOURS

Porcine ear skin was, according to Jacobi et al. (2007) reported to be the best suitable *in vitro* model for human skin in the studies on percutaneous penetration. Barbero and Frasch (2009) also reported that pigs and guinea pigs are good surrogates for human skin in *in vitro* penetration measurements. Klang et al. (2012) said that pig skin shares the same essential penetration characteristics with human skin, especially for drugs with a lipophilic character. This supports the choice of the pig skin in the thesis.

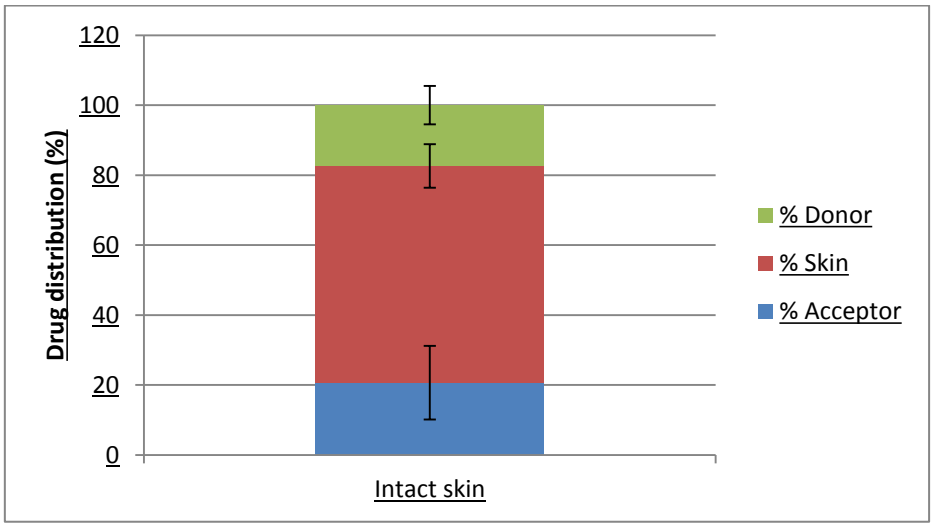

FIGURE 11: THE AMOUNT OF DRUG (%) FOUND IN THE DONOR-CHAMBER, IN THE SKIN AND IN THE ACCEPTOR-CHAMBER DURING EXPERIMENTS USING INTACT SKIN (N=2)

The result indicates that 31 ± 11 % of drug penetrated through the intact pig skin within 5 hours, as shown in Figure 10. DS has a partition coefficient of 4.5 (http://www.medicinescomplete.com/), which means it will be 4.5 times more soluble in lipophilic environment than hydrophilic. It would be genuine to think that lipophilic drugs would penetrate the SC in higher extent, but such drugs are not highly suitable. For our drug in this case, it was mostly retained in the skin during the experiments, as seen in Figure 11. The drug distribution-measurement was performed by measuring the remaining volume in the donor chamber in the FDC system after every experiment, and later do further calculations. It is probably not directly representative because of difficulty in measuring the whole solution in the chamber. But the measurement helped us giving an indication on where in the FDC system the drug was distributed. The high amount of drug in the skin is explained by the accumulation of the drug in the SC because of its low aqueous solubility. If the drug is neither hydrophilic nor lipophilic, the penetration through the skin will be easier, because of

intermediate solubility within both environments (Funke et al., 2002). This could be the explanation of why the penetration through the intact pig skin was not higher.

4.4 In vitro penetration through tape-stripped skin

The aim of performing tape stripping was to see if it was possible to damage the skin barrier and to evaluate the extent of the damage. This was controlled by checking the penetration of DS through the treated skin. The procedure involved a complete cover of the skin with tape before it was removed. The total numbers of parallels during 4 and 6 stripping were 4, and for 10 stripping the number were 8. During the first completed tape stripping-process (x4), an observation of indecent adherence between the skin and tape was seen. This was optimized by cleaning the skin with 96 % (v/v) ethanol to remove surface fat. This process was implemented when 6 and 10 stripping were performed and showed rather good adherence.

The amount of the penetrated drug after 4 stripping was approximately 27 ± 4 %, after 6 stripping 20 ± 1 % and after 10 stripping 22 ± 3 %, respectively, as illustrated in Figure 12. It should be kept in mind that 4 and 6 stripping were only performed during 1 experiment with 4 parallels, without reproduction. This could mean that our measurements were accidental. The 10 stripping was reproduced with approximately the same values, but still showed a lower value as compared to the intact skin. Since the skin used in the 6 and 10 stripping experiments was cleaned with ethanol, this could have influenced the penetration of DS. This is because ethanol can cause delipidisation and gaps between cells in the outer layers of the skin. The enhanced penetration-effect has been reported to be concentration-dependent with the maximum fluxes occurring when the ratio of ethanol to buffer is 0.6 (Akomeah et al., 2008). It is not unrealistic to assume that applying ethanol on the skin could further increase the penetration of the drug because of even more destruction, although it did not overcome intact skin penetration.

