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1. Introduction

"Frontier", "barrier", "wall" are metaphors often used by researcher describing the skin. For a citizen of the European Union enjoying the freedom offered by the Schengen treaty a frontier is something selectively permeable. If some prerequisites are met it is possible to cross this frontier – be it with good intentions or bad intentions. The same is true for the skin: Drug preparations meeting the demands made by the laws governing skin permeation can get into the body without being hampered by the skin while on the other hand substances not deemed fit to cross this barrier remain outside the body. Unfortunately in the world of today there are some unwelcome persons whom we'd like to stay out which nevertheless manage to slip in. The same is true for the skin. In real life the police and custom officers look into the matters how such undesirables managed to get in. In skin research it is the task of pharmacists to look after welcome substances and how to make their journey into the body easier. They call it drug targeting. Toxicologists have to take care of the unwanted visitors calling it dermal exposure. Pharmacists and toxicologists both look at the two side of one and the same coin: skin permeation and the mechanism regulating it.

Skin as a gateway into the body has been known and used since ancient times. Herodot of Halikarnassos (450 b.C.) describes in his "Historien" [1] p. 112 the use of ointments by the Arabs. In middle age witches' ointment, unguenti sabbati achieved some notoriety. Pig's grease containing extracts of Solanaceae's alkaloids carrying plants lead to hallucination gave birth to the famous myth of witches being able to fly. A popular myth which left a rich trace in literature – among others in Goethe's Faust They have been known since the ancient world [2]: Homer [3] describes in his Illias how Hera applies it to her body and is then able to fly over the highest mountains of the world – much to the astonishment of her husband Zeus. Apuleius – 200 a. C. – gives a detailed description of the use and effect of such a witches' ointment. These examples show how long ago application to skin was already recognized as a method for bringing pharmaceutically active ingredients into the human body. All this literature fallout would not have existed if the skin had been less permeable. While in ancient times skin permeation was mostly a matter of trial and error the interest for the underlying mechanism and the laws governing penetration and permeation have become focus of interest in the twentieth century.

2. Skin morphologically

Form defines function et vice versa. The strength of the skin barrier is rooted in its structure. Therefore an investigation about skin permeation has first to take a closer look at the general structure of the skin. Skin is the largest organ of the human body: With an average thickness of 1.2 mm skin covers an area of approximately 1.8 m². Its weight with blood sums up to 4.7 kg and its area/thickness ratio is of 150000 [4]. It is essentially a thin and wide membrane that covers our whole body. This membrane has a layered structure consisting mainly of four layers: Subcutis, dermis, epidermis and stratum corneum and their respective sublayers. The layers from the inside to the outside are [4-9]:

The **subcutis** consists mainly of connective tissue with lipocytes. This tissue shows some elasticity. The thickness of this layer depends on the weight of the person. It represents a thermal and mechanical barrier

The **dermis** also called corium fulfils a support function. Its main components are: fibrin proteins elastine and collagen which are embedded in a mucopolysaccharide matrix. Hair follicles, hair shunts, sweat glands, sebaceous glands, blood vessel and nerves lymphatic vessel are found in this tissue.

The **epidermis** consists of five layers: The stratum basale, the stratum spinosum, the stratum granulosum, the stratum lucidum and finally the stratum corneum.

The stratum basale is the deepest layer of the epidermis. It consists of a single layer of keratinocyte stem cells of columnar or cuboidal shape. The cells in this layer are mitotically active. The layer is attached to basement membrane by hemidesmosomes while the cells are attached to each other with desmosomes. The cells are subjected to continuous cell division. The differentiation process of the new cells takes place in the **stratum spinosum**.

The stratum spinosum consists of 8-10 layers of many-sided keratinocytes with spine-like projections which are responsible for the name. The differentiation process leads to a flattening of the cells and the formation of desmosomes.

The keratinisation takes place in the **stratum granulosum**. In this process the cells produce keratohyalin and store it. As soon as the keratinocytes cells are filled with keratohyalin they are transformed into cornified cells. The destruction of the nucleus is a good indicator of this process.

At the end of the transformation process the cornified cells are integrated into the **stratum corneum**. 10 to 15 layers of flat, cornified cells without a nucleus and embedded in a complex mixture of lipids [10-14]. The corneocytes are polygonal and a thickness ranging from 0.3 to 0.7 µm.

The layer that controls absorption is the outermost layer, the stratum corneum. The stratum corneum is only 15–20 µm thick but provides a very effective barrier to penetration.

3. Pharmaceutical aspects

From a pharmaceutical point of view skin is interesting for systemic and locally limited therapy. Creams, ointment, gels and transdermal therapeutic systems deliver a wide range of drugs among which are pain killer, sexual hormones and nicotine [8, 9, 15-43]. The goal of the pharmacist doing this kind of research is how to enhance formulation to reach the desired effect. The high compliance of dermal therapy and the ease of its use are major drivers behind this kind of research.

The mechanism behind skin permeation remained a mystery which was only unravelled in the midst of the twentieth century. In 1943 Rothmann [44] showed in his review that the physicochemical properties of a substance are critical for its ability to cross the skin. Higuchi demonstrated in 1960 the mathematically very simple physicochemical concepts behind skin permeation. The discovery of these principles has been a great help for fomulation science which was able to develop preparations for topical use which deliver systemically and locally a wide range of drugs [8, 9, 15-43, 45]. (The literature quoted here only represents a small sample of the overall body of literature on this subject and should only be considered as an introduction to the subject).

4. Toxicological aspect

Toxicologists look into the unwelcome side of skin permeation. Daily life in modern industrialized societies exposes humans involuntarily to many chemical substances. Some of these substances are harmless while others might affect adversely human health. There are many examples in toxicological literature for intoxication after dermal exposure: Workers handling wet tobacco leaves suffered from an illness called Green tobacco sickness (GTS) which is essentially a form of nicotine poisoning [46]. Fiorito [47] showed that dermal exposure to leather having been treated with DMSO lead to liver disease. Other examples are the use of nitroglycerin in ammunition factory which lead to cardiovascular diseases [48] and neurological damages after contact with organophosphate pesticides [49-51]. In all these cases dermal uptakes lead to systemic intoxication. The risk presented by these products was not known beforehand.

The objective of toxicological research in the area of skin research is to quantify the capacity and the amount of substances to cross the skin barrier in laboratory experiments before victims are to be deplored. This goal is supported by the REACH legislature of the European Union which forces companies releasing new substances to the market to assess – among other areas - their dermal toxicity.
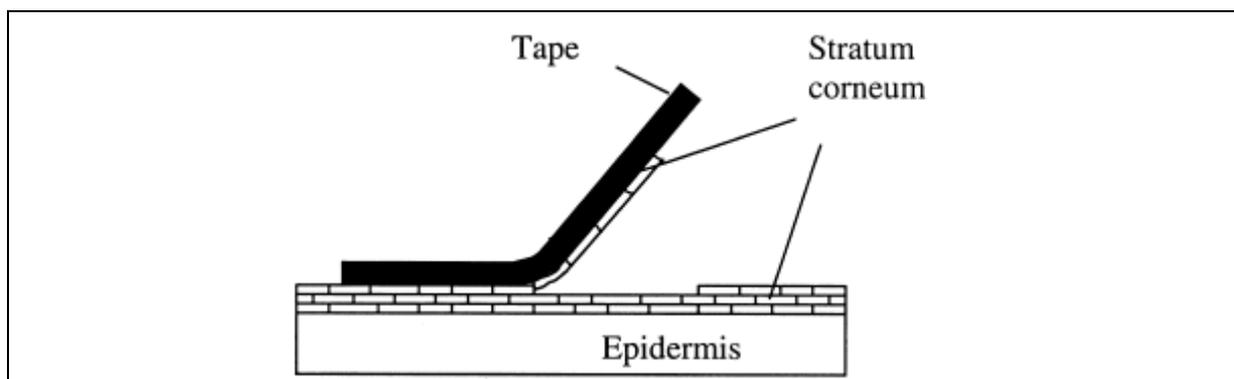
5. Testing procedures

The problems described above show that the central question for pharmacologists and toxicologists is to know how and up to which extent substances are capable of crossing the skin. Many test systems have been developed for answering these questions. An exhaustive description of all test systems would lead to far away from the scope of this work. A short summary of the most important test systems will be given instead.

6. In vivo testing

6.1 Tape Stripping

Tape stripping [52] is a technique which successively removes cell layer of the stratum corneum by applying tape strips with a definite pressure and then ripping them off. By analyzing the residues adhering to the individual tape strips it possible to draw concentration depth profiles of a drug preparation which has been applied to the skin beforehand. The technique can be used in vitro and in vivo [8, 42, 43, 53-56]

Figure 1: schematice representation of tape stripping [57]

6.2 Cutaneous microdialysis

Microdialysis as described by Schnetz [58] is an "in vivo sampling technique for measuring endogenous and exogenous solutes in the extracellular space of tissue". Originally developed to determine neurogenic transmitters in the brain of laboratory animals the principle has been adapted for dermatological research. A small probe equipped with a semipermeable hollow fiber is inserted into the dermis, parallel to the skin surface. A physiological solution is then pumped through the probe. Drug preparations are applied on top of the insertion area and the amount reaching the microdialysis tubes through the skin is assessed [59].

6.3 Skin blanching test for corticosteroids

McKenzie [60] described 1967 that locally applied glucosteroid caused skin blanching by vasoconstriction. The degree of blanching is quantified using chromametry. This procedure can be used as a method for assessing the effectiveness of glucosteroids according to the FDA [61].
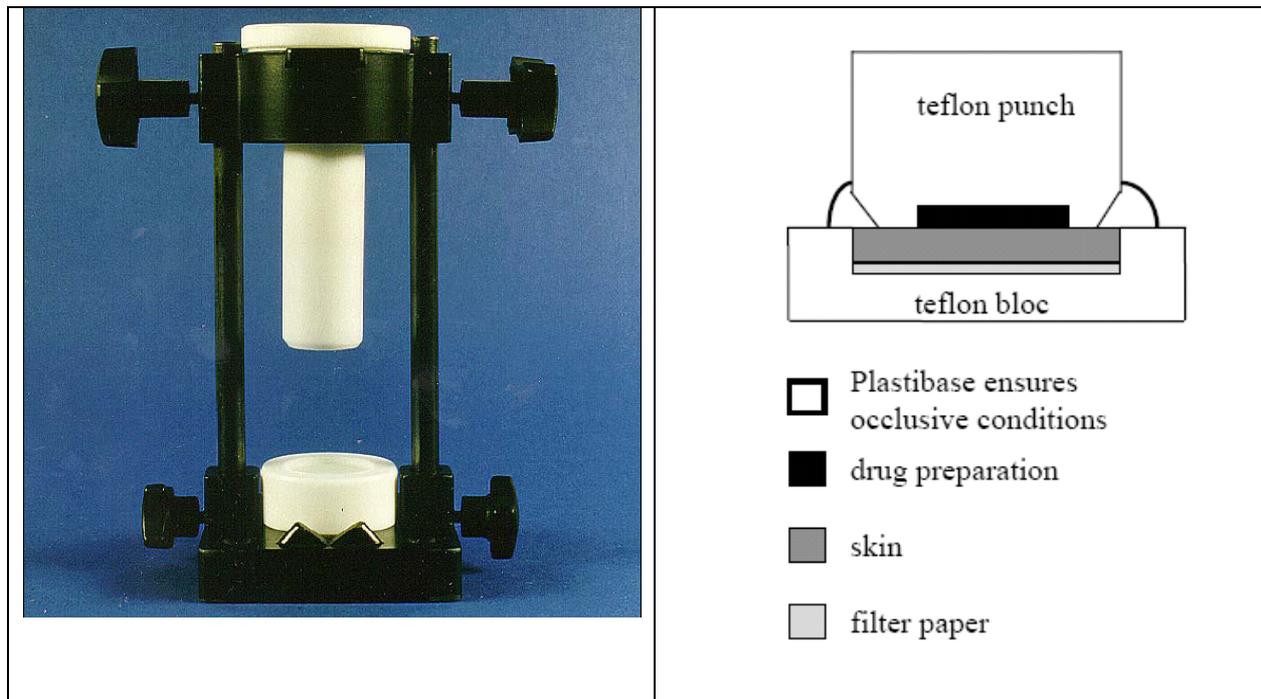
6.4 Erythema test

The ultraviolet erythema test is a method to study the strength of anti-inflammatory preparations for use in vivo. Different UV doses ranging are applied to each test subject. The aim is to obtain a measurable effect of the applied UV doses on the skin of test subject. The test fields are then treated occlusively for 48 h following irradiation. visual scoring and chromametry can then be used to determine the degree of erythema in the fields treated with anti-inflammatory preparations and the untreated, irradiated control field 7, 24 and 48 h after irradiation [62].

7. In vitro testing

7.1 Penetration systems

Schäfer [63] described in 1996 an experimental model – the Saarbrücken Penetration Model - which combines tape stripping and cryotome cutting as method for investigating the penetration of drug preparation into human skin. A drug preparation is brought down to excised skin using a teflon punch. Plastibase is used to ensure occlusive conditions. Afterwards the top layer of skin are removed using tape stripping. The deeper skin layer are cut succesively using a mycrotome. For further details please see [8, 42, 43, 53-56, 64-67].

Figure 2: Saarbrücken penetration model (pictures courtesy of Dr Ulrich Schäfer, University of Saarland)
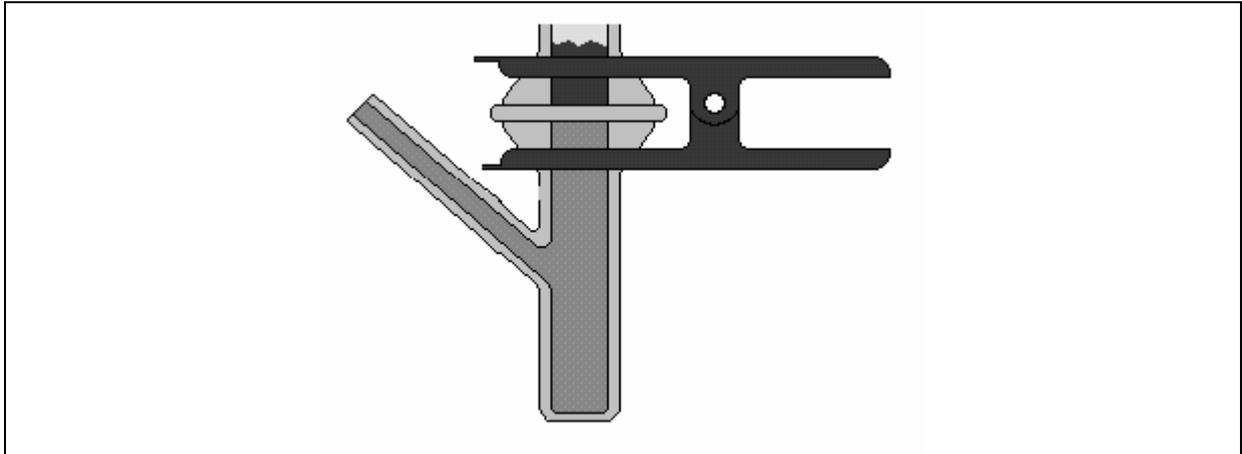

7.2 Permeation systems

Permeation system using the Franz type diffusion cells (FTDC) [68] are of special interest for this work.

The Franz type diffusion cell consists of a donor and an acceptor compartment. This setup was first described by Franz in 1968 [68]. Between these two compartments any type of membrane can be placed. A drug containing formulation is then placed in the donor compartment and then amount permeating through the membrane can be quantified by taking samples in the lower compartment through the lateral branch (see figure below).

Figure 3: The Franz Cell

The FTDC gives the user the free choice to use many different membranes and was therefore chosen as the standard tool for this work.
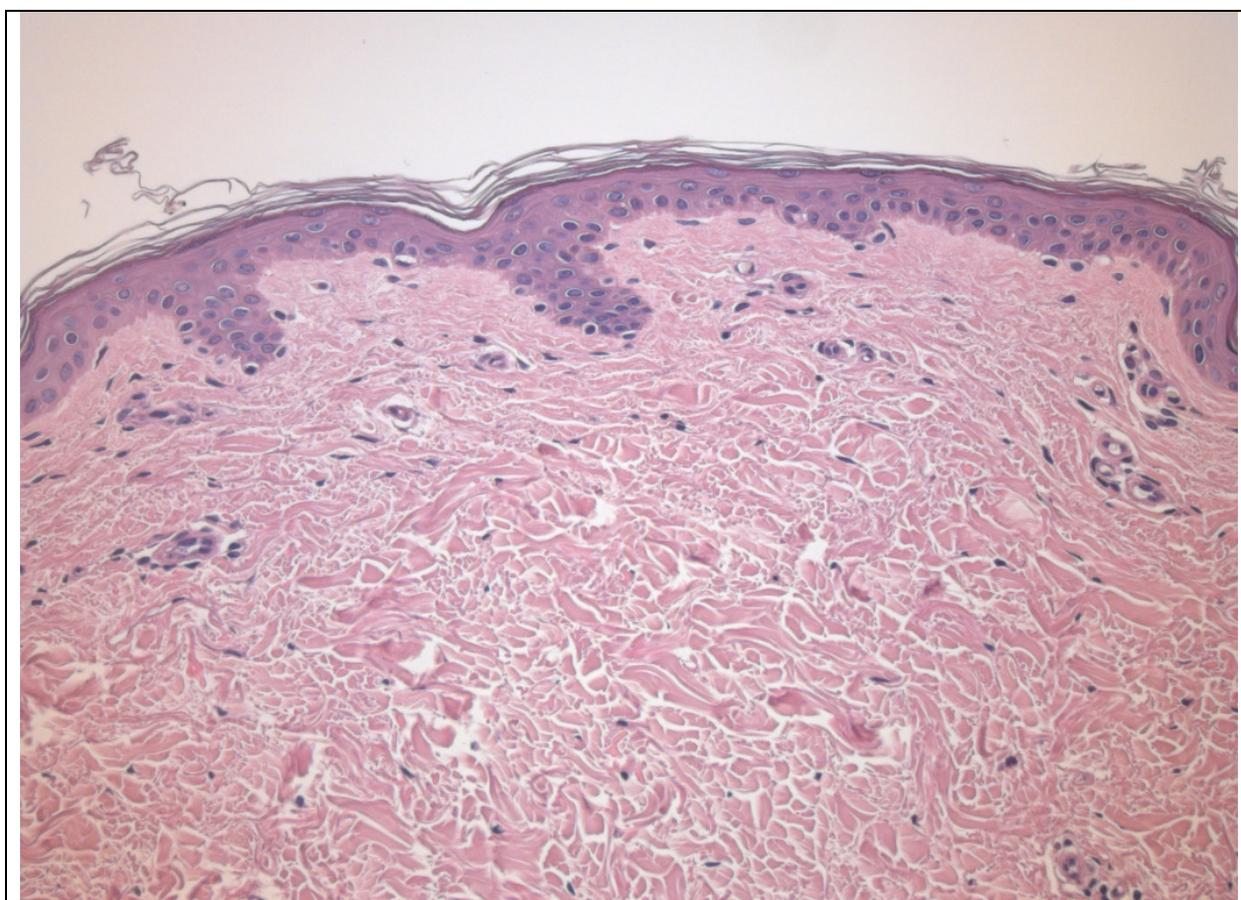
8. Membranes used in FTDC