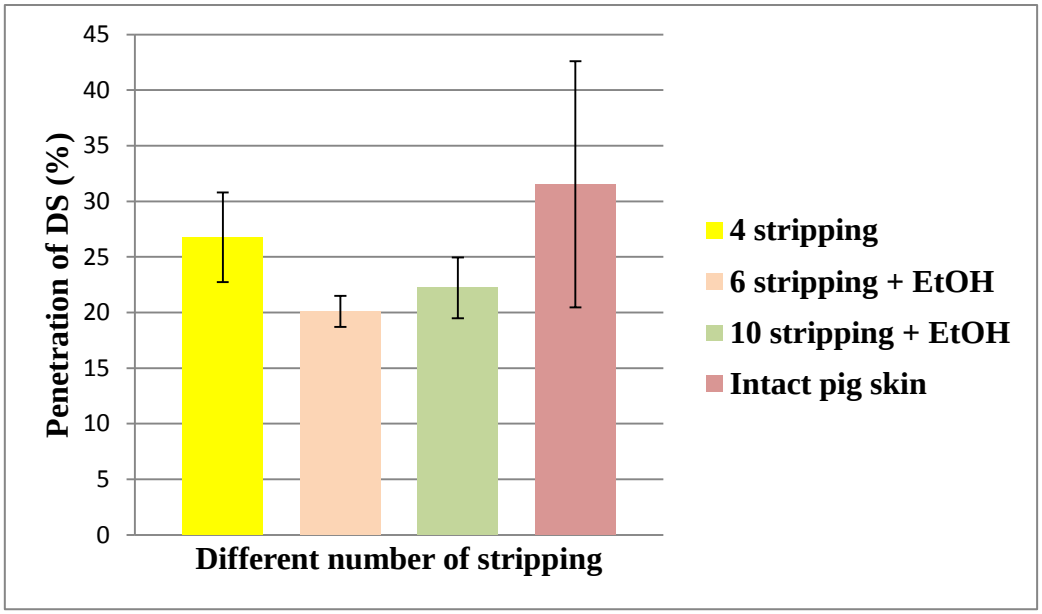

FIGURE 12: PENETRATION (%) OF DRUG THROUGH THE DIFFERENT DEGREE OF TAPE-STRIPPED SKIN (4 AND 6 STRIPPING N=1 AND 10 STRIPPING N= 2) AS COMPARED TO INTACT PIG SKIN (N= 3) AFTER 5 HOURS

Goff et al. (1992) performed 8 stripping of the pig skin and found that treated pig skin was more sensitive to treatment with 5-aminoluvulinic acid as compared to the intact skin. Morgan et al. (2003) showed a higher penetration of aciclovir through human skin when applying a maximum of 50 stripping. Since hydrophilic drugs have shown to be more affected by tape-stripping than lipophilic, due to the penetration pathways, this could be the explanation of our findings. On the other hand, a study by Rubio et al. (2011) showed that penetration of salicylic acid exhibited higher penetration through the intact pig compared to tape-stripped skin because of the slightly lipophilic character of the drug. These findings are in accordance with our findings regarding penetration through intact skin, although salicylic acid has a lower partition coefficient of 2.3 (http://www.medicinescomplete.com/) compared to our model drug with a value of 4.5. Rubio et al (2011) explained the findings with higher drug affinity towards all the skin layers. Since DS has a higher partition coefficient, it could be captured in the aqueous parts of the tape-stripped skin and not be able to fully penetrate through the skin.

Another observation during the preparation of the ears was the variety in thickness of each skin slice. It was easy to see the difference between a young and an older pig ear when purchased from the slaughterhouse. Most of the older ears were thicker and contained more fat, while the younger were thinner and had more visible blood vessels. Previous studies have also reported that pig ears can contain lesions or irregularities in the skin barrier (Klang et al.

2012). Because of the variations seen in the thickness, we decided to measure the thickness with a calliper. The thickness varied from 1.20-1.99 mm, which is approximately 0.80 mm in difference. A study by Bronaugh et al. (1982) found no correlations between ear thickness and permeability. Also, Elias et al. (1981) found no correlation in thickness of the SC, number of cell layers and percutaneous transport. They measured thickness/number of cell layers in human abdomen and leg SC and the results were probably opposite if what are expected; the compounds, salicylic acid and water, showed greater penetration through the thickest skin. Holbrook and Odland (1974) found an inverse relationship between thickness, number of cell layers and permeation. Because of contradictory results, it is difficult to draw a complete conclusion. We decided to keep these parameters in mind when comparing and evaluating the findings.

The human skin barrier function partly changes with ageing, reflecting changes in pH (between the ages 50-60) and SC hydration on specific parts of the body (Luebberding et al., 2013). The pH can give fluctuations in drug available for penetration (Konda et al., 2012). If the hydration changes, water is not present to open the compact structure of the SC (Barry, 2001). Since pig and human skin are experimentally similar (Jacobi et al., 2007), it is logical to expect that porcine skin also will be affected by ageing and penetration of drugs.

The unexpected results can also be linked to the tape-stripping procedure. Some important parameters to remember during the procedure are:

- Type of tape
- Site of application
- Application of the tape
- Application pressure
- Velocity of removal (Lademann et al., 2009).

The type of tape is also an important parameter. There are different kinds of tapes present on the market, but a tape with guaranteed uniform composition and distribution of adhesive layer on the strip should be chosen. The site of application must be uniform and areas with scares/ hair should be avoided. This is because the adherence between the tape and the skin will be less effective (Lademann et al., 2009).

The application of stripping must be standardized. If the slice is bigger than the tape, it should be marked to have control on which site of skin is treated/intact. Application pressure is important to provide optimal adherence between the tape and skin. This will assure a uniform removal of the SC. It is suggested that a combination of roller, a spatula and a constant weight during the application is optimal. The velocity is not easy to standardize, but should be uniform so equal amounts of SC are removed in each experiment (Lademann et al., 2009).

The site of application during the whole study was pig ears, but varieties in used areas on each pig slice could give different penetration properties. It is hard to standardize and we were not paying enough attention to this during the preparation of the skin. The type of tape was also the same during the whole study. The application of the tape involved complete covering of each ear before use, eliminating the error of using untreated parts in the study. The pressure during application and the velocity of the removal were done by hand and tried to be performed in a similar and constant manner. The velocity during removal is difficult to standardize, but pressure during application could have been performed by using the same weight, a spatula and a roller to assure similar adherence between the skin and tape. To control if our tape-stripping was successful, the amount of removed skin protein could have been measured by using the colorimetric protein assay (Bashir et al., 2001).