8.1 Human skin preparations

8.1.1 Full thickness skin

In this work full thickness skin was obtained by removal of subcutaneous fat by dissection after surgery. The skin was frozen until use. (For more information on storage please see [8, 63]) The major advantage of this skin is that the manipulations to which it has been subjected during preparation are minimal. This lessens considerably the risk of accidental damage to the stratum corneum barrier. All epidermal layers and the dermis are present which matches the situation in vivo more closely [69].The presence of the dermis in vitro, though, can cause a myriad of problems which have been reviewed by Netzlaff et al [70]. Summarizing one can say that a dermis in vitro represents a formidable barrier for lipophilic permeants.
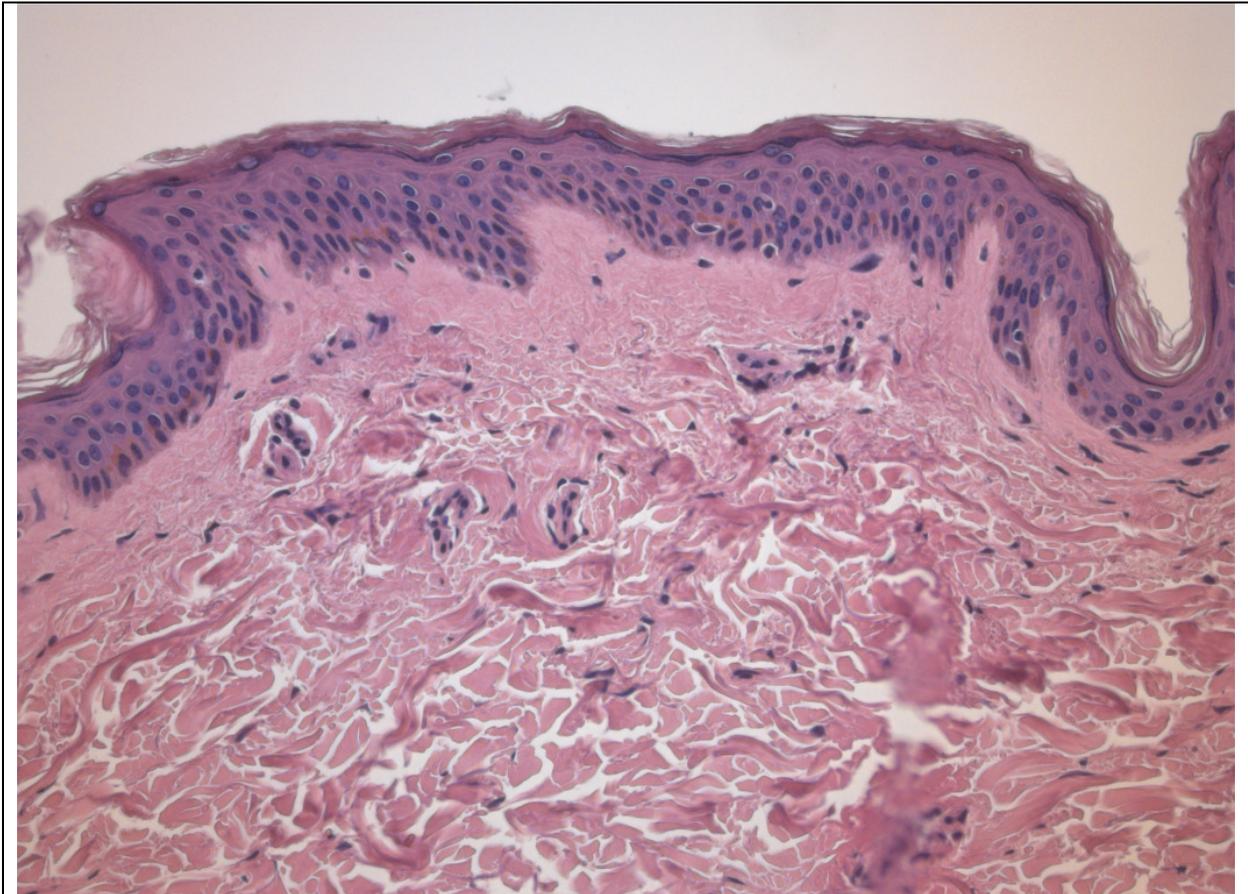
Figure 4: Full thickness skin (picture by Leon Mujis, hematoxilin-eosin, magnification = 200x)

8.1.2 Dermatomised split thickness skin

Split thickness skin is obtained by cutting full thickness skin to a defined thickness using a dermatome. OECD guideline 428 allows the use of thicknesses ranging from 200 µm to 1 mm. Dermatomised skin is robust and easy to handle. However it shows great size variation as shown by Henning [71]. Another disadvantage is that large amounts of skin have to be used when preparing it [71]. This is a major drawback compared to other types of skin preparations.

Figure 5: dermatomised skin (picture by Leon Mujis, hematoxilin-eosin, magnification = 200x)

8.1.3 Heat separated epidermis

Heat separated epidermis has first been described by Kligman [72]. Morphologically it is a clearly defined and reproducible material since it is limited to the stratum corneum and the viable epidermis. It is easy to handle and quickly prepared. The epidermis was separated by putting thawed and cleaned skin pieces in water at 60°C for 1 min, removing the skin from the water and placing it, dermal side down, on a filter paper where the SC-epidermis layer of the skin was peeled off from the dermis using forceps. The epidermis was put into PBS solution for at least 30 min in order to get a fully hydrated epidermis sheet (for further information on preparation please see [8, 73]).
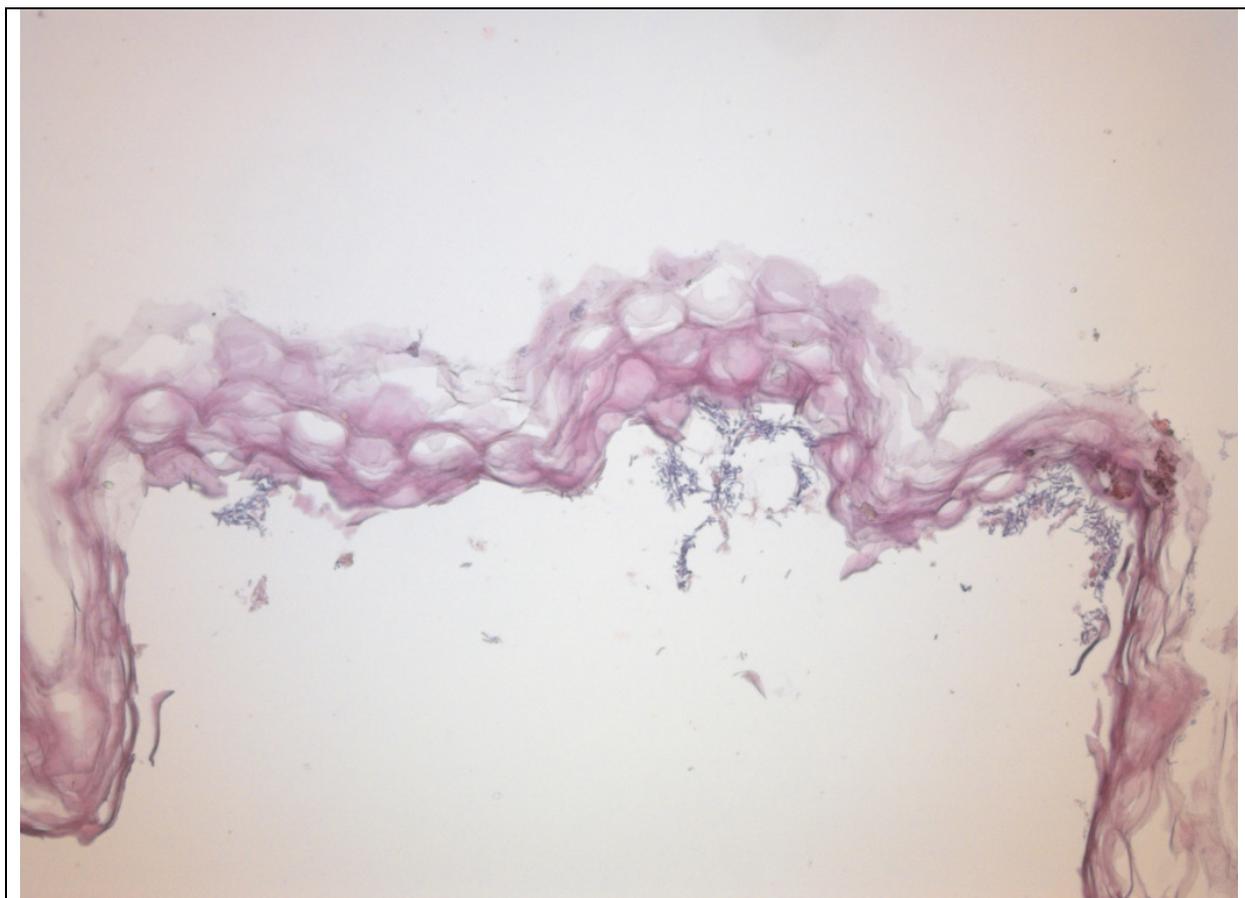
Figure 6: Heat separated human epidermis (picture by Leon Mujis, hematoxilin-eosin, magnification = 200x)

8.1.4 Trypsin isolated stratum corneum

Trypsin isolated stratum corneum – a membrane type not described in the OECD guidelines [74, 75] – is obtained by incubating skin pieces, dermis side down in a 1.0% trypsin solution in PBS buffer for 24 h at $32 \pm 0.1\,°C$. This procedure is repeated with fresh trypsin solution until the stratum corneum is fully isolated (for further detail please see [8, 54, 71]). Stratum corneum however is a very fragile membrane which requires a skilled operator in order to avoid damages to the barrier function.

Figure 7: Trypsin isolated stratum corneum (picture by Leon Mujis, hematoxilin-eosin, magnification = 200x)

8.2 Conclusion human skin preparations

Henning's work on advantages and disadvantages of different skin preparations clearly showed that heat separated epidermis and trypsin isolated stratum corneum are the best suited materials for permeation studies [71]. Dermatomised skin and full thickness skin present problems which seriously impair their usefulness for permeation experiments. Due to the ease and speed of preparation heat separated human epidermis was selected as the material of choice for this work. The only real shortcoming of human skin is its scarceness. Human skin is a limited resource which has to be used diligently.

8.3 Animal skin

Animal skin has been so far one of the favourite tools of the toxicologists for dermal toxicity testing. Their predilection for this material is due to several reasons: Animal skin is easily available and can be used in almost unlimited amounts. Human skin on the other side is scarce and the huge body of regulations linked to its use makes research with human skin difficult. Further, animal skin can be treated in a manner which mimics very closely the conditions of human exposure. Many animal species have been used for studying skin absorption, with the rat being used most. However these skins often differ from human skin as it is the case with rodent skin [76]. Pig skin on the other side reproduces closely human skin in vitro and is considered so far as the best replacement for human skin in in vitro experiments [77]. Nevertheless a heated controversy rages through western societies concerning animal testing. The public in western societies disapproves animal testing. The European Center for the Validation of Alternative Methods (ecvam.jrc.it) and ATLA (Alternatives to Lab Animals, altweb.jhsph.edu) are just two of the most prominent organisation in this movement in Europe. For years they have been trying to develop alternatives to the use of animals in research. The legislative consequence of all these efforts is the new EU regulation (76/768/EEC, Feb. 2003) which will prohibit the use of animal skin for gathering toxicological data beginning from 2009. This forces researchers to find an alternative to testing on animals. As a direct consequence human epidermis models are being developed to serve as a replacement for human and animal skin.

8.4 Reconstructed human epidermis model

Reconstructed human skin models might be the solution to all the aforementioned problems. In recent years several of these models have been developed. (For an in depth review of the most literature surrounding these models please see the general review presented later in this work.) As an industrial product they are available in unlimited amounts and their use does not raise ethical problems. Important regulations as the COLIPA-Guideline from 1995 and OECD-guideline No. 428 and OECD guideline 28 describe in vitro methods for testing penetration on human and also on artificial skin models. One major shortcoming of these models is that it has not yet been demonstrated that their barrier function is similar to the one encountered in human skin. The legislator and the relevant bodies of authorities in the member countries of the EU have not yet accepted them as a valid alternative. The formal proof has not yet been supplied that these models perform equally well as animal or human skin in dermal penetration testing. This is a *conditio sine qua non* for them to be accepted as a reliable alternative in dermal toxicity testing and is directly linked to the central question of this work.

9. Central question of this work

As mentioned earlier it must be shown that reconstructed human epidermis models are capable of replacing human and animal skin in in vitro penetration testing.
Therefore the goal of this work was to compare the barrier function of artificial human epidermal model to human skin and if possible to validate them. This is tremendous task which can not be undertaken by one single person. Therefore this work was part of a BMBF prevalidation (project number: 0312882) and validation study (project number: 0313342) on this subject. The work and the experiments of this project have been distributed throughout the entire group. This is the reason why some results are presented as publications with several partners. The data in these publications can only be presented in an intelligible way if presented with the results of the other groups. The results

have been used with the permission of the project management team. At the beginning of each chapter it has been pointed out which results were the results of the author of this work.

Some prerequisites were given: OECD standard substances had to be used in order to ensure comparability with existing literature. The OECD has tried to focus the research in the area of dermal penetration on a catalogue of substances which covers a wide range of physicochemical properties. The ambition behind this is to create a large reference data pool which might one day allow researchers to categorize the dermal penetration of new substances by comparing them to existing already investigated substances.

Another requirement was that the substances had to be applied from an aqueous donor. This is particularly a problem for highly lipophilic substances. Enhancing strategies had to be developed and their influence on the barrier function and the diffusion process had to be investigated.

To ensure maximum comparability of the results a standardized experimental setup had to be developed which could be adapted to all types of skin - artificial of different sizes, human of different preparation types. This encompasses also barrier integrity testing.

It is impossible to investigate the barrier function of the skin without taking a closer look at the intercellular lipids. An existing method for quantifying lipids has been adapted to the needs of this works and lipid profiles of all examined types of membrane have been compiled.

All these efforts were aimed toward one goal: set the groundwork for a comparative study under standardised conditions.

10. Mechanisms of skin permeation

The exact way a molecule takes through the stratum corneum has been the object of many debates for many years.
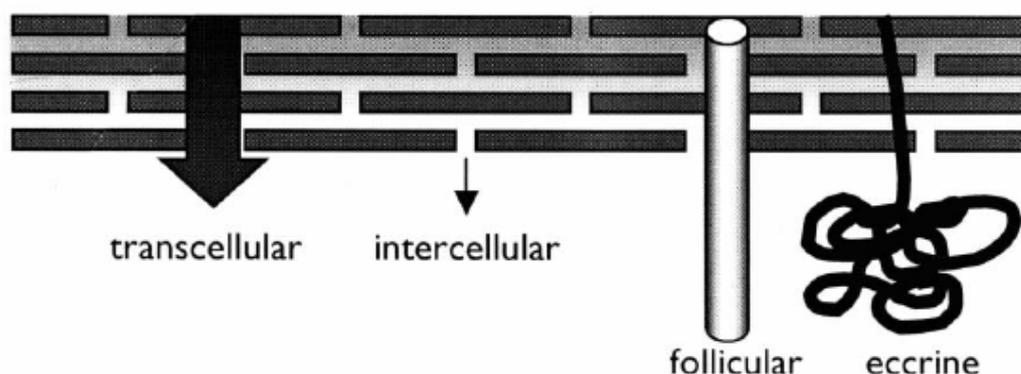
So far research has identified three major routes by which a substance can cross the stratum corneum barrier: Intercellular, transcellular and appendageal. With the follicular area accounting for approximately 0.1% of the total surface the follicular way is certainily not the most important penetration pathway for xenobiotics. [78]

Follicular transport was also considered as a possible route by Tregear [79] and Wahlberg [80]. The theory of the existence of a shunt mechanism was advanced by Scheuplein postulating that "was overtaken at longer times by general partition and diffusion through the stratum corneum" [78, 81].

This leaves the transcellular and intercellular route as main penetration and permeation pathways. There is a "weight of evidence" [78] suggesting that the intercellular pathway is predominant: In 1975 Elias said "intercellular regions of the stratum corneum comprise an expanded, structurally complex, presumably lipid-rich region which may play an important role in percutaneous transport" [82].

A classical model for describing the structure of the stratum corneum was created by Michaels in 1975 [83]. He investigated the structure of the stratum corneum and compared it to a brick and mortar wall with the cells being the bricks and the intercellular lipids being the mortar (see figure below). The lipids arrange themselves in a bilayered structure and consist of ceramides, free fatty acids, their esters and cholesterol and its sulfates [7, 84-88].

Figure 8: Brick and mortar model with the major routes of permeation [78]

transcellular   intercellular

follicular   eccrine

11. Simple diffusion Models and important parameters

The mathematical model used in this work was based on Fick's laws of diffusion [89]. Fick's laws of diffusion are the simplest way of modelling mathematically permeation through skin.
Three prerequisites are to be met:
- The driving force behind diffusion is only the concentration gradient
- Convection of the particles can be excluded
- The system is operating under isothermic conditions

Then the following equation [7-9, 71] can be applied which is called Fick's second law of diffusion:

$$\frac{\partial C}{\partial t} = \frac{\partial}{\partial x}\left(D\frac{\partial C}{\partial x}\right) = D\frac{\partial^2 C}{\partial x^2}$$

Equation 1: Fick's second law of diffusion (D = const)

This equation is essentiall a mass-balance which describes the change of the concentration of a substance in a one dimensional volume in relation to time. The first law shows that the rate of transfer J of a diffusing substance through a defined area is proportional to the concentration gradient.

Fick's first law of diffusion can be considered as one possible solution to the equation above with M being the mass of a drug in the acceptor A and the donor D over the time t. It describes basically a mass transfer. Mass transfer is the movement of matter from an area of high concentration to an area of low concentration. This movement is called diffusion. Equations based on Fick's law are often used by scientists who want to model all kind of transport processes e.g. neurons [90] or porous soils [91].

$$\frac{dM_A}{dt} = -\frac{dM_D}{dt} = P * A * (C_D - C_A)$$

Equation 2: Fick's first law of diffusion

If perfect sink (concentration in the acceptor compartment $C_A=0$) and infinite dose conditions (concentration in the donor compartment $C_d=const$) are maintained changes of the concentration gradient should be minimal. Then the following equation can be used:

$$J = \frac{dM_A}{dt} * A = P * C_D$$

Equation 3: Flux

Where J is the rate of transfer per area, M_A the mass in the acceptor C_D the concentration of diffusing substance in the donor, A the surface and P the permeability coefficient. The permeability coefficient P is a simplification of several parameters which can be assayed experimentally only with great difficulty.

$$P = \frac{D * K}{h}$$

Equation 4: the permeability coefficient

D is the diffusion coefficient of the diffusing substance, K the distribution coefficient and h the length of the diffusion pathway. One has to keep in mind that the thickness of the stratum corneum is not equivalent to the actual path length a molecule has to cross on its way through stratum corneum. Visualization of molecules crossing the stratum corneum showed that this way is much longer due to its tortuosity [92-94]
Due to this reason P is often labeled as P_{app} – the apparent permeability coefficient.
The distribution coefficient K between a barrier and the donor can approximately be described as follows:

$$K = \frac{C_{S-B}}{C_{S-D}}$$

Equation 5: the distribution coefficient

With C_{S-B} as the saturation concentration of the substance in the barrier and C_{S-D} the saturation concentration in the donor. This clearly shows the importance of solubility on the diffusion process as already outlined by Higuchi [95].
Combining equation 3 and equation 5 leads to the following equation:

$$J = \frac{D * K * C_D}{h} = \frac{D * C_{S-B} * C_D}{C_{S-D} * h}$$

Equation 6

This equation can be simplified as follows when using saturated solutions:

$$J_{max} = \frac{D * C_{S-B}}{h}$$

Equation 7

This equation describes the maximum flux, one of the most important determinants of skin penetration after topical application. It describes the amount of drug that can possibly penetrate the skin per unit time [96]. The rate at which the solute is absorbed depends on the type of the substance, the vehicle

used and "the condition of the skin"[97]. For the scientist working with skin the maximum flux (J max) of a solute is the most interesting value available for "determining the maximal dermal, toxic, or systemic effect" [97]. Therefore the equation above remains of the most important tools for evaluating skin permeability.

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Part I: Published results

The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport (summary)

The purpose of this article was to gather the most important information available over the three reconstructed human skin model which were to be used in this investigation. The literature quoted in this article showed that the models EpiSkin, SkinEthic and EpiDerm were similar to native human tissue in terms of morphology, lipid composition and biochemical markers. A large number of publications covers the testing of phototoxicity, corrosivity and irritancy. For these applications test protocols have been developed. Some of these protocols have been validated, while others are still in the validation process. The suitability of these models for permeation experiments is also very important. First results show that these models might also be useful in this area. Unfortunately the barrier function of these reconstructed human epidermis models appears to be much less developed compared to native skin. In the discussion of this publication the advantages and disadvantages of full thickness and epidermis only models are compared. The difficulties presented by a dermis in vitro are discussed here. The conclusion here is that a dermis in vitro creates additional problems for highly lipophilic substance by slowing their permeation. Based on the literature presented here the conclusion is that these models show a lot of promise but still need refinement.

My contribution to this work:

- Selection, evaluation and discussion of the presented literature

<u>**The results presented in this chapter have been published as:**</u>

The human epidermis models EpiSkin®, SkinEthic® and EpiDerm®: An evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport
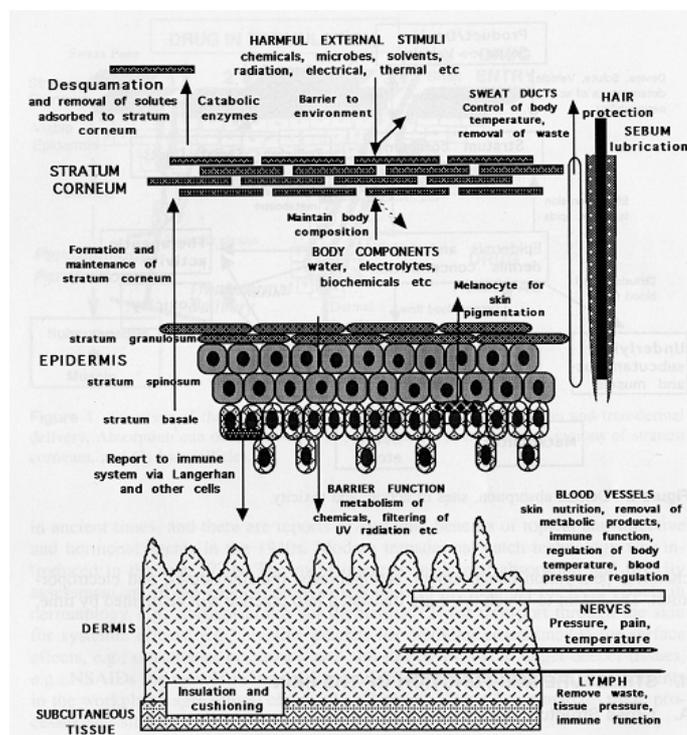
1. Introduction

In vitro models to study penetration into human skin are important tools for research and development in the pharmaceutical and cosmetic industries. Human skin is the best possible model for such in vitro studies. Possible sources for human tissue are cadaver skin, biopsy material or cosmetic surgery. However, there are a number of legal and ethical issues concerning the use of human tissues. The demand for human tissue is growing and the available amount is limited by number and by regulations. The European Union prohibits financial gain through the use of human tissue, making a widespread use no matter for which purpose very complicated. Animal skin is an alternative. However, the relevance of conclusions drawn from animal data for human skin has always been questionable. The ethical problems which arise from use of animal skin for testing purposes finally lead to the EU regulation (76/768/EEC, Feb. 2003) which, beginning in 2009, prohibits use of animals for gathering toxicological data for cosmetic ingredients. The COLIPA-Guideline [1] from 1995 and OECD-guideline No. 428 [2] describes in vitro methods for testing penetration on human and animal skin. The Draft guidance document for the conduct of skin absorption studies No. 28 clearly mentions commercially available skin models [3]. Nevertheless, it is still required that the comparability of artificial skin models and human skin has to be proven. In recent years several artificial human skin models have been developed, and some of these are commercially available. These models have undergone various testing in order to evaluate the possibility of using them to replace animal testing. For this purpose, they must mimic the relevant properties of human skin as closely as possible. Human skin consists of four layered compartments: stratum corneum, which is nonviable, the viable epidermis, dermis and the subcutaneous tissues. The functions of human skin are summarized in Fig. 1.

Figure 1: General functions of the skin reprinted from [4] by courtesy of Marcel Dekker, Inc.

Skin has two major functions: prevention of dessication and protection against environmental hazards such as bacteria, chemicals and UV radiation. The consequences for a skin model are—briefly said— that it should have a competent barrier function and show the same reaction to environmental hazards as human skin [4]. The purpose of this review is to give an overview of the most important existing artificial skin models. Existing data has been collected to cover—when possible—aspects of morphology, lipid composition and other biochemical markers, phototoxicity, irritancy testing and transport data. Models lacking published data on two or more of the aforementioned aspects have been left out of this article. To facilitate understanding of the presented data a short overview of the basic principles of the investigations and tests which have been performed with the models is given here.

1.1. Morphology

Investigations have compared the macroscopic and microscopic appeareance and ultrastructure of the models to human skin.

1.2. Lipid composition

The lipid composition of skin determines its permeability, flexibility, the partitioning of drugs into the skin and many other aspects of skin biology. Therefore, a lipid composition as close as possible to that of the native tissue is required. The methods usually used to determine lipid profiles is analytical TLC or HPTLC [5] and [6].

1.3. Biochemical markers

Biochemical markers are proteins indicative of the differentiation process. This includes the cornified envelope and its precursor proteins and the enzymes necessary for its formation. Ideally, these markers should be produced in the models in amounts similar to those found in native tissue.

1.4. Phototoxicity testing

Phototoxic substances are applied to the model and are then irradiated with UV-light of different wavenlengths and intensities to trigger a phototoxic reaction. There are several protocols describing how these tests are performed. Details of individual test protocols will be described in the text.

1.5. Irritancy testing

Irritants are applied to the model in order to see if the model shows the biochemical and histological signs of irritation. There are several protocols that have been used, and again, details will be given in the text.

1.6. Transport data

Drug formulations are applied to the surface of the model and the amount of drug which has been transported through the model is measured as a function of time. Transport data allows evaluation of barrier function.

1.7. Corrosivity testing

The model is exposed to corrosive substance and the reaction is assessed. The exact experimental setup and the protocol which has been followed are detailed in the text.

2. SkinEthic[®]

2.1. About the company

SkinEthic laboratories (Nice, France) was founded in 1992 by Martin Rosdy in Nice to develop and market the artificial human skin model SkinEthic[®]. The company offers a wide range of in vitro test system, not only the epidermal model discussed below. In addition to the epidermal model, SkinEthic[®] also offers other models, such as, e.g. reconstituted human corneal, oral, gingival, esophageal epithelium, etc.
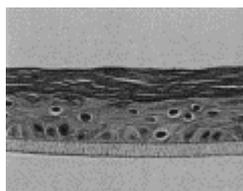
2.2. General

The technical data and safety sheet from the SkinEthic Laboratories describes the SkinEthic[®] epidermal model [7] as 'epidermis reconstituted by air lifted culture of normal human keratinocytes for 17 days in chemically defined medium on inert polycarbonate filters'.

2.3. Morphology

The general epidermal structure of the SkinEthic[®] model is highly similar to human epidermis. Stratum corneum, stratum granulosum, stratum spinosum which are the major structures of human skin can all be found in the SkinEthic[®] model [8]. The first description of the model in the literature revealed desmosomes, keratohyalin granules and lamellar granules [7]. Hemidesmosomes with plaques, components of the lamina densa, anchoring filaments and a structure which looks closely like a basement membrane were identified by electron microscopy [9]. However, Ponec et al. could not confirm these findings. This group only encountered hemidesmosomes but no anchoring fibril [10].
When it comes to the number of viable epidermal and stratum corneum cell layers, the SkinEthic[®] models seems to differ from native tissue. Lipid droplets have been found through all layers of the SkinEthic[®] model, which are not found in native tissue. The highest frequency of these droplets was encountered in stratum basale. The extrusion of lamellar bodies was retarded in some SkinEthic[®] cultures. The research group also discovered characteristic electronmicroscopic structures as alternating electron-dense and electron-lucent lipid lamellar sheets in the intercellular space of the stratum corneum in the SkinEthic[®] model [6]. (Fig. 2: morphology of the SkinEthic[®] model).

Figure 2: Morphology of the SkinEthic[®] model [15], reprinted with permission of S. Karger AG, Basel.

2.4. Lipid composition

For labeling skin lipids in the part lipid composition we use the nomenclature according to Motta et al. [11] side by side with the nomenclature used by the quoted author. In most cases, the original authors named the ceramides according to the fraction numbers with ceramide 1 corresponding to the least polar fraction.
The major subclasses of ceramides and their precursors, the gucosylceramides, as described in the literature [12], [13] and [14] are present in the SkinEthic[®] model (Table 1), although the model seems to have a higher ceramide 2 (Cer[NS]) content than native tissue. Ceramide 7 (Cer[AP]) is missing. The general lipid composition of the SkinEthic[®] model comes close to that of native tissue [15].

Table 1: Lipid composition of SkinEthic[®] model compared with native human tissue [15]

Lipid class	SkinEthic[®] mean±SD (n=4)	Native tissue mean±SD (n=3)
Phospholipids	17.0±10.6	36.5±4.1
Sphingomyelin	2.8±1.3	8.9±1.6
Phosphatidylcholine	6.4±3.8	11.2±0.8
Phosphatidylserine	1.1±0.7	3.9±0.3
Phosphatidylinositol	1.8±1.2	2.2±0.8
Phosphatidylethanolamine	4.9±4.0	10.3±0.8
Cholesterolsulfate	3.8±2.0	5.0±1.6
Glucosphingolipids	3.0±1.7	5.0±0.4
Ceramides	26.5±12.2	12.1±1.8
Free fatty acids	6.9±3.9	7.8±1.2
Cholesterol	19.5±9.5	17.7±3.2
Lanosterol	4.3±3.1	–
Di-/triglycerides	12.6±8.6	8.9±3.7
Cholesterolester	6.5±4.4	7.0±0.4

Data are presented as percent of total lipids. In the original publication the last line was by mistake also labeled as cholesterol. This error could be clarified with the help of the author [15]. It should be noted that in the native tissue, the stratum corneum is maintained at a constant thickness, so the ratio of ceramides, which are found mainly in the stratum corneum, to phospholipids from the viable epidermis is constant. In the culture model, there is no desquamation, so the stratum corneum becomes progressively thicker. This could explain why the proportion of phospholipids is lower in the culture model compared to native epidermis and the proportion of ceramides is higher. Also, it should be noted that the origin of the di-/triglycerides in native skin is questionable [5]. This may reflect contamination with sebaceous lipids or subcutaneous fat.

2.5. Biochemical markers

Keratin 1, 10, SPRR, SPRR3, loricrin, involucrin and transglutaminase are all present in the SkinEthic[®] model. In analogy to human skin loricrin is present in the stratum granulosum. Involucrin and transglutaminase were encountered in suprabasal layers [6]. Keratin 6 and SKALP are not found in human epidermis but are present in the SkinEthic[®] model [6]. Some words to explain those findings: Yoshida describes SKALP as an epithelial serine protease inhibitor in psoriatic epidermis. It is a heat-stable, cationic protein with an apparent molecular weight of 9–11 kDa. SKALP is not found in normal epidermis but only in differentiating cells in psoriasis and healing wounds. It has an anitinflammatory effect [16]. Loricrin, a small proline rich-protein (SPRR) and involucrin are protein precursors of the cornified envelope, which are cross linked by a transglutaminase in the final stages of keratinization. The proper expression of these enzymes and their substrates is a condition sine qua non for the formation of a competent barrier [17]. Unfortunately Ponec et al. [6] and [18] did not specify the anatomic site, gender or age of the source of the human reference tissue used. This may have been helpful for comparison with other published lipid compositions.

2.6. Applications

2.6.1. Phototoxicity testing

The relevance of the SkinEthic[®] model for the assessment of phototoxicity has been shown by comparing experimental results to in vivo data from tolerability studies. Following the protocol described by Liebsch [19], 13 nonphototoxic and phototoxic compounds were applied to the model and exposed to UVA radiation. The model proved capable of discriminating between phototoxic and nonphototoxic compounds [20]. Medina used the leakage of LDH, as a marker for a decrease in cell viability, and increased IL 8 release and expression of IL 8 mRNA as a tools to quantify the phototoxicity of test compounds. It was shown that phototoxic compounds could be correctly identified using the SkinEthic[®] model [21]. A testing strategy for evaluation of phototoxic hazards using the SkinEthic[®] model has been described by Jones et al. [22].

2.6.2. Irritancy testing

Sodium lauryl sulfate, calcipotriol and trans-retinoic acid were applied to both human skin and the in vitro model for 24 h. Afterwards the level of cytokine expression and inflammatory skin reaction was measured. Results obtained with the SkinEthic[®] model correlated with results from intact human skin [23]. In 2002 a study was conducted to determine the reproducibility of data obtained from in vitro irritation testing using the commercially available reconstructed human epidermal models, including the SkinEthic[®] model. A protocol was established based on the measurement of cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The release of proinflammatory mediators and enzymes after different times of exposure to sodium lauryl sulfate (SLS) were quantified. The viability results showed that the SkinEthic[®] model was potentially the most sensitive model to SLS, but also the least reproducible [24]. Another study showed that the release of interleukin-1α and interleukin-8 by the SkinEthic[®] model correlated with the results from the MTT assay and also could be used to classify sensitizing and irritating compounds [25].

2.6.3. Transport data

The speed of transport across the SkinEthic[®] model was examined and compared to human skin using lauric acid, caffeine and mannitol as penetrants. It was found that lauric acid was the best permeant of the group followed by caffeine and mannitol. This rank order correlates with findings in human skin. Variations in transport rates were low compared to those seen with human skin [26].
In another investigation the fluxes of terbinafine, clotrimazole, hydrocortisone and salicylic acid through human skin were compared using the SkinEthic[®] model. The substances were applied at a concentration of 1% in propylene glycol or in propylene glycol/water 9:1 (for salicylic acid). The penetrants were chosen to provide a large range of polarity. Terbinafine showed a high flux through the SkinEthic[®] model while it showed almost no transport through dermatomed human skin (The authors did not specify the thickness to which the skin was cut.) The drug concentration in the model after the end of the experiment was 55-fold higher than in human skin. Clotrimazole was transported almost nine time faster through the model than through the native tissue. The concentration in the model was up to 200 times higher compared to native skin. Also hydrocortisone penetrated faster through the model than through its native human counterpart, and the concentration was 25 times higher than in human skin. Only with salicylic acid similar concentrations were found in the model and human skin. The transport, however, was 6–12 times higher through the model. It was concluded that the SkinEthic[®] model does not provide sufficient barrier function to be useful for transport studies [27].
The transport of caffeine from w/o/w multiple and o/w emulsions through the SkinEthic[®] model and human skin was compared by another group. The cumulative absorbed amount from the o/w emulsion was always higher than from the w/o/w emulsions in human skin and the model. The amount which permeated through the model after 24 h was close to 25 times higher than the amount that permeated through human skin after 6 h [28]. In another study, the effects of albumin on the absorption of drugs and their metabolism in the model was examined and discussed with respect to results from excised pig skin [29]. The drug preparations studied included solid lipid nanoparticles, an ethanolic solution of testosterone and 0.25% cream preparations of prednisolone 17-ethylcarbonate, prednisolone 21-carbonate and prednicarbate. The addition of albumin to the acceptor reduced steroid permeation, especially while testing formulations with low prednicarbate uptake. Furthermore the authors showed that the metabolic pattern of prednicarbate was affected by the presence of BSA when using the reconstructed human epidermis equivalents.

3. EpiSkin[®]

3.1. About the company

The company known today as L'Oreal was founded in 1909 by Eugène Schueller, a chemist, and has become one of today's biggest cosmetic companies. It is active in the investigation of the skin and artificial skin models. Investigative work with skin models is carried out at the research center of L'Oreal in Aulnay-sous-bois.
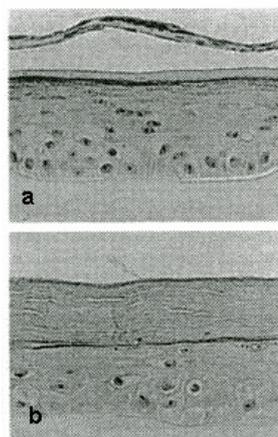
3.2. General

The EpiSkin[®] model was first developed by E. Tinois and was bought by L'Oréal in April 1997. The EpiSkin[®] kit is currently marketed in the form of 12 well plates. It consists of a 'type I bovine collagen matrix, representing the dermis, surfaced with a film of type IV human collagen, upon which is laid, after 13 days in culture, stratified differentiated epidermis derived from second passage human keratinocytes' [30]. This model is used for studies of irritation. To produce a model more suitable for drug penetration, the keratinocytes are cultured for 20 days prior to transfer to the collagen substrate [6] and [31].

3.3. Morphology

The EpiSkin[®] model shows all of the epidermal layers seen in its native counterpart. The stratum corneum of the EpiSkin[®] penetration model shows a significantly increased number of cell layers compared to most native stratum corneum samples, and as a consequence, is thicker. The cells of viable portion of the EpiSkin[®] model are organized somewhat differently than the cells in native epidermis. There are sudden changes in cell shape in the suprabasal compartment. Basal cells tend to be cubical in shape, while the upper cell layers are relatively flat. Extrusion of lamellar body contents is disturbed in EpiSkin[®] cultures. Granular cells with keratohyalin are present but of irregular shape. Intrabatch variation is low, but there is somewhat greater interbatch variation. As in the SkinEthic[®] model characteristic electron microscopic structures as alternating electron-dense and electron-lucent lipid lamellar sheets in the intercellular space of the stratum corneum are found [6], [10] and [18] (Fig. 3: morphology of the EpiSkin[®] irritation model (c) and penetration model (f)). Obviously the penetration model has a thicker and tighter packed stratum corneum than the irritation model.

Figure 3: Morphology of the EpiSkin[®] irritation model (a) and penetration model (b) [15], reprinted with permission of S. Karger AG, Basel.