4.5 In vitro penetration through chemically treated skin

Since the results from the tape-tripping as a method to induce damage to the skin barrier failed to increase the penetration of DS, we tried to apply harsher treatment. The used solutions were a strong acid and a strong alkali, represented by 1 M HCl and 1 or 3 M NaOH, respectively. The solutions were poured over the outer layer of the skin, and time of exposure measured from the moment when the whole surface was covered. Time of exposure was set to be either 39 seconds or 5 minutes. The results of the penetration of drug through the differently chemically-treated skin slices are shown in Figure 13.

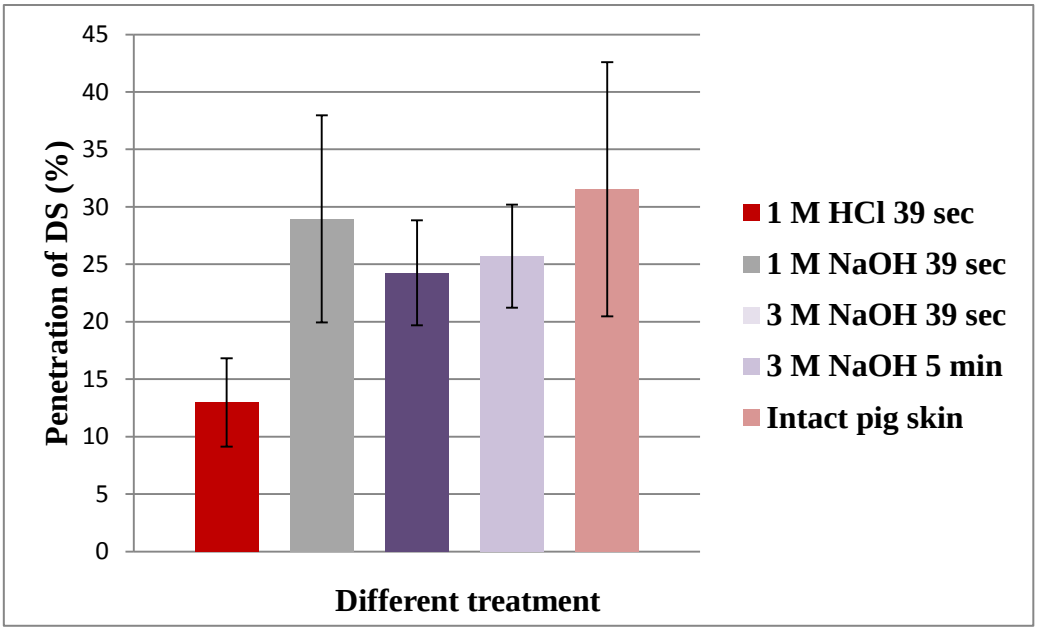

FIGURE 13: PENETRATION (%) OF DRUG THROUGH THE CHEMICALLY DAMAGED SKIN (N= 2) AS COMPARED TO INTACT SKIN (N=3) AFTER 5 HOURS

4.5.1 Effect of acid-induced damage on skin penetration

First we tried a 1 M HCl-solution during a total number of 7 parallels. The results of penetration of DS through the acid-damaged skin are presented in Table 2. Since this is a strong acid, we expected the penetration to be rather higher than through the intact skin. But the penetration of DS through the acid-treated skin was 13 ± 4 %. The explanation of this can also be caused by variations in skin slices according to thickness mentioned in the last section.

TABLE 2: DRUG PENETRATION THROUGH THE SKIN TREATED WITH 1 M HCL FOR 39 SECONDS (N= 2) COMPARED TO INTACT SKIN (N=3) AFTER 5 HOURS

Penetration of DS through acid-damaged skin			
	Parallels	**Penetrated drug (%)**	**SD**
Intact pig skin	12	31.53	11.06
1 M HCl 39 seconds	7	12.97	3.83

It is also interesting to see the retention of the drug left in the skin after treatment, shown in Figure 14. Most of DS was retained in the skin, while only a smaller amount reached the acceptor chamber. Compared to other treatments, this was the treated skin with most drug distributed within the skin. This could indicate that HCl is a highly suitable chemical to

induce severe damages to the skin and not only the surface lipids as expected. The acid could have penetrated in the deeper underlying tissues making almost complete damage to the present structures. This can make it almost impossible for DS to penetrate and made it rather retain in the skin between the destroyed lipids.

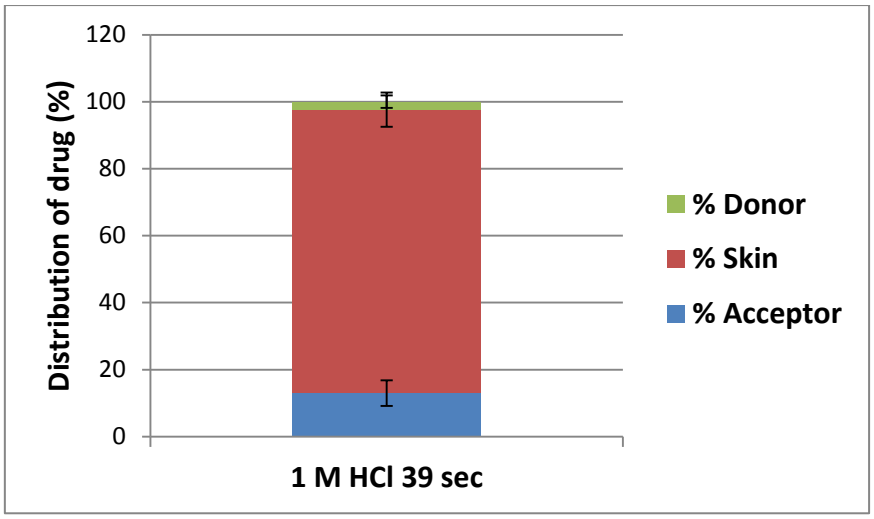

FIGURE 14: THE AMOUNT OF DRUG (%) FOUND IN THE DONOR-CHAMBER, IN THE SKIN AND IN THE ACCEPTOR-CHAMBER DURING EXPERIMENTS USING ACID-DAMAGED SKIN (N=2)