3.4. Lipid composition

Ponec examined the lipid composition of the EpiSkin[®] irritation and penetration models (Table 2). All major epidermal classes were found in both models. The phospholipid content found in the EpiSkin[®] irritation model was very close to that of human epidermis. However, in the EpiSkin[®] penetration model the amount of phospholipids was low. The precursors of ceramides, glucosphingolipids, were found in comparable amount as in human tissue in both models. Ceramide amounts in the EpiSkin[®]

models differed from those found in native epidermis. While ceramide 7 (Cer[AH]) was missing, ceramide 5 (Cer[AS]) and 6 (Cer[AP]) were unusually low and ceramide 2 (Cer[NS]) was present in a much larger amount than in normal human epidermis. The amounts of free fatty acids and of cholesterol esters were lower than in native tissue. Lanosterol which is not found in significant amounts in human epidermis was found in the EpiSkin® models. Large variations in lipid composition from batch to batch were encountered [10] and [15].

Table 2: Lipid composition of the EpiSkin® model compared to native human tissue [15]

Lipid class	EpiSkin® irritation model mean±SD (*n*=5)	EpiSkin® penetration model mean±SD (*n*=3)	Native tissue mean±SD (*n*=3)
Phospholipids	33.0±12.5	13.7±6.9	36.5±4.1
Sphingomyelin	4.7±1.3	2.2±0.9	8.9±1.6
Phosphatidylcholine	10.8±2.7	5.2±2.5	11.2±0.8
Phosphatidylserine	2.6±1.6	0.9±0.7	3.9±0.3
Phosphatidylinositol	3.6±2.5	1.5±0.6	2.2±0.8
Phosphatidylethanolamine	11.2±5.4	4.0±2.5	10.3±0.8
Cholesterolsulfate	2.0±0.5	1.4±0.5	5.0±1.6
Glucosphingolipids	3.4±1.4	1.2±0.4	5.0±0.4
Ceramides	18.5±6.9	25.1±4.2	12.1±1.8
Free fatty acids	1.8±0.5	2.0±1.3	7.8±1.2
Cholesterol	17.6±4.2	17.5±2.3	17.7±3.2
Lanosterol	1.3±0.5	2.8±1.5	–
Di-/triglycerides	20.9±2.9	34.0±6.4	8.9±3.7
Cholesterolester	1.5±0.5	2.3±0.8	7.0±0.4

Table 2 shows a great difference between native tissue and the Episkin models regarding their amounts of di-/triglyceride 8.9 vs 20.9%. The reasons for these variations cannot be explained. The subsequent question which arise from these findings is of course if it does influence the barrier properties of the models. Correlations of the amount of certain lipid classes with the transport of drugs have been attempted several times [32], [33] and [34]. Elias et al. [32] outlines that the transport is more closely linked to the total amount of lipids present in skin than to single lipid classes. A prediction of how the higher triglyceride amount affects the barrier function can therefore not be formulated. An elevated production of triglycerides and retention of lipids within the cornified cell has been associated with hyperproliferation and impaired barrier function. This is seen in a number of hyperproliferative diseases or conditions including atopic dermatitis, psoriasis and essential fatty acid deficiency. This association has been demonstrated in a non-commercial epidermal keratinocyte air–liquid culture system [35].

3.5. Biochemical markers

In the EpiSkin® penetration model Keratin 1 and 10 are present in stratum spinosum and stratum granulosum. Keratin 6 is present in all layers. SKALP is found in the upper stratum spinosum and–as SPRR2 and SPRR3–in the stratum granulosum. Loricrin is absent while involucrin and transglutaminase are present in all suprabasal layers. These results are similar to those from the EpiSkin® irritation model. One difference is that in the EpiSkin® models SPRR3 is missing, and loricrin is present in the EpiSkin® irritation model [15].

3.6. Applications

3.6.1. Phototoxicity testing

In studies to examine the suitability of the model for phototoxicity the effects of several weak phototoxic, 6-methylcoumarin and ofloxacin, were compared to the effect of chlorpromazine, a strongly phototoxic substance. SLS and sulisobenzone served as negative controls. The substances were applied topically, and after 1 h incubation the cultures were exposed to UVA at a non-cytotoxic dose. After incubation for another 18 h the MTT viability test was performed, and the IL1α- released into the culture medium was quantified. The phototoxic compounds combined with UVA lead to an increase in cell mortality and a rise in IL1α-release, and thereby demonstrated the ability of the model to be used for identification of phototoxic substances [36].

3.6.2. Irritancy testing

Several studies have been undertaken to investigate if the model responds to irritants in a manner similar to native skin. In one of these investigations the model was subjected to several surfactants (Table 3).

Table 3: Surfactants used for irritation testing with EpiSkin[®]

Nr	Chemical name
1	Polyoxyethylene sorbitan monooleate
2	Polyoxyethylene sorbitan mono monolaureate (Tween 20)
3	Pentadecanol (etherified)
4	Industrial polyoxyethylene sorbitan monolaureate
5	Dodecanol (etherified)
6	1,2-Dodecanediol (etherified)
7	Blend of decanol and dodecanol (both esterified)
8	Octyl phenoxypolyethoxy ethanol
9	Dodecyl mercaptans (etherified 25 r)
10	Dodecyl mercaptans (etherified 15 r)
11	Dodecyl mercaptans (etherified 20 r)
12	Blend of sodium and magnesium laurylethersulfate
13	Triethanolamine acylamidopolyglycolehtersulfate
14	Sodium dodecyl sulfate (SDS)
15	Sodium dodecylether sulfate
16	Ammonium dodecyl sulfate
17	Triethanolamine dodecyl sulfate
18	Sodium lauryl *N*-, ethylglycinate
19	Coprah amphoteric alkylimidazolium dicarboxylate (MIRANOL)

Nr	Chemical name
20	Cocobetain derivative
21	Hexadecyltrimethylammonium bromide (CTAB)
22	Tetradecyltrimethylammonium bromide
23	Octodecylmercaptans (etherified)

The release of cytokines (Interleukin 1α) and eventual impairment of the barrier function were measured. The damage of the epithelial barrier was measured using the permeability marker fluorescein. The data obtained was compared with historical data from in vivo ocular irritancy testing and subjected to a statistical analysis. A correlation was shown for the cytotoxic potential ($r=0.93$; $n=23$; $P<0.00001$) and the impairment of the barrier function ($r=0.87$; $n=20$; $P<0.00001$). A correlation was also shown for cutaneous irritation ($r=0.81$; $n=20$; $P<0.0001$) [31]. In another study the concentrations of surfactants necessary to cause irritation were determined and compared with in vivo human skin data from the literature [37]. The results showed that the concentration necessary to trigger a reaction in the model was smaller than required in vivo. The authors suggested that this reflected the impaired barrier function of the model [38]. Another example of the use of the EpiSkin[®] model in the investigation of irritation in human skin is the work of Cotovio et al. [39]. These investigators exposed the model to 10 ppm of ozone and monitored the formation of protein carbonyls by an ELISA method. Oxidative stress was also assessed using the fluorogenic probe, 2′,7′-dichlorofluorescin diacetate. Comparison with human keratinocytes cultures showed correlating results. The EpiSkin[®] model was subsequently used to test the potential of compounds for reduction of oxidative stress in human skin. The results showed that the EpiSkin[®] model was susceptible to oxidative stress induced by air pollutants. Faller et al. used the EpiSkin[®] model in a comparison of human in vivo and in vitro skin irritation caused by cosmetic products. Formulations representing 22 different cosmetic products were tested in vivo and in the model. Cell viability as judged by the MTT test and the release of IL-1 α and cytosolic lactate dehydrogenase were measured. There was a good correlation for these measured parameters between skin in vivo and in vitro EpiSkin[®] model [40].

The European Centre for the Validation of Alternative Methods (ECVAM) supported a prevalidation study on in vitro tests for acute skin irritation triggered by chemicals. The performance of the EpiSkin[®] method for predicting skin irritation was judged as insufficient in phases 1 and 2 of the project during which reproducibility and transferability were examined. On the base of these results, the existing protocol was refined. The new protocol showed good results when it came to sensitivity, specificity and accuracy [41].

3.6.3. Transport data

Several studies have examined transport through the EpiSkin[®] model. In one case the penetration of caffeine from different vehicles into the EpiSkin[®] model was examined. The penetration of caffeine and α-tocopherol acetate from a w/o-emulsion, an o/w-emulsion, a liposomal dispersion and a hydrogel were compared with EpiSkin[®] and human skin. The EpiSkin[®] model showed the same permeability rank order as human skin [42]. The model was more permeable to both drugs than human skin. Furthermore transport through the model reached its maximum earlier than through human skin. The vehicles affected the skin bioavailability in human skin only slightly. The bioavailability in the EpiSkin[®] model varied among the formulations. α-Tocopherol acetate absorption from the hydrogel was lower than from the other preparations. After prolonged application of the hydrogel on the skin model, permeation dropped to an insignificant level. The author relates this to an interaction between the lipids in the EpiSkin[®] model and alcohol and Carbomer 950 in the preparation [42]. Experiments carried out by the same group showed that the model was more permeable than human skin to mannitol [43].

3.6.4. Corrosivity testing

In 1998 the results of an ECVAM supported international validation study on in vitro tests for skin corrosivity using the EpiSkin® model were published. The objective of the study was to find an in vitro test method that could discriminate between corrosive and non-corrosive substances. Testing of 60 different chemicals was conducted in three different laboratories. Intralaboratoy and interlaboratory reproducibilities were acceptable. The model managed to meet the test criteria and correctly identified corrosive and non-corrosive test chemicals. [44].

4. Epiderm®

4.1. About the company

MatTek Corporation—Ashland, Ma, USA—was founded in 1985. EpiDerm was introduced in 1993 to the market. The company is offering several other types of models, such as, e.g. reconstructed epidermis containing melanocytes, reconstructed corneal epithelium, etc. Recently a full thickness skin (EpidermFT®) model has been released. Unfortunately, at this time there are essentially no published studies using this model. This article will therefore focus on the EpiDerm® model which has been widely covered in the literature. Two subtypes of the Epiderm® models are covered in the literature: the hydrocortisone-free culture and the percutaneous absorption culture [45].
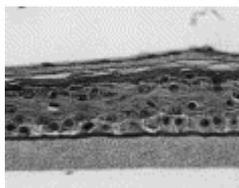
4.2. General

The Epiderm® model was first described by Cannon et al. in 1994 [45]. The data sheet from the MatTek Corporation describes the EpiDerm® skin model as 'normal, human derived epidermal keratinocytes (NHEK) which have been cultured to form a multilayered, highly differentiated model of the human epidermis'.

4.3. Morphology

The general morphology of the model is comparable to that of normal human epidermis. One difference is that, since the keratinocytes are grown on polycarbonate filters, there are no Rete ridges, which anchor dermis and epidermis in native tissue. All stratas are present in the MatTek penetration model. The number of viable cell layers ranges from 6 to 8, and in the irritation model from 7 to 14, which leads to an epidermal thickness 28–43 µm for the first model and 83–100 µm for the second. Basement membrane is described as patchy for both models and hemidesmosomes are present in 50% of the cultures. The models do not differ in the stratum basale. The cell shape is described as columnar to round. Intracellular lipid droplets, which are absent from native epidermis, are encountered in the MatTek cultures. In the stratum spinosum the cells are flattened, as in native epidermis. Intracellular lipids in the stratum corneum of the culture model are present, but their organization is highly variable. The lamelar bodies in the stratum granulosum appear normal, and the keratohyalin granules appear rounded to stellate in shape. The stratum corneum contains 16–25 loosely packed cell layers with an overall thickness of 12–28 µm. The extrusion of lamellar body contents at the SG/SC interface is complete [10], [15] and [18] (Fig. 4: morphology of the EpiDerm® penetration model).

Figure 4: Morphology of the EpiDerm® penetration model [15], reprinted with permission of S. Karger AG, Basel.

4.4. Lipid composition

The lipid profile of this epidermal model includes all major lipid classes in amounts similar to normal human epidermis (Table 4). The model shows a slightly elevated amount of glucosylceramides. The ceramide profile is overall comparable to human epidermis with all classes of ceramides present but with some deviations in relative proportions. The amount of ceramide 2 (Cer[NS]) was higher than in human epidermis, while ceramides 5 (Cer[AS]) and 6 (Cer[AP]) were present in relatively low amounts. Ceramide 7 (Cer[AH]) was absent. As in other models, contents of cholesterolesters and free fatty acids were lower than in native tissue [15].

Table 4: Lipid composition of EpiDerm® model compared to native human tissue [15]

Lipid class	EpiDerm® irritation model mean±SD (*n*=5)	EpiDerm® penetration model mean±SD (*n*=3)	Native tissue mean±SD (*n*=3)
Phospholipids	36.5±2.7	30.4±1.1	36.5±4.1
Sphingomyelin	8.2±1.5	6.3±0.3	8.9±1.6
Phosphatidylcholine	13.6±2.4	10.7±0.2	11.2±0.8
Phosphatidylserine	3.2±0.7	2.3±0.1	3.9±0.3
Phosphatidylinositol	4.3±0.8	3.7±0.9	2.2±0.8
Phosphatidylethanolamine	7.1±1.6	7.4±0.3	10.3±0.8
Cholesterolsulfate	5.8±1.2	5.7±1.6	5.0±1.6
Glucosphingolipids	9.5±1.3	5.8±0.1	5.0±0.4
Ceramides	18.5±3.5	28.9±0.3	12.1±1.8
Free fatty acids	2.6±0.5	3.1±0.6	7.8±1.2
Cholesterol	14.8±1.3	17.9±0.9	17.7±3.2
Lanosterol	1.2±0.5	1.0±0.1	–
Di-/triglycerides	10.5±2.2	6.9±0.8	8.9±3.7
Cholesterolester	2.7±1.1	2.1±0.4	7.0±0.4

4.5. Biochemical markers

Keratin 1, 10 and 6 are present in both the EpiDerm® irritation and penetration models in all suprabasal layers. In the irritation model Keratin 6 is only present intermittently in the lower stratum spinosum. SKALP is present in the stratum spinosum and—as SPRR2—in the stratum granulosum. SPRR3 is present in the stratum granulosum in the irritation model but missing from the penetration model. Loricrin is encountered in the stratum granulosum. Involucrin is present in all suprabasal layers in both models. Transglutaminase is present in all suprabasal layers in the penetration model, but in the irritation model transglutaminase is only present in the upper suprabasal layers [15].

4.6. Applications

4.6.1. Phototoxicity testing

Liebsch et al. transferred a test protocol which was originally applied by the german ZEBET (Centre for Documentation and Evaluation of Alternatives to Animal Experiments) for phototoxicity testing with the Skin2 model to the EpiDerm® model. The principle of the test is to apply test materials topically to the model at five different concentrations and then evaluating the reaction with and without a non-cytotoxic dose of UVA and visible light, which simulates sun. One day after irradiation, the cytotoxicity is determined using the MTT assay. The model correctly identified phototoxic compounds [19].

4.6.2. Irritancy testing

In an interlaboratory comparison 16 surfactant containing formulations were tested on different batches of EpiDerm® in three different laboratories. The results from the different labs were then compared mutually and with human in vivo data. The correlation was good and showed that the model can be used to identify irritants [45].

Studies were conducted to identify biochemical markers of skin irritation that are measurable before physiological signs of irritation occur. SLS and tritiated water were applied topically to the EpiDerm® model and to excised human skin. The level of irritation was determined by measuring IL-1α mRNA levels. The response of the EpiDerm® cultures differed significantly from that of human skin. This difference was attributed to the suboptimal barrier function of the model compared to human skin. It was concluded that the model is suitable for screening possibly irritating substances. Because the barrier function is limited, the concentrations necessary to trigger a reaction are lower than they would be in excised human skin [46].

A prevalidation study was conducted to examine the interlaboratory and intralaboratory repeatability of irritancy testing using the EpiDerm® model. The results showed good intralaboratory reproducibility of the assay; however, there were statistically significant differences among the different laboratories [47]. In this study, the substances of Table 5 were used.

Table 5: Irritants used in the EpiDerm® irritation prevalidation study

Nr	Chemical name
1	1-Bromohexane
2	Tetrachloroethylene
3	2-Ethoxyethylmethacrylate
4	*n*-Butyl propionate
5	Alpha-terpineol
6	Heptanal
7	Tallow polypropylene polyamine
8	1,6-Dibromohexane
9	Sodium metasilicate (10%)
10	Sodium bisulphite
11	Methyl palmitate
12	1-Bromopentane
13	3,3′-Dithiodipropionic acid
14	4,4′-Methylene bis (2,6-ditert-butyl)phenol

A comparison of 22 cosmetic formulations were tested for irritation potential in vivo on human skin and on the model. A good correlation was found, indicating that the model could be useful for assessment of the irritation potential of cosmetic compounds [40].

4.6.3. Transport data

Dreher et al. [42] compared the penetration of caffeine from different vehicles into the EpiDerm[®] model. The penetration of caffeine and α-tocopherol acetate from the range of formulations described above in Section 3.3 were compared. The results were similar between human skin and the EpiDerm[®] model. Also, the rank order of permeability was the same in the EpiDerm[®] model and human skin. Permeability for both drugs was higher in the model than in human skin. The maximum rate of transport was reached earlier in the model than in human skin. With human skin, bioavailability was only slightly influenced by the different vehicles; however, with the EpiDerm[®] model bioavailability varied significantly among the different preparations. A lower amount of α-tocopherol acetate was absorbed from the hydrogel than from the other preparations. Prolonged application of the hydrogel on the skin model lead to a drop in permeation. As noted previously, this decrease in apparent permeability was attributed to an interaction between the culture model lipids and the alcohol and Carbomer 950 in the formulation. In another study transport experiments with mannitol through the EpiDerm[®] model were presented. The results demonstrated that the model is more permeable to mannitol than normal human skin [43]. Further transport experiment were carried out using the lipophilic drug flufenamic acid. The drug was applied to the model in either a wool alcohol ointment (0.1125%) or in Soerensen phosphate buffer pH 7.4 (0.1125% solution). Transport across the model was around forty times higher from the solution than from the ointment. The permeability of the reconstructed skin was five times higher compared to human epidermis [48].

4.6.4. Corrosivity testing

Fentem describes the completion of a successful prevalidation study on the use of the EpiDerm[®] human skin model for corrosivity testing. Experiments performed to optimize test conditions are presented [49].

5. Conclusion

The SkinEthic[®], EpiSkin[®] and EpiDerm[®] skin models are well documented in the literature. At the moment they come close to reproducing human skin, but only in certain aspects. Their general structure, composition and aspects of biochemistry bear a close resemblance to human skin. These skin models are useful in toxicity testing, including phototoxicity testing, and to an extent for drug transport studies. Although considerably more permeable than human skin on average, the culture models appear to be more consistant in permeability and responsiveness than human skin, which is highly variable. The biggest limitation of all three commercially available models is the still relatively weak barrier function. Several possible reasons for this have been suggested, including impaired desquamation [50] and the presence of unkeratinized microscopic foci [51]. In any case, the elevated permeability relative to native human skin obviously limits the value of these skin models for transport studies and may lead to some false positives in toxicity studies. It is the stated goal of all manufacturer to fit their skin models with a barrier similar to human skin in vivo, but it is not foreseeable if or when they will suceed. While this review focuses on epidermis-only models one must not forget the role of the dermis in permeability studies. Nakamura et al. [52] outline that full thickness models match more closely the situation in vivo. The presence of a dermis in vitro solves some problems, but at the same time creates other problems which are adressed by Roberts and Walters [4]. Transport experiments with lipophilic substances through epidermis models might suggest a higher transport through the skin than it is actually the case in vivo because of the lack of a dermis which in vivo is a efficient barrier for lipophilic substances. The lack of a dermis in vitro could lead to higher transport in vitro which would not correspond to the amounts actually encountered in vivo. But just by adding a dermis to a skin model this problem would only partially be solved. Brain et al. [53] give a more detailed description of the problems encountered here. The dermis in vivo is continously perfused by the subcutaneous vasculature, which penetrates the dermis. This system can rapidly remove permeants reaching the epidermal-dermis junction. In the in vitro situation this system is no longer present. Another problem is that in vitro, the aqueous environment of the dermis will slow the penetration of lipophilic compounds. In vivo this barrier is circumvented by the capillary bed. The authors conclude that the use of dermatomed, epidermal or SC membranes is more appropriate for examining particularly lipophilic permeants. Summarizing one can say that adding a dermis to an epidermal only model as Apligraf[®]

from Organogenesis (California, USA) or EpiDermFT from Mattek (Ashland, USA) is certainly interesting for investigating highly lipophilic compounds. However, before they can be put to good use the abovementioned problems of the dermis in vitro have to be solved. Nevertheless, the present commercially available skin models represent a major improvement over what was available just a few years ago. But, it is still not foreseeable if there will be validated models for all necessary testing to completely replace animals by the 2009 ban of animal testing. Because appropriate altenative methods are needed for the development of new products by the pharmaceutical and cosmetics industries, further improvement of reconstructed human epidermis models still represents an important challenge.

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TEWL measurements as a routine method for evaluating the integrity of epidermis sheets in static Franz type diffusion cells in vitro. Limitations shown by transport data testing (Summary)

The purpose of this investigation to investigate the suitability of transepidermal water loss (TEWL) measurements as method for assessing in vitro the barrier integrity for human heat separated epidermis (HSE). The reason behind this is that the OECD guideline 428 (OECD 2004) requests integrity testing before performing permeation experiments. Integrity testing is not necessarily the measurement of the TEWL. The Guidance document 28 recommends the measurement of TEWL among measurement of the electrical resistance or the use of tritiated water as a permeation marker.

Many factors influence the outcome of TEWL measurements when using heat separated human epidermis. Since this investigation was focused on the ability of detecting minor perforations of epidermis sheet a model system consisting of a Teflon membrane mounted in Franz diffusion cells (FDC) filled with phosphate buffer saline (PBS) was set up. The TEWL of the Teflon membrane was first measured with the intact membrane. Successively up to five holes were added by puncturing the membrane with a needle. After each puncturing the TEWL was measured. With this setup all other influence other than the perforation could be eliminated from the measurement. The results showed that only the TEWL of intact and punctured Teflon membrane differed significantly. However the amount of holes did not show a significant influence.

The next step was to repeat the experiments with human skin. From three donators intact human HSE and punctured HSE were compared. No significant difference of the TEWL was found. Permeation experiments with flufenamic acid (FFA) using the same epidermis as in the experiment described above showed a significantly higher diffusion rate through punctured HSE. In order to investigate if the TEWL was able to detect large deterioration of the epidermis opposed to puncture holes HSE was stripped three, seven and 15 times prior to heat separation. Afterwards permeation experiments with these skin and an intact control group were performed. Only the TEWL values of intact HSE and HSE stripped 15 times differed significantly. However, seven and 15 times stripping resulted in significantly higher diffusion rate in the permeation experiments. The conclusion is that TEWL measurements are capable of detecting a weakening of the overall barrier. Small and locally strictly limited damages of the stratum corneum barrier, e.g. puncture holes which already influence drug diffusion are not detected. For this reason, TEWL measurements appear to be of limited use as a barrier integrity test for human HSE in in vitro test systems.

My contribution to this work

- Development of the concept of two types of damage to the stratum corneum: locally limited perforations and general weakening of the overall barrier (e.g. by tape stripping)

- Development of the test assay with the Teflon membrane

- Adaptation of an existing tape stripping set up for use with heat separated epidermis

- Performing of all presented experiments and subsequent statistical evaluation

<u>**The results presented in the following chapter have been published as:**</u>

TEWL measurements as a routine method for evaluating the integrity of epidermis sheets in static Franz type diffusion cells in vitro. Limitations shown by transport data testing

1. Introduction

The measurement of transepidermal water loss (TEWL) is a well established method in dermatology to assess the integrity of the skin barrier in vivo [1]. When skin is damaged, its barrier function is impaired resulting in higher water loss. A frequent problem in the routine practice of diffusion experiments in Franz type diffusion cells (FDC) is the lack of suitable methods for routinely assessing the integrity of epidermal sheets inside the diffusion cell before the experiment has started. However, the OECD guideline 428 [2] requests integrity testing before performing permeation experiments and the Guidance document 28 [3] specifies this statement by recommending the measurement of TEWL, electrical resistance or the use of tritiated water as permeation marker. In comparison with the other methods, TEWL measurements have the advantage that no solutions have to be added to perform the barrier integrity test other than those used in the permeation experiments. Therefore, this investigation was focused on the potential of TEWL measurements to be used for routine testing for the integrity of human HSE prepared for in vitro permeation experiments in a Franz type diffusion cell. Furthermore, the influence of the degree of skin surface damage on drug permeation was addressed. For this purpose the stratum corneum (SC) barrier was damaged by punctuation and stripping with adhesive tape. TEWL measurements and permeation experiments were carried out using the same membrane in both experiments. As model drug flufenamic acid was chosen.

2. Materials and methods

2.1. Materials

The following chemicals and equipment were used: flufenamic acid (Sigma-Aldrich, Deisenhofen, Germany); phosphate buffer solution pH 7.4, McIlvaine citric acid-phosphate buffer pH 2.2 (all components from Merck, Darmstadt, Germany); methanol (VWR International, Leuven, Belgium); Teflon membrane (thickness 1 mm) (Arthur Krüger, Brasbüttel, Germany)

2.2. Human heat separated epidermis

Human skin from caucasian female patients, excised during abdominal plastic surgery, was used with the approval of the ethic commission of the Caritas hospital Lebach, Germany. The subcutaneous fatty tissue was removed immediately after excision. The cleaned skin was stored at –26 °C until use. Previous studies had shown that the skin is stable over 3 and 6 month [4]. This has been confirmed by other laboratories [5] and [6]. Skin discs with a diameter of 25 mm were punched out and thawed prior to putting them in water at 60 °C for 1 min. Afterwards the SC-epidermis layer of the skin was peeled off from the dermis using forceps [7]. The epidermis sheets were spread out in Petri dishes filled with PBS for at least 30 min.

2.3. Punctured human heat separated epidermis

Before heat separation the skin disks were punctured with a needle with a diameter of 1 mm in the center of the diffusion area in order to simulate damage sustained during surgical extraction or preparation. After heat separation the presence of the hole was checked with a magnifying glass.

2.4. Stripped human heat separated epidermis

Tape stripping has often been used to induce barrier disruption on human skin [8], [9] and [10] and was therefore used in this investigation to inflict varying, but defined damage on the stratum corneum barrier. The skin was stripped prior to heat separation. The numbers of adhesive tapes used were fixed to three, seven and 15; the stripping procedure is described in detail by Wagner [11]. Stripping more than 15 times could not be performed due to the high fragility of the resulting HSE sheet.

2.5. Experimental design for TEWL measurements

For TEWL measurements a TEWAMETER Multi Probe Adapter 5 (Courage and Khazaka, Köln, Germany) was used and positioned in a cabinet drier set to 25±1 °C. The FDC setup was transferred to the drier. Using a telescopic arm the TEWL sensor was placed into the opening of the FDC. Another sensor (ambient condition sensor, Courage and Khazaka, Köln, Germany), for humidity and temperature, was added, too. Both sensors were linked to a computer. During measurements the relative humidity (49±5%) in the cabinet drier was subtracted constantly from the TEWL value. The drier was closed and the measurements were initiated when the temperature was stable at 25±1 °C, usually 30 min later. The TEWL was then measured every 15 s over 7 min resulting in 28 data points.

2.6. Testing and characterization of the system via Teflon membranes

To examine whether an increasing damage of a membrane was reflected in a higher TEWL value, a Teflon membrane was placed in the FDC to act as an artificial diffusion barrier. To tightly seal the acceptor from the donor compartment, petrolatum was applied between the Teflon membrane and the upper and lower part of the FDC. Subsequently, the lower part of the FDC was filled with PBS. Preliminary experiments had shown that the distance between sensor and membrane surface had to be reduced to 3 mm. Due to this reason a cut off upper part of a FDC was used. The experiments were carried out as follows: Six FDCs were mounted with intact Teflon membranes and the first set of TEWL measurements was performed. Then the membranes were punctured with a needle and the TEWL was measured again. This procedure was repeated until a total of five holes was reached.

2.7. Experimental setup using human heat separated epidermis

The experimental setup had to be optimized to fulfill the special requirements necessary while working with human HSE. Preliminary experiments demonstrated that it took around 15 min for topically adhering water to evaporate (Fig. 1). To avoid this problem the experimental system was left without cover for 30 min in the cabinet drier. Topically adhering water could evaporate and an equilibrium was reached. The TEWL measurements were initiated afterwards.
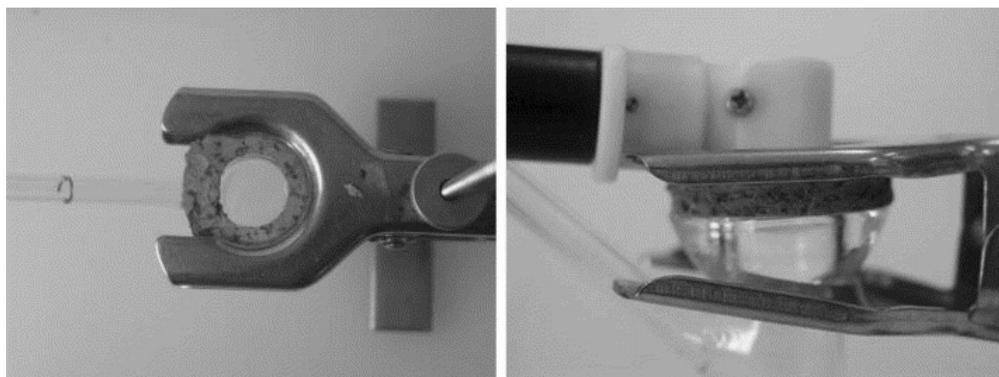
Figure 1: TEWL values over time obtained from a not correctly dried HSE sheet.

To be able to use the same experimental setup firstly for TEWL measurement and secondly for permeation experiments the following procedure was adopted: The upper part of the FDC was replaced by a cork ring and a ring of a dialysis membrane. At the center of that dialysis membrane an opening with a diameter of 16 mm, slightly larger than the diffusion area in the permeation experiments, had been punched out. This membrane ring was placed on top of the HSE. The whole

system was then secured with a horse shoe clamp to avoid accidental displacement. The cork ring and the clamp assured that the HSE was securely placed on top of the FDC. The cork ring kept the HSE from being damaged by the clamp. The dialysis membrane ring allowed the removal of the cork ring after the TEWL measurements were carried out without the HSE sticking to the cork ring. The opening at the center of the cork ring was large enough to place the sensor 3 mm above the surface of the epidermis membrane. The distance was controlled by using a micrometric ruler. The experimental setup is shown in Fig. 2.

Figure 2: Experimental setup of TEWL experiments.

2.8. Permeation experiments

For the following diffusion experiments the cork ring was replaced by the original upper part of the FDC. The same HSEs as in the TEWL experiments were used. Permeation experiments were carried out in Permegear static type 6G-01-00-15-12 Franz cells (Perme Gear, Riegelsville, PA, USA); receptor volume: 12.1 ml; donor volume: 0.5 ml 1250 µg/ml of Flufenamic acid in PBS buffer; diffusion area: 1.768 cm^2; acceptor: PBS buffer. At defined time intervals 0.4 ml samples were drawn and replaced by fresh PBS. For further details see Wagner [12].

2.9. Calculation of Papp value

The linear branch of the permeation data was determined using correlation analysis. A minimum of six data points in the linear branch were taken to calculate the flux J [µg/(cm^{2}s)] by linear regression. The flux J was then divided by the concentration in the donor [µg/cm^3] in order to calculate the apparent permeation constant Papp [cm/s].

2.10. Drug analysis

From each sample 50 µl were injected directly onto an isocratic HPLC system consisting of a Dionex ASJ 100 automated sample injector, an UVD 170S detector, a Dionex P580 pump, Chromeleon 6.50 SP2 build 9.68 and a 5 µm LiChrospher$^®$ 100/RP-18 column/12.5 cm×4 mm (Merck-Hitachi, Darmstadt, Germany). An 80/20 (V/V) mixture of methanol/McIlvaine citric acid–phosphate buffer pH 2.2 was used as a mobile phase. At a flow rate of 1.2 ml/min the retention time of FFA was 3.5±0.2 min. The detector was set at 284 nm. Unknown FFA concentrations were calculated against known standards via the method of area under the absorption-time curves. The method provided good linearity (r=0.999) over a concentration range of 50–2000 ng/ml.

2.11. Statistical evaluation

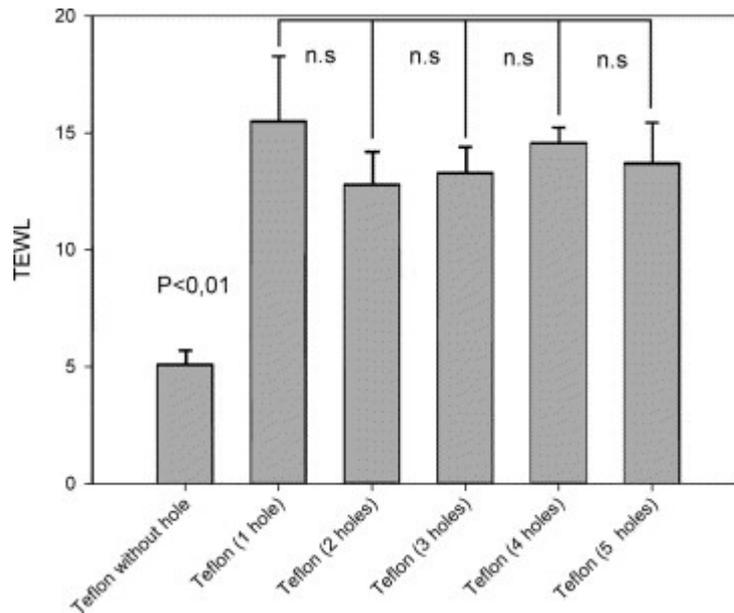All statistical evaluations were done with Sigmastat 3.0.1. (SPSS Inc., Chicago, USA).

3. Results

3.1. Experiments with Teflon membrane

As shown in Fig. 3, the TEWAMETER could only distinguish between an intact and a punctured Teflon membrane. A significant increase of the TEWL value was observed after the first hole but did not rise any further adding more holes (Fig. 3).
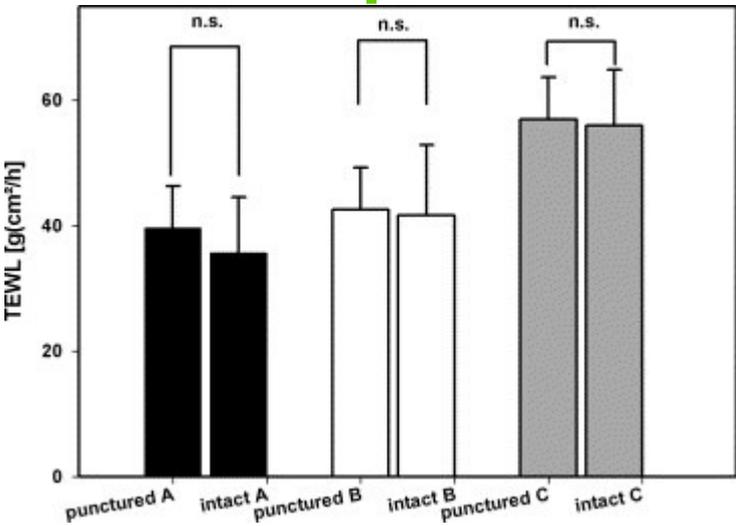
Figure 3: Results of experiments with Teflon membranes (all groups *n*=6).

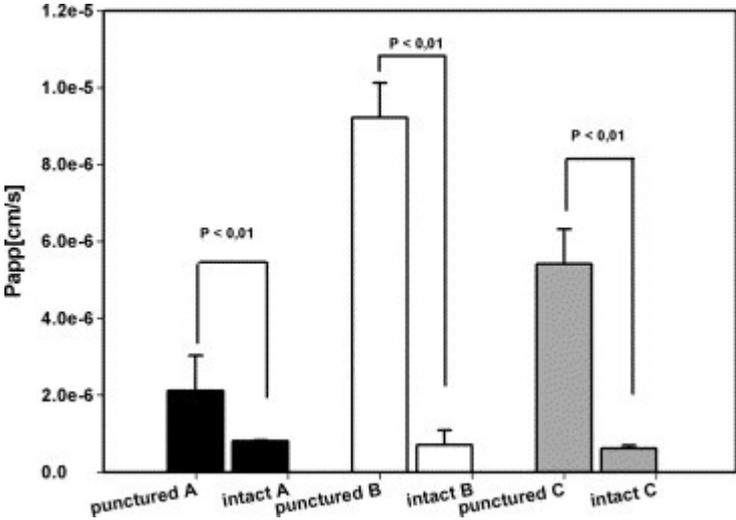## 3.2. Experiments with punctured epidermal sheets

Considering the results with the Teflon membrane six FDCs were prepared with intact human HSE and six FDCs with HSE sheets each punctured with one hole to compare their TEWL values. This experimental design was repeated with HSE sheets of two additional donors (Fig. 4). Independent of the skin used no significant differences could be found (t-test with alpha=0.05: set A: $P=0.075$; set B: $P=0.375$; set C: $P=0.282$). The TEWL measurement was not suitable to identify those small skin damages.

Figure 4: TEWL values of punctured and intact HSE (all groups *n*=6).

Furthermore, permeation experiments with FFA were carried out with set A, B and C to investigate the impact of the punctuation on drug diffusion (Fig. 5).