However, a study by Petitot et al. (2010) showed that unlike concentrations with HNO_3 increased percutaneous penetration of uranyl nitrate through rat skin, with a time of exposure of 30 minutes. We also regarded 30 min as too long exposure time in respect to the real life situation, and think that time in seconds (as in our case) is a more realistic exposure time. The difference from our experiment and the mentioned study is the characteristics of the used acid, but most important the character of the drug. Hydrophilic drugs are reported not to be affected to greater extent as compared to lipophilic drugs when chemicals are applied, but it can vary dependent on which chemical is used. It is clear that more studies regarding penetration of lipophilic drugs acid-treatment skin are needed, also including the different time scale.

4.5.2 Effect of alkali-induced damage on skin penetration

The penetration of DS through the base-damaged skin varied from 24 to 29 %, dependent on the alkali concentration and time of exposure. The total number of parallels was 8 for 1 or 3 M NaOH exposed for 39 seconds, and 7 parallels for 3 M NaOH exposed for 5 minutes. These values are similar to the values for the intact pig skin (seen in Figure 13). Interestingly, a short time of alkali-exposure seems to be the most effective (29 %), but this could again be

affected by the variations in the ears. It can be genuine to think that the alkali has penetrated to the deeper layers accomplished to affect the skin lipids by making them slide away from each other, thereby making the penetration easier.

The skin treated with alkali showed a clear change of colour during the treatment. It turned out to be slightly brown-dark red, as shown in Figure 15. The consistency of the skin became softer and made it challenging to prepare. These observations indicate that something had happened to the skin and the lipid composition during the treatment. The drug was even distributed in the donor-chamber, in the skin and the acceptor-chamber, as seen in Figure 16. This can reflect that the treatment could have been irregular, destroying both the SC and the underlying lipids in some part of the ears, hindering further passage through the skin. While other parts of the ear was less affected and allowed penetration. A study by B-Blasco et al. (1997) supports our findings regarding reduced penetration; the penetration of lipophilic drugs was moderately less affected by the alkali-exposure of the skin than hydrophilic compounds, although they applied another chemical (SLS).

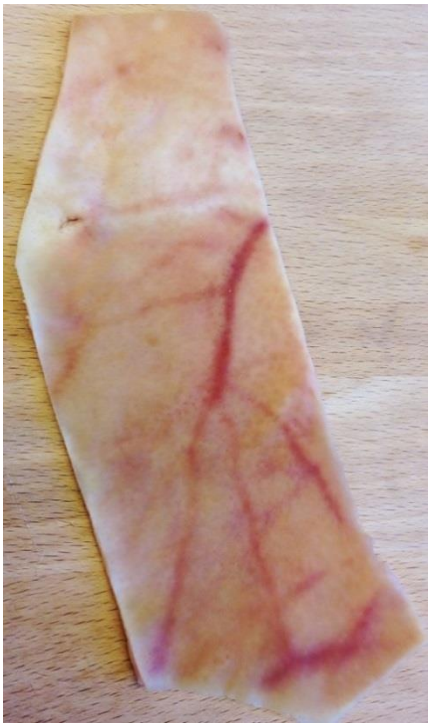

FIGURE 15: A PICTURE OF A SKIN SLICE AFTER 5 MIN TREATMENT WITH 3 M NAOH, ILLUSTRATING THE DIFFERENCE BETWEEN INTACT AND TREATED SKIN

Our study is in agreement with previously published studies on 10 N NaOH-treated skin by Petitot et al. (2007), who treated the rat skin for 30 minutes, 2 and 6 hours, respectively. The authors found no significant changes in uranium concentration in the blood or urine in the alkali-damaged rat skin as compared to the controls. This is probably reflected in smaller effects in the barrier properties during the treatment. The early study of Berenson and Burch (1951) also reported no changes in the water diffusion rate through 0.1 M NaOH-treated skin. The mentioned studies have all tested hydrophilic drugs, while our model drug is lipophilic. However, the results are comparable; no obvious changes are seen in drug penetration, independently of drug properties. The variation in these findings is interesting as it could reflect that the animal species can also contribute to variation in the results. More studies are needed before making a final conclusion on how the NaOH will affect the penetration of lipophilic drugs.

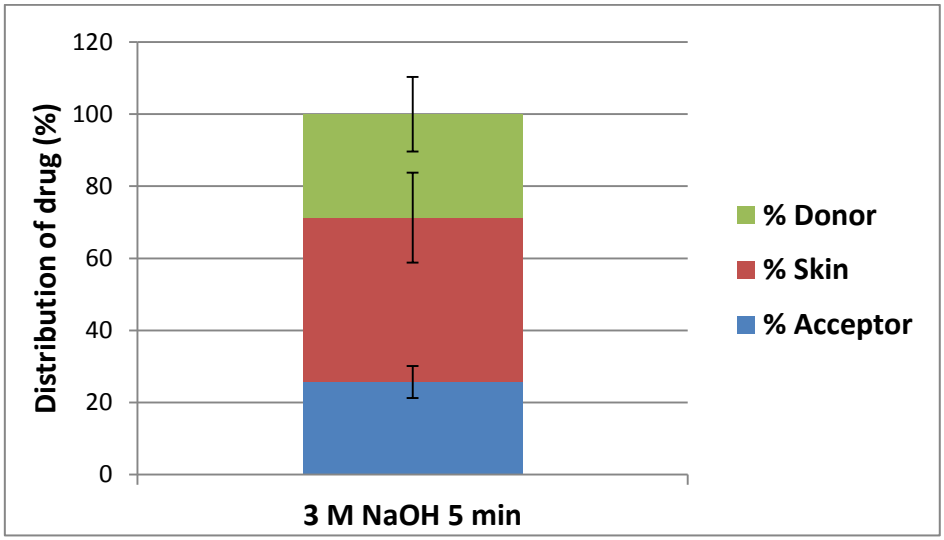

FIGURE 16: THE AMOUNT OF DRUG (%) FOUND IN THE DONOR-CHAMBER, IN THE SKIN AND IN THE ACCEPTOR-CHAMBER DURING EXPERIMENTS USING BASE-DAMAGED SKIN (N=2)

4.6 In vitro penetration through burned skin

Burnings are reported to induce severe damages to the SC and possible removal/damage of the SC (Chiang et al., 2012). Since treatment with different chemicals did not increase the skin penetration of DS, we decided to induce the damage in the skin barrier by burning. The process involved the use of propane flame to burn the skin for a total of 25 seconds during a total number of 6 parallels. The results are presented in Figure 17.

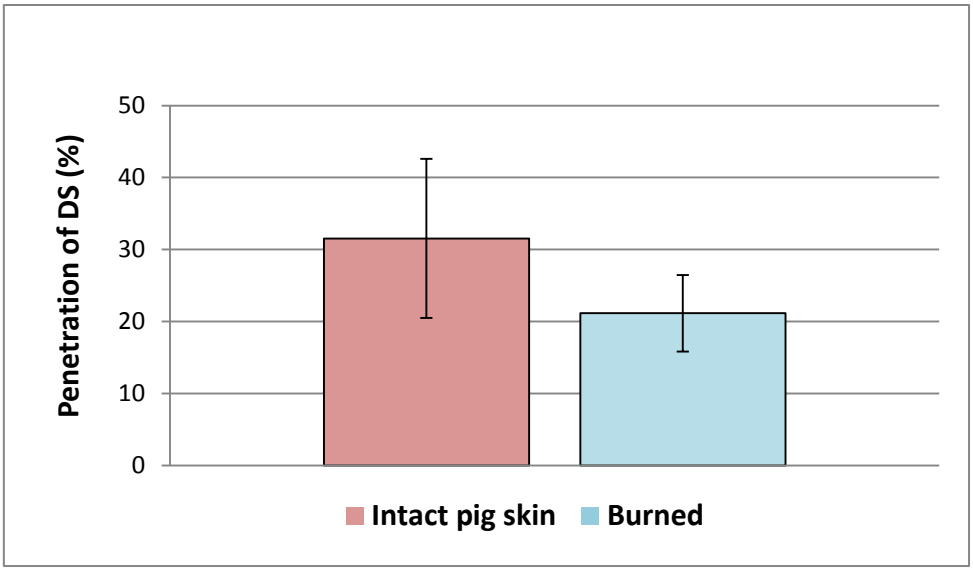

FIGURE 17: PENETRATION (%) OF DRUG THROUGH BURNED SKIN (N=2) AS COMPARED TO INTACT SKIN (N= 3) AFTER 5 HOURS

The amount of drug able to penetrate through the burned skin was 21 % ± 5, still less than through the intact skin. This can be explained by total destroying the lipids in the skin, decreasing the penetration of DS through the skin. However, a study by Papp et al. (2009) showed that by applying epinephrine on burned skin showed higher serum concentrations of the drug. They explained the findings by increased absorption properties in burned skin. Since epinephrine is a hydrophilic drug, it is genuine to think it will be more affected than lipophilic drugs, as it is for tape-stripped/chemically treated skin.

Our findings could be explained by the procedure itself, giving less influence on the exposed area. The process was performed with the approximately same distance between the skin and the flame all the time. The flame was moved around the surface in smaller circles. This could have limited the treatment of the middle part of the ear, but the areas exposure to treatment was easy to see, as shown in Figure 18. The time of exposure could have been prolonged, but the borderline between a damaged skin and carbon soot is difficult to determine. If the burnings were causing soot, it could affect the UV-absorption when measuring the drug concentration afterwards. The results can also, as mentioned earlier, have been affected by the variations in the ear thickness. If the SC was thicker and contained more fat, it would need more exposure of flame to cause enough reduction of the SC.