Figure 5: Results of permeation experiments using intact and punctured HSE as barrier (all groups *n*=6).
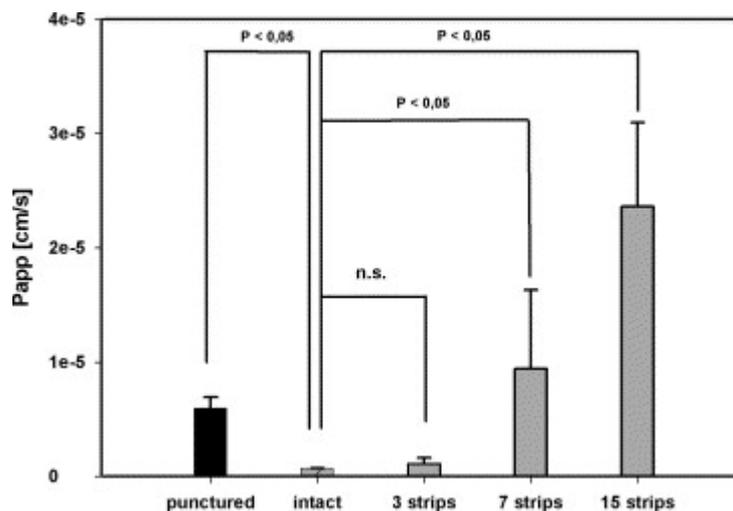

In each set, the Papp values of punctured HSE were compared with the intact HSE control group using a t-test (t-test with alpha=0.05; set A: P<0.01; set B: P<0.01; set C: P<0.01). A statistically significant difference was found in all three data sets.
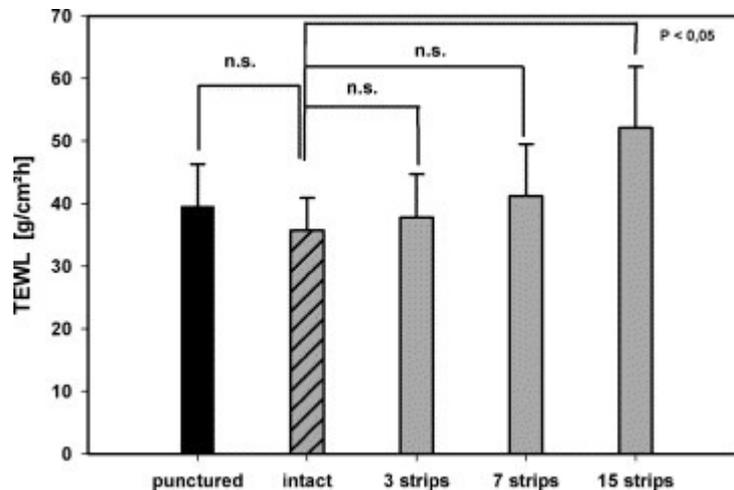
3.3. Experiments with stripped skin

In this experimental series, a control group with undamaged HSE (n=6) was compared with a stripped epidermis group consisting of HSE sheets stripped 3 (n=6), 7 (n=6) and 15 (n=6) times prior to heat separation. The TEWL values of all HSE membranes are shown in Fig. 6. A statistically significant difference was only found between intact HSE and HSE stripped 15 times (for statistical comparison ANOVA one way [13] was used [alpha=0.05; P<0.05; null hypothesis=no difference between the experimental groups]). The Holm-Sidak method was taken to identify significantly differing groups (P=0.05). A statistically significant difference was only found between intact HSE and HSE stripped 15 times.

Figure 6: TEWL values of intact, punctured and stripped HSE (all groups *n*=6).

The results of the subsequent permeation experiments are shown in Fig. 7. The Papp values of all treatments were compared with Kruskal–Wallis Analysis of Variance on Ranks (null hypothesis=no difference in the distribution of values between the different groups). The Kruskal–Wallis ANOVA on Ranks is a non-parametric test that does not require assuming all the samples were drawn from normally distributed populations with equal variances [13]. The application of this test was necessary since one group of data was not normally distributed. The null hypothesis was rejected. Therefore, the groups demonstrated a significant difference (P<0.05). Now, a Holm-Sidak procedure could be used (overall significance level=0.05) to find out which groups differed significantly. A statistically significant difference was determined between intact HSE, punctured HSE and HSE stripped seven and 15 times. No difference could be detected between intact HSE and HSE stripped only three times.

Figure 7: Papp values of permeation experiments with intact, punctured and stripped HSE (all groups *n*=6).

4. Discussion

The TEWL is a well established method for testing the integrity of the barrier function of the SC of human skin in vivo [14], [15], [16], [17], [18], [19] and [20]. Therefore, this method is often recommended in the literature [3] as a barrier integrity test for in vitro permeation experiments. The first experiments with the TEWAMETER showed that in order to be able to use TEWL measurements as a barrier integrity test in static FDCs the experimental parameters had to be adjusted. Two factors proved to be of crucial importance: First, reproducible data were only obtained when the sensor of the TEWAMETER was positioned 3 mm above the membrane. Second, the experiments had to be performed in a cabinet drier to avoid air turbulences and set at constant temperature (in our case 25 ± 1 °C). One method to place the sensor is shown in Fig. 2. Another difficulty encountered during this test phase was the water adhering on the HSE's surface. To overcome this problem an equilibrium time of at least 30 min was necessary (Fig. 1). When these factors were taken into account it became possible to distinguish intact Teflon membranes from punctured Teflon membranes as shown in Fig. 3. The lipophilic Teflon membrane was used in order to make sure that water could only cross the barrier at the puncture holes. Although only the influence of the holes on the TEWL should be addressed, it was not possible to differentiate between membranes with a different degree of damage, e.g. one to five holes. Since, water droplets could be seen on top of the holes while performing the experiments a possible explanation might be that this water covers the surface of the membrane with a thin water layer—independently of the number of holes. Experiments with punctured HSE sheets (one hole) of three different donors showed no statistical significant difference between damaged and intact HSE concerning the TEWL value (Fig. 4). One explanation might be, that due to swelling processes, the holes were sealed [3]. Similar findings had been reported for holes in guinea pig skin resulting from the hairs [21]. In contrast to the TEWL results, permeation experiments with punctured HSE demonstrated a strong influence on drug diffusion, which show that the integrity of the barrier was compromised. As indicated in Fig. 5 the Papp value for the damaged HSE sheets is 2–10 fold higher compared to the intact HSE (ratio depending on skin donor used). This clearly illustrates that TEWL measurements in vitro are not predictive for drug permeation in vitro even though a local damage of the epidermal barrier function is present. In the experiments with tape stripped HSE, which focused on reducing the barrier function over the whole surface of the HSE, a statistically significant difference of the TEWL values could only be detected between intact HSE and HSE stripped 15 times (Fig. 6). These results are in accordance with results reported by Pirot [22] who showed that the TEWL rises exponentially when the skin barrier is gradually removed by tape stripping. Furthermore, our findings are in line with results reported by Chilcott who found a significant increase of the TEWL with full thickness pig skin stripped 15 times [23]. In addition, for three and seven times stripping only a slight increase of the TEWL value could be identified. However, these values were not statistically significant different from

the intact HSE. Concerning the permeation experiments with gradually weakened barriers produced by tape stripping the application of seven and 15 strips caused a statistically significant increase of the Papp values (Fig. 7). This effect was more pronounced for the 15 times stripping experiments than for the seven times one. The weakening of the barrier of the SC by three strips could not be detected as statistically significant different from intact HSE by transport experiments. However, a trend to higher Papp values with a broader standard deviation was apparent, indicating a minor damage of the barrier. In summary, TEWL measurements could only detect some relatively severe damage of human HSE sheets in vitro, e.g. induced by tape stripping 15 times. Lighter damage, e.g. done by stripping with three or seven tapes only or by puncturing one hole, could not be determined. However, all damaging procedures, except stripping with three tapes resulted in an increased drug permeation in vitro across human HSE sheets.

5. Conclusion

The OECD guideline 428 [2] requests skin integrity tests for in in vitro diffusion experiments and suggests among other methods the measurement of the TEWL as a possible tool to assess the integrity of the SC barrier. However, the data presented here suggests that this method used in the described experimental design is very limited in its potential to correctly evaluate the barrier integrity in in vitro permeation test systems based on static FDCs. Only a relatively strong damage of the barrier function could be detected, while drug transport was already influenced by slight barrier changes, which a barrier integrity test should be capable of detecting. Therefore, the TEWL measurement does not seem to be an appropriate tool to routinely check skin integrity in human HSE in vitro.

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Permeability of the reconstructed human epidermis model Episkin in comparison to various human skin preparations (Summary)

The major aim of this was to develop a robust and versatile experimental setup for small diameter reconstructed human epidermis models.

The secondary aim was to find out which of several human skin preparations is the best choice for performing permeation studies.

A modification for the Franz diffusion cell had to be developed for this purpose. The experimental setup was first tested using the insert and methylene blue solution and a dialysis membrane. No influence of the insert could be detected and experiments with human skin and the model could be undertaken. The human skin preparations used were as follows: Full thickness skin (FTS), split thickness skin (STS), heat-separated epidermis (HSE), and trypsin isolated stratum corneum (TISC). Afterwards experiments with Episkin using the insert were done.

The comparative experiments showed then that HSE is the best suited material due to its clear morphological structure and ease of preparation. The experimental data gathered with Episkin showed the following results: In general the Episkin model showed a higher permeability than its human counterpart. Permeation data with HSE and Episkin were then gathered using caffeine and testosterone. Both test compounds permeated much faster through Episkin than through HSE. Moreover, opposed to Episkin, HSE differentiated between the two test compounds. Additionally the lipid profiles of HSE and Episkin were analyzed and showed significant differences in terms of cholesterol, ceramides and triglycerides contents, whereas cholesterol esters and fatty acids were not different. Even if the current model is a remarkable progress compared to what was available several years before Episkin is obviously not capable of replacing human skin for in vitro permeability experiments.

My contribution to this work

- All experiments for the evaluation of the Episkin adaptor with Methylene Blue and dyalisis membrane

- Permeation Experiments with RHEs

- Statistical data analysis

- Experiments in the plastic filter inserts

- Evaluation of all experimental data (permeation experiments and lipid profiles) from all partners

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Permeability of the reconstructed human epidermis model Episkin[®] in comparison to various human skin preparations

1. Introduction

Reliable data about permeation of compounds through human skin are necessary for pharmaceutical, cosmetical and toxicological research. However, a formal standardisation of skin absorption experiments is still missing, although documents on conducting skin permeation studies are reported [1], [2] and [3]. Nevertheless, the differing procedures influence the results and make comparisons difficult. Recently an improvement was the release of the OECD guideline 428 [4] in 2004 and the guidance document 28 [5]. Herein some long sought clarification and standardisation for *in vitro* testing has been provided by setting the regulatory base for conducting *in vitro* skin permeation studies. The OECD guideline 428 clearly states that human skin preparations – full thickness skin (FTS), split thickness skin (STS), heat-separated human epidermis (HSE) – can be replaced *in vitro* by reconstructed human epidermis (RHE) models. However, the same guideline requests the proof that data gathered with RHE are equivalent to data collected with human skin. Epiderm (MatTek, USA), Skinethic (Skinethik, F) and Episkin[®] (L'Oreal, F) are so far the most used reconstructed epidermis models for investigative purposes. The areas in which RHE are used are manifold: Phototoxicity, irritancy and corrosivity testing are the most thoroughly investigated areas although permeation data are limited (see [6] for a review). For Epiderm and Skinethic a larger body of literature on permeation studies exists [6] but for Episkin[®] only two publications about permeation can be found [7] and [8]. The lack of permeation studies with Episkin[®] is striking. The reason for this might be that the Episkin[®] model is different from Epiderm and Skinethic in two important points. (i) In contrast to EpiDerm and Skinethic, which are cultivated on inert filter membranes, the Episkin[®] model is cultured on a layer of collagen. The manufacturer specifies further that as a result of the collagen layer no shrinking of the cell layers occurs and therefore the cell layers are tight. (ii) Episkin[®] is only delivered in a diameter of 12 mm or smaller whereas Epiderm and Skinethic are also available in a 24 mm format, which fits static Franz diffusion cell (FTDC) with 15 mm orifice which e.g., have been used in a prevalidation study of the German BMBF testing skin permeation (see [9] and [10] and http://www.foerderkatalog.de; Project Number 0313342). To gather permeation data for the different reconstructed epidermis models with the same diffusion cell an adaptation is necessary. The aim of this study therefore was (i) to develop an adapter which makes it possible to use Episkin[®] in static 15 mm FTDC, and (ii) to identify the best suited preparation of native human skin as reference for reconstructed epidermis equivalents in permeation studies under the same experimental conditions. Full thickness skin, dermatomised skin, heat-separated human epidermis and trypsin isolated stratum corneum (the latter not being mentioned in OECD guideline 428) were compared. As relevant criteria for the selection of the reference material were considered the barrier function *in vitro*, ease of preparation, and robustness of the skin preparation. As skin lipids are widely assumed to play an important role for skin barrier function [11], [12], [13] and [14], lipid analysis of the most suitable human skin preparation and Episkin[®] was also performed.

2. Materials and methods

If not further specified, all substances used were of highest analytical degree available and were used without further purification.

Purified water was prepared by means of a Millipore Milli Q Synthesis system (Heidelberg, Germany).

2.1. Test compounds

Both test compounds were obtained from Sigma–Aldrich, Deisenhofen, Germany. All other chemicals were from Merck, Darmstadt, Germany.

4-Androsten-17β-ol-3-on (testosterone); molecular weight [g/mol]: 288.4; log K_{ow}: 3.48 [15].

Caffeine; molecular weight [g/mol]: 194.2 log K_{ow}: –0.08; pK_a: 1.4 [15].

2.2. Buffer solutions

All buffer substances presented here were of analytical grade (Merck, Darmstadt, Germany).

PBS buffer, pH 7.4: 1 L contains KCl 0.2 g; NaCl 8.0 g; KH_2PO_4 0.2 g; $Na_2HPO_4 \times 2H_2O$ 1.44 g; or Na_2HPO_4 1.1486 g; in purified water.

Buffer, pH 2.6: 1 L contains phosphoric acid 1.16 ml; KH_2PO_4 4.08 g; in purified water.

2.3. HPLC analysis of the test compounds

2.3.1. HPLC system

Pump: Dionex P580 Pump; Autosampler: Dionex ASJ 100 automated sample injector; Detector: UVD 170S detector; Column oven: Dionex STH 585 column oven; Software: chromeleon 6.50 SP2 build 9.68.

A LiChrospher$^®$ 100/RP-18 (5 µm) column/125 × 4 mm (Merck–Hitachi, Darmstadt, Germany) with a Lichrocart 4 × 4 mm guard column, LiChrospher$^®$ 100/RP-18, (5 µm) were used as stationary phase for all substances.

2.3.2. Test conditions

Testosterone: mobile phase: methanol/water 70:30 (v/v); retention time: 4.8 min ± 0.2 min; detection wavelength: 250 nm; flow rate: 1.2 ml/min; injection volume: 50 µl; detection limit 15 ng/ml; quantification limit 50 ng/ml; calibration from 50 to 5000 ng/ml ($r^2 = 0.999$).

Caffeine: mobile phase: buffer, pH 2.6/acetonitrile; 90:10 (v/v); retention time: 5.1 ± 0,2 min; detection wavelength: 262 nm; flow rate: 1.2 ml/min; injection volume: 50 µl; detection limit 15 ng/ml; quantification limit 50 ng/ml; calibration from 50 to 8000 ng/ml ($r^2 = 0.999$).

Methanol and acetonitrile were of HPLC grade (Merck, Darmstadt, Germany).

Drug quantification in samples was done by using the external standard method.

2.4. Lipid separation and quantification

2.4.1. Materials

All chemicals used for lipid separation and quantification were of analytical grade. The following materials and chemicals from Merck/Darmstadt, Germany were used: Diethyl ether, *n*-hexane, glacial acetic acid 100%, methanol (Lichrosolv), chloroform (Lichrosolv), petrolether, isopropanol, HPTLC plates (silicagel 60 non-fluorescent, Merck Nr. 105641) copper sulfate-pentahydrate, phosphoric acid (85%).

The following materials and chemicals from Sigma/Deisenhofen, Germany, were used as standard and reference material: For ceramides: ceramide III, ceramide IV; for triglycerides: triolein; for fatty acids: oleic acid; for cholesterol: cholesterol; for cholesterol esters: cholesteryl oleate.

Fat free cotton was obtained by Soxhlet extraction with chloroform–methanol (2:1; v/v).

2.4.2. Separation and quantification method

The method presented here was based on the method developed by Hauck [16] and [17] including modifications according to Wertz [18]. Lipids are extracted from freeze-dried samples using a mixture of chloroform and methanol (2V + 1V). The extract is filtered, dried under a stream of nitrogen, and the residue dissolved in a volume of the solvent mixture to obtain about 15 µg total lipids per µl.

Depending on the amount of the lipid component indicated by a first overview chromatogram, the amount applied to the HPTLC plates has to be varied from 1 to 5 µl or the sample has to be diluted to be within the range of the corresponding standard solutions.

HPTLC separation was performed on silica gel plates at room temperature using the following solvent systems:

1. For the analysis of ceramides (separation from free fatty acids and sterols): chloroform–methanol–glacial acetic acid (190:9:1, v/v/v). Development 15 cm.

2. For the analysis of cholesterol and fatty acids: n-hexane–ether–glacial acetic acid (80:20:10, v/v/v). Development 15 cm.

3. For the analysis of triglycerides, sterol esters, and n-alkanes: first development (10 cm) with n-hexane–ether (80:20, v/v) and after drying, second development (15 cm) with petroleum ether.

The lipid substances were detected by charring with copper sulfate–phosphoric acid reagent (copper sulfate-pentahydrate 10.0 g; phosphoric acid (85%) 10.0 g, purified water 80.0 g) by heating the plate from 110 to 160 °C (approximately 15 min). After charring plates were scanned using a flatbed scanner (Epson expression 1680 pro) at 150 dpi and quantified with the TNIMAGE program (GNU public license; downloaded at www.icewalkers.com/linux/Software/5250/Tnimage.html) using the method of the external standard. Standard substances were applied in a range from 0.7 to 15 µg (Triolein and oleic acid) or 0.7 to 7 µg (sterols and ceramides).

2.5. Permeation experiments in standard Franz diffusion cells

Permeation experiments were carried out in Permegear static type 6G-01-00-15-12 Franz cells (Perme Gear, Riegelsville, PA); receptor volume: 12.1 ml; donor volume: 0.5 ml; orifice diameter: 15 mm (diffusion area: 1.76 cm^2); acceptor: PBS buffer. At defined time intervals 400 µl samples were drawn and replaced by fresh PBS. For further details, see Wagner [19]. The temperature was 32 ± 1 °C as required by OECD guidelines [4] and [5]. The total experimental time was set to 24 h for the various skin preparations and reduced to 6 h for Episkin$^®$ to guarantee sink conditions due to the higher drug permeation with this model.

2.6. Preparations of human skin

Excised human skin from female Caucasian patients, who had undergone abdominal plastic surgery, was used, approvals from the Ethic Committee of the 'Caritas-Traegergesellschaft Trier e.V.' as well as written consent of every donor and the medical personal in charge according to national regulations being on file.

2.6.1. Preparation of full thickness skin (FTS)

After excision the subcutaneous fatty tissue was removed and the remaining tissue stored at –26 °C. For further details, see Wagner et al. [20].

2.6.2. Preparation of split thickness skin (STS)

Skin sections with a thickness of 500 ± 100 µm were prepared from the thawed (full thickness) skin samples using an Aesculap GA 630 dermatome (Aesculap, Tuttlingen, Germany).

2.6.3. Preparation of heat-separated epidermis (HSE)

Based on a procedure reported by Kligman and Christophers [21] the epidermis was separated by putting thawed and cleaned full thickness skin pieces in water at 60 °C for 90 s. After removing the skin from the water and placing it, stratum corneum side up, on a filter paper, the SC-epidermis layer of the skin was peeled off from the dermis using forceps. The epidermis was put into PBS solution for at least 30 min in order to get a fully hydrated epidermis sheet.

2.6.4. Preparation of trypsin isolated human stratum corneum (TISC)

In order to obtain TISC sheets, skin pieces were punched out of FTS, thawed, and cleaned with isotonic Ringer solution. The skin pieces were transferred, dermis side down, into a Petri dish, which contained a 0.15% trypsin solution in PBS buffer. They were incubated for 24 h at 32 ± 1 °C. This procedure was repeated with fresh trypsin solution until the stratum corneum was fully isolated [22].

2.7. Human skin equivalent Episkin®

The technical data sheet provided by the manufacturer describes the Episkin® model as "type I collagen matrix, representing the dermis, surfaced with a film of type IV collagen, upon which is laid a stratified and differentiated epidermis derived from human keratinocytes".

The Episkin® kit (J13, 1.07 cm²) was shipped from (L'Oreal, Paris, F) for delivery on Tuesday or Wednesday morning and was used within 24 h post-arrival. After integrity check of the kit (red colour of the agar medium and temperature indicator) the Episkin® models were removed from the nutrient agar and transferred into 12-well plates filled with maintenance media provided by the manufacturer. After storage overnight in an incubator (37 °C, 5.0% CO_2) the Episkin® model was punched out in total from the plastic insert and using a punch of 11 mm in diameter and afterwards was transferred onto the standard FTDC equipped with a special adapter (see Section 2.8).

The following batches were used for this investigation: 03-EPIS-036(J13); 04-EPIS-014(J13); 04-EPIS-015(J13); 04-EPIS-018(J13); 04-EPIS-021(J13); 04-EPIS-022(J13); 04-EPIS-031(J13).
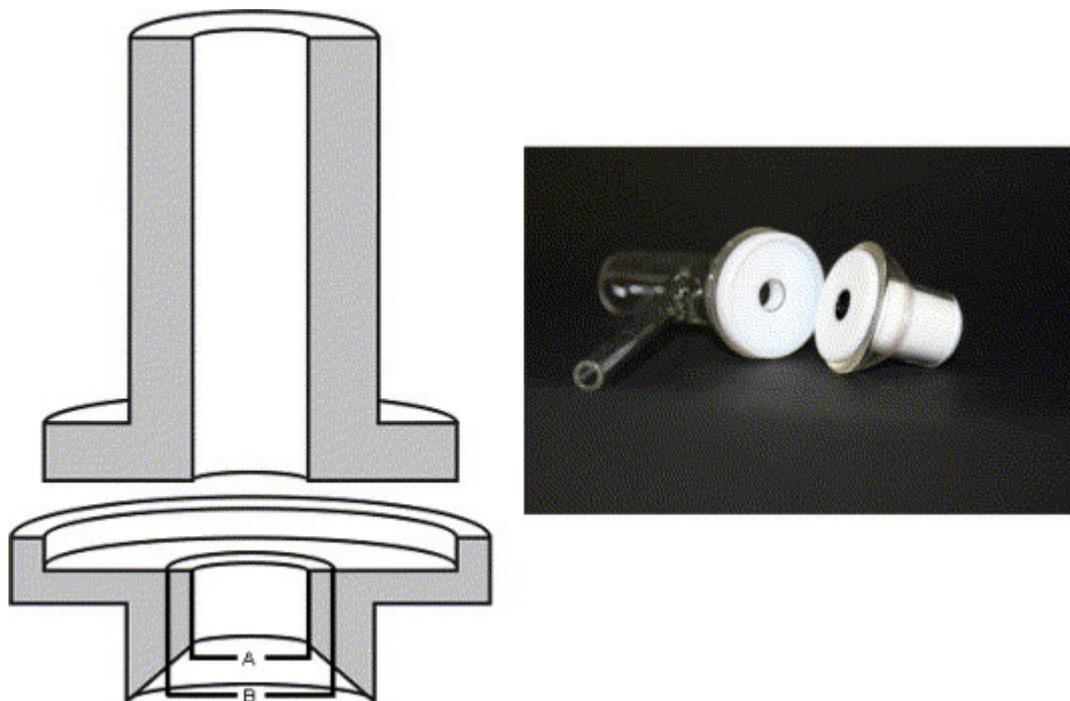
2.7.1. Separation of the collagen layer

The collagen layer was separated from the epidermal layer by removing the safety ring fixing the collagen layer to the plastic insert. Using forceps the collagen layer could now be pulled free from the plastic insert and transferred to the Franz cell.

2.8. Permeation experiments with Episkin® adapter

To fit the Episkin® model in the standard diffusion cell of 15 mm orifice a reduction of the diffusion area was necessary. The insert made of Teflon (Fig. 1) reduced the diffusion area of the Franz cell from 1.76 cm² (15 mm) to 0.385 cm² (7 mm) and the receptor compartment volume from 12.1 to 11.4 ml. The upper part was elongated until it surpassed the upper part of the Franz cell. There it could be tightened with Parafilm and aluminium foil. To have the same amount of donor per surface area as in the standard FTDC the donor was reduced to 110 µl.

Figure 1: Schematic representation of the Episkin® adapter. (A=7 mm, B=10.5 mm).

2.9. Experiments in the plastic filter insert

To obtain hydrostatic equal levels the liquid inside and out the plastic insert the donor volume was adjusted to 150 µl and the receptor volume to 1500 µl, respectively. At predetermined time intervals samples of 200 µl were drawn from the receptor compartment and immediately replenished with preheated receptor medium. During the whole experiment the Episkin® inserts were kept in an incubator at 32 °C without carbon dioxide to have experimental conditions similar to the FTDC experiments. Furthermore, to obtain a proper mixing of the receptor phase and to minimize unstirred water layers the receptor phase was stirred by means of a magnetic bar (4 × 1.5 mm) at 400 rpm.

2.10. Drug preparation and dosage

For the various human skin preparations caffeine was applied in a concentration of 10,000 µg/ml in PBS whereas for the Episkin® model only a concentration of 1000 µg/ml was used. The higher concentration for the various skin preparation was utilized to reduce the lag-time and the lower concentration with the Episkin® to avoid dilution of the samples for the HPLC analysis. Testosterone was used in a concentration of 40 µg/ml in PBS with 2% Igepal for all experiments. Igepal (Sigma–Aldrich, Deisenhofen, Germany) was added as a non-ionic solubilizer to enhance the solubility of testosterone following the recommendation of the OECD guideline [4]. Schreiber et al. [9] have shown that Igepal in this concentration does not influence the barrier function of the skin and Episkin®. With all concentrations applied 'infinite dose' conditions were realized.

2.11. Permeation experiments with methylene blue for evaluation of the adapter

Transport experiments were carried out analogously in Franz type diffusion cells as described above. Donor concentration 1 mg/ml methylene blue (Sigma–Aldrich, Deisenhofen, Germany); Separation membrane: Dialysis membrane (Methocell, London, UK; 10,000 Da molecular weight cutoff); sampling time points every 2 min. All experiments were repeated six times. The temperature was set to 32 ± 1 °C.
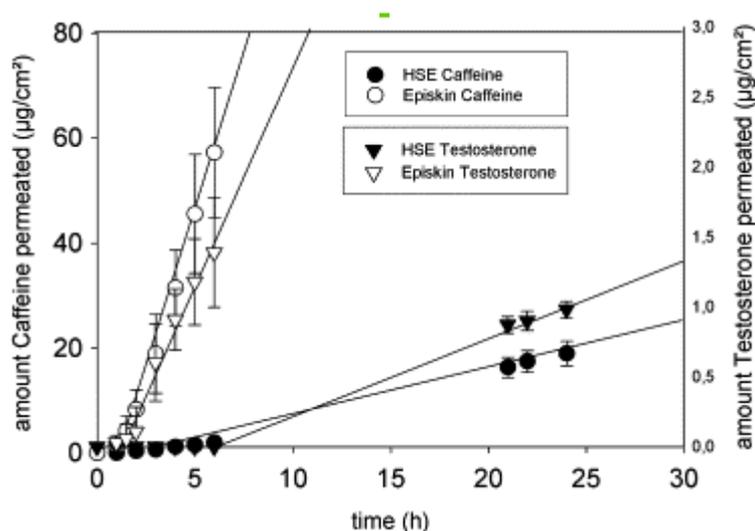
2.12. Data analysis

The apparent permeation coefficient (P_{app}) was extracted from the [µg/cm^2] vs. time curves of the single experiments by fitting a line through the linear range of the curve using a linear regression model. At least 4 data points were used for calculation. Exemplary representative curves for HSE and Episkin$^®$ are shown in Fig. 2. The slope of this linear curve yields the flux J of a substance through the membrane, which together with the donor concentration c_d is used to calculate the P_{app} as follows:

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

These calculations were done using SigmaPlot 9.0 (SPSS Inc., Chicago, IL 60606, USA).

Figure 2: Representative permeation curves for caffeine and testosterone (means ± SD); right scale testosterone; left scale caffeine.

2.12.1. Statistical analysis

Statistical analysis was performed with Sigmastat 3.1. (SPSS Inc., Chicago, IL 60606, USA).

3. Results

3.1. Evaluation of the Episkin$^®$ adapter

To evaluate the Episkin$^®$ adapter, experiments were carried out with a well-defined hydrophilic dialysis membrane to reduce the variability of the diffusion barrier first (see Section 2.11). Two groups were compared: The first group consisted of six FDTCs without the Episkin$^®$ adapter while in the second group six cells with the Episkin$^®$ adapter were used. The resulting P_{app} values from both groups ($P_{app} \times 10^5$ [cm/s] ± SD: with adapter: 6.75 ± 0.52; $n = 6$; without adapter: 6.20 ± 0.33 $n = 6$) showed no statistically significant differences (t-test; $P < 0.05$).

3.2. Barrier properties of the collagen layer

For these experiments the collagen layer was separated from the epidermal model and transport experiments with both test compounds were performed. The test compounds permeated much faster through the collagen layer than through the complete model indicating that the rate-limiting step is the permeation through the stratum corneum and epidermis like layer of the model (Table 1).

Table 1: Results of all permeation experiments

		Caffeine $P_{app} \times 10^7 \pm$ SD [cm/s]	n	Testosterone $P_{app} \times 10^7 \pm$ SD [cm/s]	n
In vitro human skin preparations	Full thickness skin (FTS)	0.14 ± 0.014	4	Not detectable	4
	Split thickness skin (STS)	0.36 ± 0.06	4	3.53 ± 0.51	4
	Trypsin isolated stratum corneum (TISC)	0.34 ± 0.026	4	2.72 ± 0.25	4
	Heat-separated epidermis (HSE)	0.26 ± 0.023	4	3.76 ± 0.26	4
Reconstructed human skin equivalent Episkin®	Experiments in Franz diffusion cells with adapter	28.77 ± 9.93	24	22.25 ± 5.57	22
	Experiments in plastic inserts direct	20.1 ± 4.3	3	16.6 ± 1.4	3
Collagen layer	Experiments in Franz diffusion cells with adapter	228 ± 14	6	121 ± 11	6

3.3. Experiments in the plastic insert of Episkin®

For aqueous solution of caffeine and testosterone, experiments with Episkin® in the plastic insert were performed and compared to the data gathered with Episkin® in the Franz cells with adapter (Table 1). No significant difference was found either for caffeine or for testosterone (t-test; $P > 0.05$).

3.4. Permeation through different human skin preparations

Several types of skin preparation for in vitro investigations are specified in the OECD guideline 428 [4] documents: FTS, STS and HSE. In addition, TISC was also included in this investigation. The experiments conducted in this part were all performed using skin from one donor to ensure maximum comparability and avoiding any interindividual variation. The results are summarized in Table 1. From all in vitro human skin preparations FTS showed the lowest permeation for caffeine, while transport of testosterone was not even detectable. For the other skin preparations TISC, HSE and STS permeation data of caffeine and testosterone were each of the same scale. Moreover in the latter three skin preparations testosterone showed a significantly higher permeation (approximately 10×) in comparison to caffeine (t-test: $P < 0.05$).
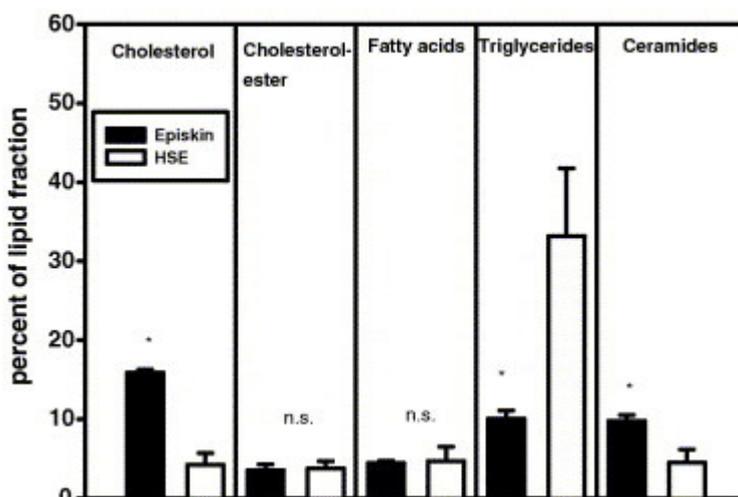
3.5. Comparison of HSE diffusion experiments and Episkin® experiments

The data for HSE (Table 1) show a lower permeation of caffeine compared to testosterone. Notably while HSE is differentiating between both compounds, Episkin® (Table 1) is not differentiating between both compounds (t-test; $P > 0.05$). These differences cannot be attributed to the different applied concentrations of the caffeine donor solutions because the presented P_{app} value for the 10,000 µg/ml donor solution is in accordance with the P_{app} value of $0.6 \pm 0.4 \times 10^{-7}$ (cm/s) for 1000 µg/ml donor solution reported in the German prevalidation study [10]. Moreover, the mean P_{app} values for caffeine and testosterone as observed in the Episkin® model are approximately 100 or 10 times higher than for HSE.

3.6. Comparison of the lipid contents of the Episkin® model and HSE

The lipid profile of the Episkin® model and heat-separated epidermis are shown in Fig. 3. Pairwise comparison of lipid classes showed that there are significant differences in cholesterol (t-test; $P < 0.05$), ceramide (t-test; $P < 0.05$) and triglyceride (t-test; $P < 0.05$) amounts. On an average, the Episkin® model contained approximately two times more cholesterol, one-third of triglycerides and two times more ceramides than human skin. No differences were found for cholesterol ester (t-test; n.s) and fatty acids (t-test; n.s.).

Figure 3: Results of skin lipid analysis as percent of lipid fraction. * Significant different ($P < 0.05$), n.s. not significant different ($P > 0.05$); HSE $n = 66$; Episkin® $n = 15$.

4. Discussion

One of the major drivers for the development of reconstructed human skin models are to replace human and animal skin in *in vitro* testing. The OECD guideline 428 [4] clearly allows their use for these purposes. Before accepting them as a valid replacement, however, it has to be shown that the permeation data gathered with reconstructed human skin models is equivalent to the data generated with human skin as requested by the OECD guideline. Depending on the preferences of the manufacturer RHEs come in different sizes whereas the Episkin® model being an example for a relative small size epidermal model ($d = 12$ mm). Since a 15 mm Franz diffusion cell has been introduced as standard in a national multicentre prevalidation study of skin penetration in Germany (see [9] and [10] and http://www.foerderkatalog.de; Project Numbers 0313342 and 0313339) it was a goal of this study to find a possibility of using these FTDC for a smaller RHE such as Episkin® also. As shown by the permeation data with the methylene blue dye the use of the adapter does not influence the permeation data and therefore the use of the same diffusion cell for different sized separation membranes is possible. These results were in accordance with the theory of Fick [23] and data by Chilcott [24] showing that differences of the size of the diffusion area do not have a significant influence on the permeation data as long as a perfect sink is maintained in the acceptor chamber.

Furthermore, the experiments performed with the Episkin® model directly in the plastic insert showed similar results as in the Franz cell and therefore this setup might be a reasonable alternative especially for high throughput experiments. However, it must be pointed out that these experiments had to be carried out with stirring of the receptor phase to minimize unstirred water layers, which may be critical concerning rate-limiting of permeation if using low water soluble drugs. Keeping this in mind additional advantage is given by the fact that no damage of the cell layers can occur because no removal of the model from the plastic insert is needed to transfer it onto the FTDC. Another important point in this investigation was the collagen support layer of the Episkin® model on which keratinocytes are growing to build the epidermis like structure. Since the collagen layer is thicker than the epidermal layer the question arises if this collagen layer represents a barrier for the test compounds. The barrier function of the model should be located in the stratum corneum like epidermis layer as in the case in its human counterpart. The rate-limiting step of the diffusion should thus not be the crossing of the collagen layer. As a consequence, the transport over the collagen layer should be significantly faster than over the whole model. As shown in Table 1 for both test substances the diffusion over the collagen layer was much faster than through the whole model indicating that the barrier function in the Episkin® model is located rather in the cell layers than in the collagen support. In contrast to the experiments with the Episkin® model by using the collagen support layer alone caffeine permeation is much faster than testosterone permeation. The reason may be that collagen has a high testosterone binding capacity, which is likely due to the high protein binding of testosterone in comparison to caffeine. Therefore, caution should be taken when using a reconstructed epidermis model with collagen support membrane together with drug substances having a high protein binding capacity. The next point of interest was which type of skin preparation should be used for comparison. FTS did not appear useful because no transport of testosterone into the receptor fluid could be detected during 24 h. Although HSE, TISC and STS performed equally well regarding the permeation of the test substances, HSE was finally selected due to the following reasons. The main advantages of HSE are the well-defined morphological structure, the speed and ease of preparation, and the economical use of human skin. Furthermore, the data set generated with HSE (Table 1) showed P_{app} values consistent with data published by other authors [15]. As shown by Table 1, one clearly notices that Episkin® is more permeable than human heat-separated epidermis. However, the reduced barrier function is not solely a problem of the Episkin® model. Many epidermal models currently on the market are afflicted with the higher permeability [6] and [10], however, the effects are different. Par example, this is shown by the German prevalidation study [10] where different rankings of the permeability for various commercially available reconstructed epidermis models related to caffeine and testosterone are reported: Caffeine: SkinEthic, Episkin® > EpiDerm. Testosterone: SkinEthik > EpiDerm, Episkin®. Several possible reasons for this have been suggested, including impaired desquamation [25] and the presence of unkeratinized microscopic foci [26]. In any case, the elevated permeability currently limits the value of reconstructed epidermis models for transport studies and may lead to some false positives results in toxicity studies. Furthermore, in contrast to Episkin®, HSE shows a clear distinction between both test compounds with respect to permeation. The reason for this may be attributed to reduced barrier of the Episkin® model making differences in the permeation behavior of the two test compounds invisible. Moreover, the binding of testosterone to the collagen layer of the Episkin® model may diminish the differences in permeation of caffeine and testosterone by acting as an additional receptor compartment. This will result in a reduced drug amount permeated through the model. Lipids play an important role in the barrier function of skin. In Episkin® the same major classes of skin lipids were found in comparison to HSE. The high amounts of triglycerides found in human skin were not surprising since skin originated from plastic surgery is always contaminated with triglycerides as shown by Wertz et al. [27]. Thus, the lower amounts of triglycerides in the Episkin® model were not considered as a problem. The differences in the amounts of the different lipid classes between Episkin® and HSE in our investigation are corroborating the findings of Ponec et al. and Boelsma et al. [28], [29] and [30]. The assumption that the reduced barrier function of Episkin® is somehow linked to the lack of lipids is tempting. Also in our studies there was no obvious correlation between the skin lipid pattern and permeability data obtained for RHE vs HSE. If and how the lack of certain kinds of lipids does affect the barrier function is difficult to answer. Correlation of the amount of certain lipid classes with the transport of drugs has been attempted several times [13], [31], [32] and [33], however, no clear results exist.