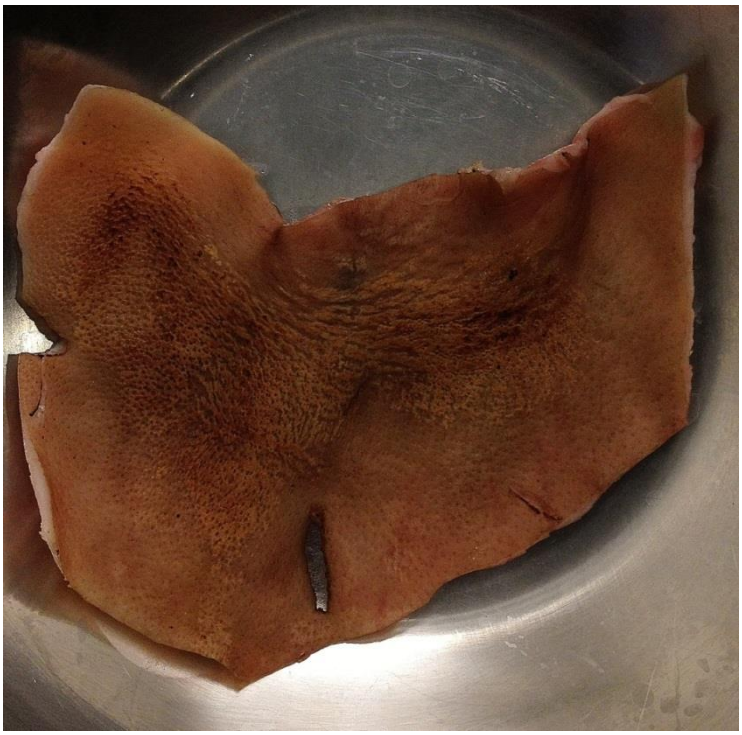

FIGURE 18: A PICTURE OF A SKIN SLICE AFTER 25 SECONDS BURNING

The burning has also been reported as not sufficient to induce significant effect on the permeation when temperatures of 70 °C were used (Boosalis et al., 1987). Before we treated the skin, the flame was left to stabilize for about half a minute. Even though the temperature was not measured, it can be assumed that the temperature was maintained the same during the treatment.

4.7 An overview of the different treatments used to induce damage in skin barrier

None of the treatments in our study showed higher penetration of DS as compared to intact skin, as summarized in Figure 19. At this stage, it is rather hard to conclude whether the treatments were not successful, or the choice of the drug was not optimal, or potentially both.

During this study, a drug with a lipophilic character was used to determine penetration-changes through the skin slices, but a more hydrophilic drug could, in theory, have passed the skin easier (Funke et al., 2002). This is also demonstrated in studies performing both tape-stripped and chemically-treated skin (Akomeah et al., 2008). Although, our study is providing necessary information in how a drug with a more lipophilic character could act on treated

skin. Based on the number of experiments and parallels performed, it is not enough to draw a final conclusion, but rather see a trend.

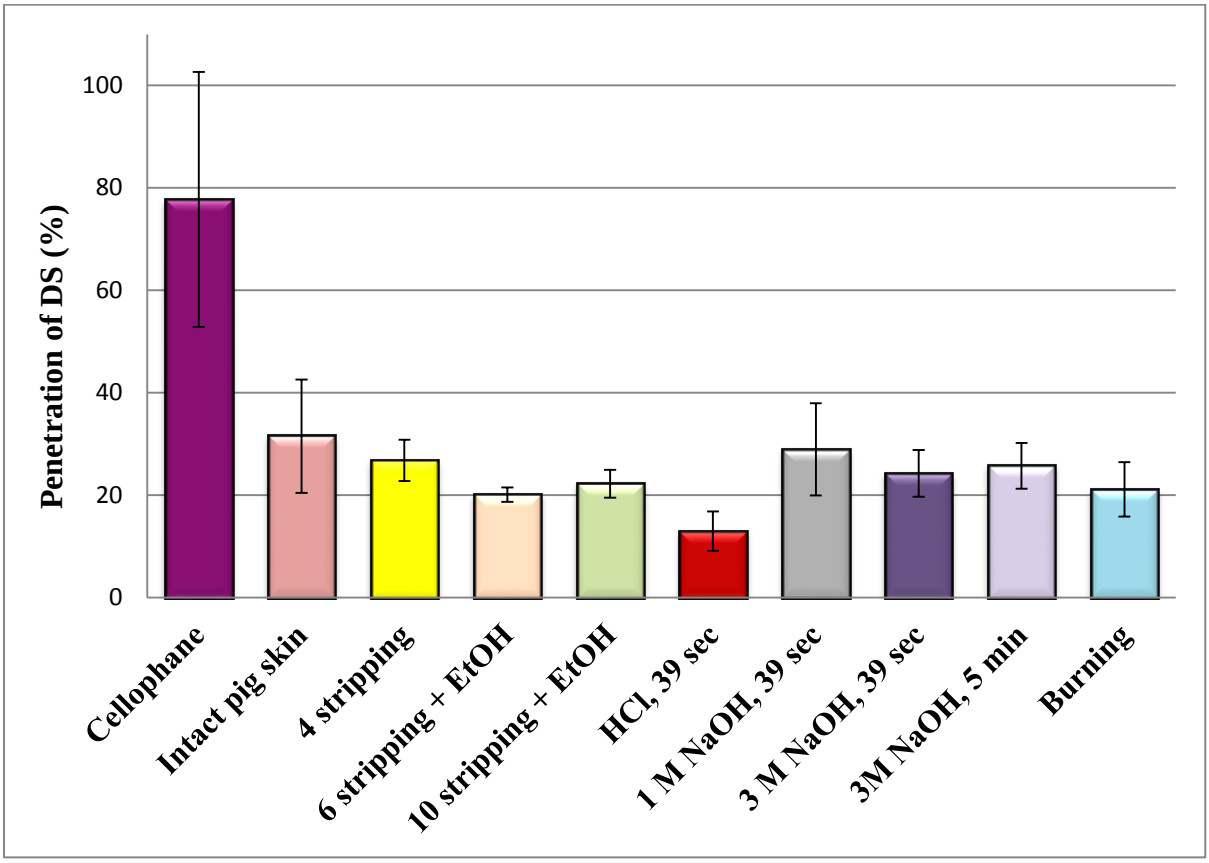

FIGURE 19: A SUMMARY OF ALL TYPES OF TREATMENT OF PIG SKIN AND THE RESULTS OF PENETRATED DRUG (%) THROUGH INTACT AND TREATED SKIN AFTER 5 HOURS

The overall trend in our study seems to show that the treatments are failing to exceed the penetration of DS through the intact skin, but a remarkable difference in reducing the penetration is seen when using the acid, probably explained by extreme destruction of the SC. But the varying results can also be explained by the differences in each skin slices used in each parallel regarding thickness, application site and general quality. This is a parameter that is hard to control, because the pigs are different in age and not chosen by us from the slaughterhouse. It can also mean that our treatments have been highly successful in reducing the amount of lipids in the skin, making the lipophilic DS pathway through the skin less favourable.

The findings also strongly support our hypothesis that, in order to evaluate the penetration of drugs through skin with compromised barrier properties, one needs to rather develop a

suitable and reproducible *in vitro* model to determine the transdermal drug's behaviour on the skin.

4.8 The effect of the different volume of large liposomes on the top layer

When the human skin barrier is diseased, the skin composition is abnormal and the volume of lipids could be reduced, as for AD. This could probably affect the skin penetration of drugs (Chiang et al., 2012). The large liposomes in the PVPA represent the SC. Our hypothesis was that when the volume of the large liposomes is reduced, something will happen to the permeation of DS. We expected to see an increased permeation because the PVPA will mimic a reduced human skin barrier.

This experiment was performed by following the procedure in the section 3.2.5. The difference was made in the last part where the large liposomes are added. A normal volume for making an intact barrier is 100 µl, but we experimented by reducing this to 50 and 25 µl. To be able to make comparison, an original barrier containing 100 µl was also made in the same batch. The experiment was accomplished by doing two experiments containing 2 batches with 16 and 48 inserts representing barriers with different volumes of liposomes extruded through 1200 nm added. Table 3 shows the number of parallel in each batch.

TABLE 3: THE NUMBER OF PARALLELS IN EACH BATCH WITH DIFFERENT VOLUME OF BIGGER LIPOSOMES

	100 µl	**50 µl**	**25 µl**	**Total number**
1.batch	4	6	6	16
2. batch	9	18	21	48

The mean P_{app} was 0.382 ± 0.267 10^{-6} cm/s for the original PVPA with 100 µl. For the barriers containing 50 and 25 µl of liposomes the P_{app} was 1.676 ± 0.486 10^{-6} cm/s and 3.525 ± 0.877 10^{-6} cm/s, respectively. The results on permeability showed that the P_{app} is increasing with decreasing volume of liposomes extruded through 1200 nm added, as shown in Figure 20.

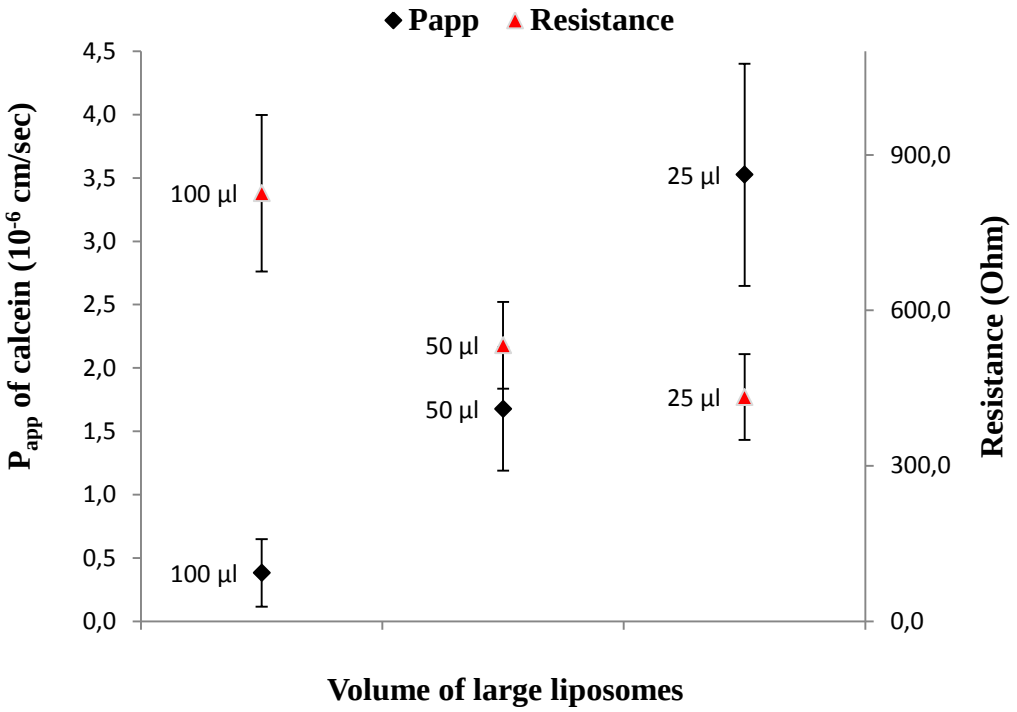

FIGURE 20: PERMEABILITY AND RESISTANCE OF THE PVPA WHEN THE VOLUME OF BIGGER LIPOSOMES WAS REDUCED TO 25 AND 50 µL COMPARED TO THE ORIGINAL VOLUME OF 100 µL (N= 2) AFTER 5 HOURS

The differences between the P_{app}-values are obviously remarkable. This is also seen in the Figure 20, where the permeability values are spread in different regions of the graph. When looking at the added SD, they are not overlapping each other. This means the results are unlike. To ensure if our assumption was right, we performed an ANOVA-test. This test showed a difference between the groups. We also wanted to check exactly where the differences were present by doing a t-test, but because the varying number of parallels in each batch, it is not directly correct to make a comparison on this basis. However, it is possible to do a t-test with the presented values, and it showed a statistical significance between all the groups (<0.05 level), meaning that 100 µl was different from 50 and 25 µl, 50 µl was different from 100 and 25 µl and 25 µl different from 100 and 50 µl.

Since the reduction in volume showed a statistical significant difference, it means our hypothesis regarding increased penetration of DS through the barriers were successful. This opens up a big opportunity to even make the barriers more representative to different degrees of damage.

But to make this a quick and effective model, it is also necessary to check the stability for making it less labour intensive (Engesland et al., 2013). This can make the barriers be used for industrial production.

4.9 Stability of the PVPA

Any *in vitro* model, able to mimic barrier properties of the skin, needs to be stable over required time. The stability of the inserts used in PVPA model was tested according to section 3.2.6. The aim was to test long-term stability of the barriers upon storage in the freezer. This was accomplished by testing the integrity of the barriers by using a 5 mM calcein solution. The experiment included inserts stored in the freezer for 9, 3 and 2 months. The number of parallels in each batch was 5 for 9 and 3 months, but the number for 2 months was 6.

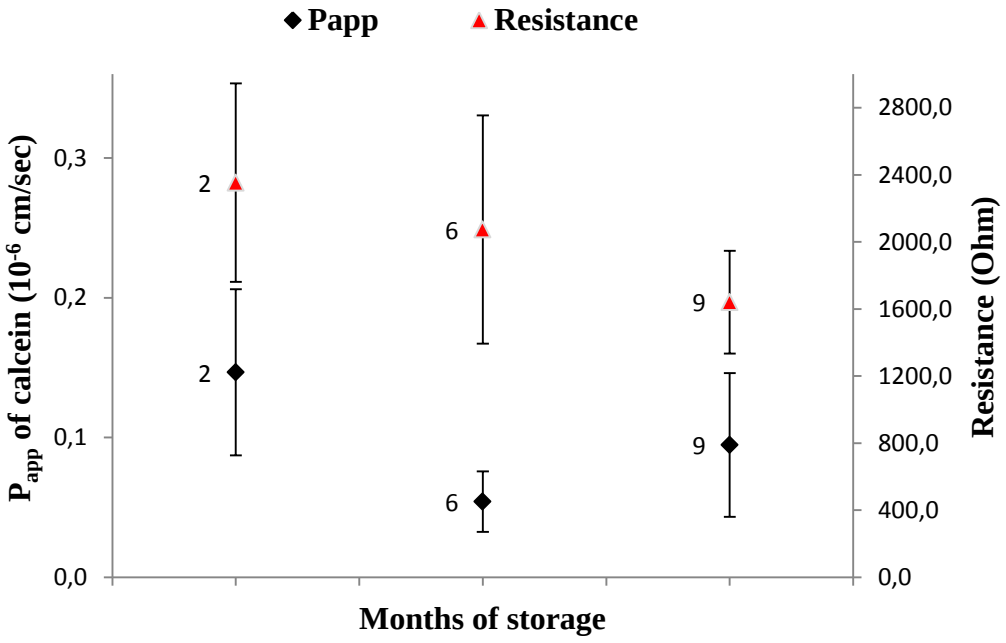

FIGURE 21: PERMEABILITY AND RESISTANCE OF THE PVPA BARRIERS STORED AT - 70 °C FOR 2, 6 AND 9 MONTHS (N=1), RESPECTIVELY AFTER 5 HOURS

The inserts stored in the freezer for 9 months showed a low P_{app} of 0.095 ± 0.051 10^{-6} cm/s, for 3 months at 0.054 ± 0.022 10^{-6} cm/s and for 2 months at 0.147 ± 0.059 10^{-6} cm/s (Figure 21). It is difficult to conclude whether the P_{app} is different as the SDs was rather large and quite overlapping. However, the trend during this study seems to be that of a decreasing resistance value is seen with increasing time of storage. But a final conclusion cannot be taken

on the basis of the resistance only, since the measurement only is used for identifying the integrity of the barriers.

More studies are needed for making a final conclusion if the promising barriers can be produced in bigger batches, but the previous section confirms our aim to induce different degree of leakiness to the barriers were managed.

5. Conclusions

During this study we have examined the *in vitro* penetration of DS through the intact and damaged pig skin. To induce the damage in the pig skin, we applied the tape-stripping, chemical disruption by using acid and alkali solutions and finally burning. However, all of the treatments failed to increase the penetration of the model drug, DS.

In order to explain the findings, we have identified the factors that can affect the outcomes in studies measuring drug penetration through animal tissue, especially pig ears. The ears can vary in thickness, due to the age of the animal and quality of slice preparations, which can result in varying outcomes. The mentioned factors also affect the reproducibility of the experiments and are an additional reason to focus on the development of more reliable *in vitro* models.

Contrary to the *in vitro* experiments, we accomplished to produce reproducible results on drug penetration through the PVPA. It is a straightforward model and is quite easy to induce changes to, which clearly can be used in skin penetration studies. We also managed to induce different degrees of leakiness to the barriers by modifying the preparation process. This was reflected by increased permeation of DS through the barriers.

As a summary, the PVPA barriers seem to be better model to study the penetration through the compromised skin.

6. Perspectives

The modified PVPA has a potential in providing insight on permeability of drugs through diseased skin. However, based on our preliminary data, several points need to be taken into the consideration:

- More studies on how the permeability is affected by the change of volume with bigger liposomes on the top of the filter are needed. When different levels of leakiness are introduced in the PVPA barriers, the correlation to the different degrees of damaged skin barrier in *ex vivo* experiments using another animal origin skin could be performed
- Find other parameters related to PVPA preparation which could be modified, for example ethanol concentration, freeze-thaw cycle or centrifugation
- Conduct the experiments with parallels to reveal long term stability of the inserts

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