5. Conclusion

A standardised experimental setup for small diameter reconstructed human epidermis (RHE), e.g., Episkin® fullfilling the requirements of the OECD guideline 428 has been developed allowing to perform permeation studies in 15 mm static Franz type diffusion cells. Heat-separated human epidermis (HSE) seems to be the best suited human skin preparation for these kinds of experiments. However, regardless if STS, HSE or TISC is used the barrier function of the Episkin® model is still not comparable to human skin preparations. Permeability values for caffeine and testosterone are at least 10 times higher in comparison to HSE. In addition, permeability differences of the two test compounds could only be demonstrated with human skin preparations, but not with Episkin®. We conclude that RHEs like Episkin® represent a remarkable step towards the development of alternative methods, but at present it cannot yet be fully considered as replacement for human skin in *in vitro* permeation studies. Further experiments with a broader panel of substances with different physico-chemical characteristics have to show the potential of RHEs for regulatory purposes according to [1], [2], [4] and [5].

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Suitability of bovine udder skin and pig skin as a replacement model for human skin in percutaneous permeation experiments (Summary)

The aim of this publication was to evaluate the barrier of bovine udder skin in comparison to pig and human skin. Due to upcoming EU regulation an alternative to pig and human skin has to be found. Bovine udder skin might a possible solution to this problem. In order to assess the effectiveness of bovine udder skin the barrier strength of bovine udder was compared against four different substances (benzoic acid, testosterone, caffeine and flufenamic acid). Further, its histology and lipid profile was compared to those of pig skin and heat-separated human epidermis. In the permeation experiments pig and human skin performed equally well, while bovine udder skin seemed to exhibit a weaker, but less variable barrier against caffeine, benzoic acid, testosterone and flufenamic acid. The histology of all examined skins showed high similarities. The lipid profile of the skin of all three species contained the major lipid classes cholesterol, ceramides, cholesterol ester, fatty acids and triglycerides. There were no major morphological differences between the species. The conclusion of this work is that bovine udder skin is a possible alternative for human skin in skin permeation studies.

My contribution to this work:

- All experiments with human skin
- Statistical evaluation of the experimental data (permeation experiments and lipid contents)

The results presented in the following chapter have been published as:

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Comparison of Bovine Udder Skin with Human and Porcine Skin in Percutaneous Permeation Experiments

1. Introduction

Detailed and reliable information on percutaneous absorption is crucial both for modern pharmaceutical and cosmetic product development and for toxicological risk assessment. The use of skin from the target species is regarded as the most adequate approach to gathering such information, due to known species differences in the epidermal barrier. However, since human skin is not readily available, alternative models are regularly used. As newer approaches, such as the use of reconstructed human epidermis models, still show a much less developed penetration barrier than human skin (1, 2), skin from various animal species is widely accepted as suitable replacements and its use is recommended by the OECD (3, 4). The predictability of excised animal skin for human skin has been subject to numerous investigations (5–9), and most attention has been paid to skin from rats and pigs. Due to significant follicular transport, skin from rats and other laboratory animals exhibits a much weaker barrier than human skin (10). Pig skin has been shown to closely resemble human skin in many aspects (see [11] for review), it seems to be preferred by many investigators, and is a well-established model in skin permeation experiments (5, 11–13). Unfortunately, porcine skin from the regular slaughtering process is unsuitable for skin permeation experiments, as it is scalded and scraped in order to remove hair and scurf from the carcass. Therefore, special arrangements have to be made in order to obtain untreated material, which is difficult in many big slaughterhouses. For these practical reasons, bovine udder skin can be a cheap and readily available alternative, as it is an untreated waste material, which can be gathered directly from the industrialised butchering process. Its use does not require animals to be sacrificed solely for experimental purposes. While bovine udder skin was found to be histologically inconspicuous and to exhibit the normal constitution of mammal skin (14), the bovine surface lipid profile has been described as unusual and mainly composed of wax esters (15). Pitman and Rostas (16) compared the barrier strength of human and bovine skin by using levamisole as a model drug. They reported that dorsal cattle skin was 400 times more permeable to levamisole than human skin. On the other hand, bovine udder skin was found to be valuable for modelling the *in vivo* percutaneous permeation with an isolated perfusio procedure (17–20).

In order to establish the usefulness of bovine udder skin as a model for the prediction of human percutaneous absorption, we used a standardized, *in vitro* infinite dose procedure (see 7, 21 and http://www.foerderkatalog.de; BMBF project numbers 0312881-4 and 0313338-43) to examine udder split skin in comparison with two well-accepted standard models: human heat-separated epidermis (HSE) and porcine split skin. We determined the permeation coefficients and the within-batch and between-batch variations of four test compounds (benzoic acid, caffeine, flufenamic acid, and testosterone), which covered a wide range of lipophilicities (logk = –0.08–4.88), for each model. As the skin's morphology may considerably influence the barrier characteristics of skin from different species, we conducted histological examinations of bovine udder skin with particular reference to the epidermal thickness and number and penetration depth of hair follicles, as these may act as shunt routes for transepidermal transport. In order to relate differences in the epidermal lipid profiles to interspecies variations in skin barrier strength, we also quantified the main lipids in skin samples from humans, pigs and cows. Particular attention was paid to lipid classes which are thought to be crucial for the skin barrier function (22–30), and which to our best knowledge have not been examined in cattle in the case of cholesterol, cholesterol esters and ceramides.

2. Materials and Methods

2.1 Test compounds

The following test compounds were obtained from Sigma-Aldrich, Deisenhofen, Germany:

— Caffeine: molecular weight = 194.2g/mol; $logK_{ow}$ = –0.08; pKa = 1.4 (31).
— Benzoic acid: molecular weight = 122.1g/mol; $logK_{ow}$ = 1.9; pKa = 4.2 (31).
— 4-Androsten-17ß-ol-3-on: molecular weight = 288.4g/mol; $logK_{ow}$ = 3.48 (31).
— Flufenamic acid: molecular weight = 281g/mol; $logK_{ow}$ = 4.88; pKa = 3.9 (32).

2.2 Buffers and other solutions

All buffers were of analytical grade (Merck, Darmstadt, Germany).

— Sorensen phosphate buffer solution (pH 7.4): 1l contains 2.0g K_2HPO_4, 9.2g Na_2HPO_4 x $2H_2O$ and deionised water (Millipore Milli Q Synthesis, Heidelberg, Germany).
— McIlvaine buffer (pH 2.2): 1l contains 20.8g citric acid, 0.4g Na_2HPO_4 x $2H_2O$ and deionised water.
— Phosphate-buffered saline (PBS) buffer (pH 7.4): 1l contains 0.2g KCl, 8.0g NaCl, 0.2g KH_2PO_4, 1.44g Na_2HPO_4 x $2H_2O$ or 1.1486g Na_2HPO_4, and deionised water.
— Phosphate Buffer (pH 2.6): 1l contains 1.15ml phosphoric acid, 4.08g KH_2PO_4 and deionised water.

Acetonitrile (HPLC grade) was also obtained from Merck.

2.3 High performance liquid chromatography (HPLC) system

Pump = P580 pump; Autosampler = ASJ 100 automated sample injector; detector = UVD 170S detector; column oven = STH 585 column oven; software = chromeleon 6.50 SP2 build 9.68. All from Dionex Corporation, Sunnyvale, CA, USA.

2.4 HPLC analysis of the test compounds

A 125 x 4mm LiChrospher® 100/RP-18 column (Merck-Hitachi, Darmstadt, Germany) with a 4 x 4mm LiChrospher® 100/RP-18 guard column (5µm; Merck-Hitachi) was used as the stationary phase for all the test substances.

— Caffeine. Mobile phase: buffer pH 2.6/acetonitrile, 90:10 (v/v); retention time = 5.1 +/– 0.2 minutes; detection wavelength = 262nm; flow rate = 1.2ml/minute; calibration from 25 to 10,000ng/ml (r^2 = 0.999); injection volume = 50µl.

— Benzoic acid. Mobile phase: acetonitrile/buffer pH 2.6, 72:28 (v/v); retention time = 3.8 +/– 0.2 minutes; detection wavelength = 228nm; flow rate = 1.2ml/minute; calibration from 50 to 4000ng/ml (r^2 = 0.999); injection volume = 50µl.

— Testosterone. Mobile phase: methanol/water, 70:30 (v/v); retention time = 4.8 +/– 0.2 minutes; detection wavelength = 250nm; flow rate = 1.2ml/minute; calibration from 50 to 5000ng/ml (r^2 = 0.999); injection volume = 50µl.

— Flufenamic acid. Mobile phase: methanol/McIlvaine buffer pH 2.2, 80:20 (v/v); retention time = 3.1 +/– 0.2 minutes; detection wavelength = 284nm; flow rate = 1.2ml/minute; calibration from 25 to 5000ng/ml (r^2 = 0.999); injection volume = 50µl.

Test substances in samples were quantified by the external standard method.

2.5 Materials used for lipid separation and quantification

All the chemicals used for lipid separation and quantification were of analytical grade. The following materials and chemicals were obtained from Merck: Diethylether, n-hexane, glacial acetic acid (100%), methanol (Lichrosolv), chloroform (Lichrosolv), petrolether, isopropanol, HPTLC plates (silicate 60, nonfluorescent, Merck Nr. 105641), copper sulphate pentahydrate, phosphoric acid (85%).

The following materials and chemicals were from Sigma: ceramide III, ceramide IV, triolein, oleic acid, cholesterol, cholesterol ester.

Other: fat-free cotton (Hartmann, Heidenheim, Germany; fatty compounds removed through Soxhlet extraction with chloroform-methanol [2:1; v/v]).

2.6 HPTLC of skin lipids

Skin samples from three individuals of each species were freeze-dried to yield a dry weight of at least 20mg, which were then extracted with 5ml chloroform-methanol (2:1, v:v) for 12 hours. The extract was filtered and evaporated under a gentle stream of nitrogen. The residue was weighed and redissolved in chloroform-methanol (2:1, v:v) for application to silica gel plates. The HPTLC method presented here is based on that of Hauck (33) and Loth *et al.* (34), and was improved by taking into account that of Wertz *et al.* (35) and suggestions made by Phil Wertz, University of Iowa.

HPTLC separation was performed at room temperature by using the following solvent systems.

1. For the analysis of ceramides, free fatty acids, and sterols: chloroform-methanol-glacial acetic acid (190:9:1, v/v/v).

2. For the analysis of sterols, fatty acids, and other acidic substances: n-hexane–ether–glacial acetic acid (80:20:10, v/v/v).

3. For the analysis of triglycerides, sterol esters, and n-alkanes: first development with n-hexane-ether (80:20, v/v). Drying and second development with petroleum ether.

4. For the analysis of triglycerides and sterol esters: first development with n-hexane-ether (40:16, v/v), drying, and second development with petroleum ether.

The lipid substances were detected by charring with copper sulphate-phosphoric acid reagent (copper sulphate pentahydrate 10.0g, phosphoric acid [85%] 10.0g, dematerialised water 80.0g) by heating the HPTLC plate from 110 to 160°C.

After charring, the plates were scanned by using a flatbed scanner (Epson expression 1680 pro) at 600dpi, and quantified with the TNIMAGE program (GNU public license downloaded from:
www.icewalkers.com/linux/Software/5250/Tnimage.html), by using the external standard method. The lower limit of quantification with this method is 10ng.

2.7 Preparation of human skin

Human HSE (36) closely mimics percutaneous absorption *in vivo* (37-39). The human skin used here was excised from female Caucasian patients who had undergone abdominal plastic surgery. This procedure was approved by the ethics committee of Caritas-Traegergesellschaft Trier e.V. The skin was taken from three patients in good health and with no medical history of any dermatological disease. After excision, the subcutaneous fatty tissue was removed. The epidermis was separated by putting thawed and cleaned skin pieces in water (60°C) for 1 minute, then removing the skin from the water and placing it,

dermal side down, on filter paper, where the stratum corneum and epidermal layer of the skin were peeled away from the dermis with forceps and subsequently examined in diffusion cells. For further details, see Wagner *et al.* (40).

2.8 Preparation of bovine and porcine skin

As the presence of a dense pelage makes the heat separation technique liable to produce leaks, it is not usual to use HSE from animal skin. For this reason, dermatomised animal skin was used, as recommended by the OECD (3).

Udder skin samples were obtained from healthy lactating Holstein Frisian cows that had been slaughtered according to legal requirements. Only lightly pigmented organs were chosen. Skin flaps of about 10 x 20cm were taken from the lateral udder wall, 5cm dorsal of the hairless teat. Porcine skin samples were taken from animals that were sacrificed for other purposes at the Hannover Medical School. Samples were taken from the lateral abdominal region.

Each flap was cleaned with water and stored at 20°C until required for use. 1000µm-thick dermatomised split skin samples were then prepared, whose thickness was controlled by using a pocket thickness gauge (Model 7309, Mitutuyu Corp, Tokyo, Japan). Samples were not included, if they contained deviations of more than 100µm.

2.9 Morphological examinations

Cross sections of bovine udder skin from three individuals were prepared for histological examination from tissue samples embedded in TissueTec® (Miles Inc, Elkhart, IN, USA) and stained with haematoxylin and eosin. For each skin sample, the epidermal thickness and the penetration depth of at least 24 follicles were measured by using a microscope. In order to facilitate a general comparison of the skin morphology of the three species examined here, cross sections of human and porcine skin were also prepared by using the same method. The hairs were clipped from the porcine and bovine udder skin, and the number of follicles per cm² from three individuals was counted by using a grid and a magnifying glass.

2.10 Test substance preparation and dosage

All the test substances were dissolved in phosphate buffer and applied to maintain unchanged concentration differences between donor and acceptor chamber throughout the whole experimental period (infinite dose conditions). The following concentrations were used: caffeine and benzoic acid – 1000µg/ml; flufenamic acid – 750µg/ml; and testosterone – 40µg/ml. The solubility of testosterone in the buffer was enhanced by the addition of 2% of the non-ionic solubiliser, Igepal® (Sigma-Aldrich). The suitability of all the media had previously been tested, to confirm the solubility of test substances in their specific media (for details, see 21).

2.11 Permeation experiments

Permeation experiments were conducted by using skin samples from at least three individuals, with at least three repetitions per individual, in Permegear static type 6G-01-00-15-12 Franz cells (Perme Gear, Riegelsville, PA, USA): receptor volume = 12.1ml; donor volume = 0.5ml; diffusion area = 1.767cm²; acceptor = PBS buffer. 0.4ml samples were taken at defined intervals and replaced with fresh PBS.

2.12 *Data analysis*

The apparent permeation coefficient (P_{app}) was calculated from the amount ($\mu g/cm^2$) of permeated substance over time in the individual experiments by linear regression over the linear range of the curve. The slope of this linear curve is equivalent to the substance flux J ($\mu g/cm^2 s$) through the membrane, which, together with the donor concentration c_d, is used to calculate the P_{app}, as follows:

$$P_{app} = J/c_d$$

2.13 Statistics

In order to compare the variabilities of repeated measurements on preparations from different individuals, the within-batch (intra-individual, s_w) and between-batch (inter-individual, s_b) precisions were calculated, as follows:

$$[1] \quad s_w = \sqrt{\frac{\sum(f_{wi} \cdot s_{wi}^{\ 2})}{\sum f_{wi}}}$$

$$[2] \quad s_b = \sqrt{\frac{\sum(\overline{X}_{wi} - \overline{X}_t)^2}{f_b}}$$

where: s_w = within-batch precision; s_b = between-batch precision; $s_{wi}^{\ 2}$ = the variance of repeated measurements at day i; f_{wi} = degrees of freedom of repeated measurements at individual i; f_b = degrees of freedom of individuals; $\overline{X}_{wi}$ = the mean of repeated measurements at individual i; and $\overline{X}_t$ = the mean of all measurements.
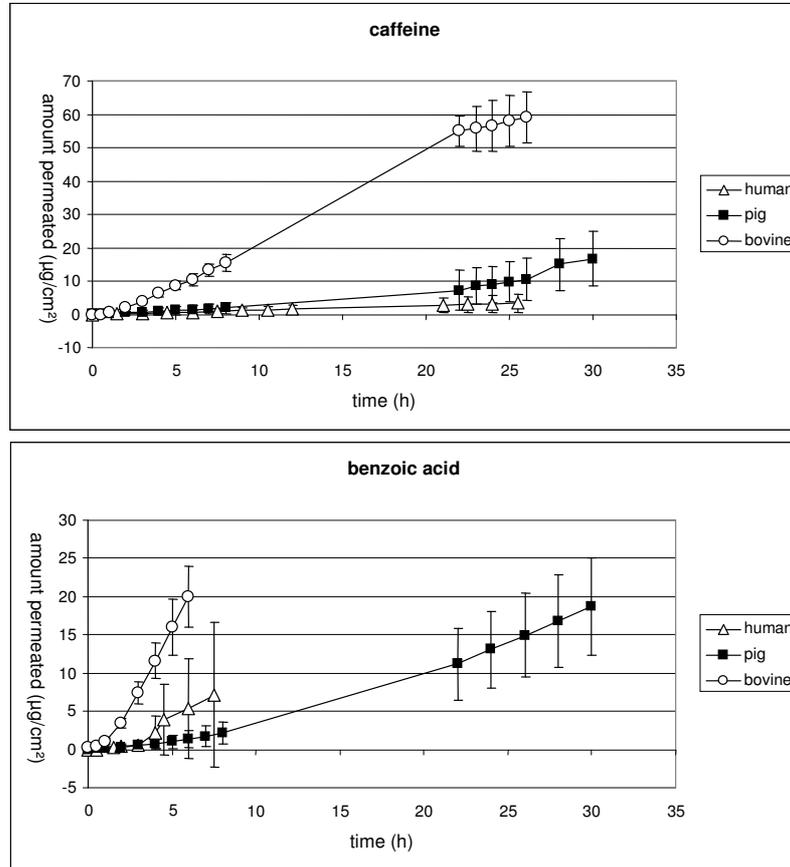
3. Results

Mean amount permeated versus time curves and permeation coefficients from the three different species are shown in Figure 1. The individual and mean P_{app} values are given in Table 1, along with their calculated within-batch and between-batch precisions. Except for caffeine, where porcine skin was almost four times more permeable, human and porcine skin were found to have comparable epidermal resistance, taking into account the high variations in their permeability coefficients (see Figure 2). Compared to human skin, bovine udder skin was found to be about two to three times more permeable, except in the case of caffeine, where it was almost 20 times more permeable. Compared to pig skin, bovine udder skin showed a two-fold to five-fold weaker resistance for all four test compounds. The highest within-batch and between-batch variations were observed with HSE, followed by pig skin. Bovine udder skin exhibited only relatively minor within-batch and between-batch variations for all four test compounds.

As shown in Table 2 and Figure 4, the highest amounts of overall lipids, triglycerides, fatty acids, ceramides, and cholesterol ester, were found in human skin. Considerably lower amounts were detected in skin from the two animal species, but all the major classes of epidermal lipids were present in all three species. The concentrations of triglycerides, fatty acids, and ceramides were highest in human skin, followed by pig and then bovine skin (human > pig > bovine). The relative concentration of cholesterol ester was highest in human skin, followed by bovine and then pig skin (human > bovine > pig). The amounts of cholesterol were comparable in all three species.

The histological examination of bovine udder skin revealed the typical mammalian skin morphology (see Figure 5 for representative sections of skin from the different species). Table 3 summarises our own results and other published results on the morphological parameters of skin from the different species. In our own examinations, the epidermal thickness of bovine

udder skin was in the range of 54 to 92µm, and there were between 207 and 338 follicles per cm², as compared to 30 to 36 follicles per cm² in porcine skin. The penetration depth of the bovine hair follicles was 1476 ± 152µm (mean ± SE).

Figure 1: Permeation versus time curves for the four test substances with human, pig and bovine skin (Values are mean ± SD)
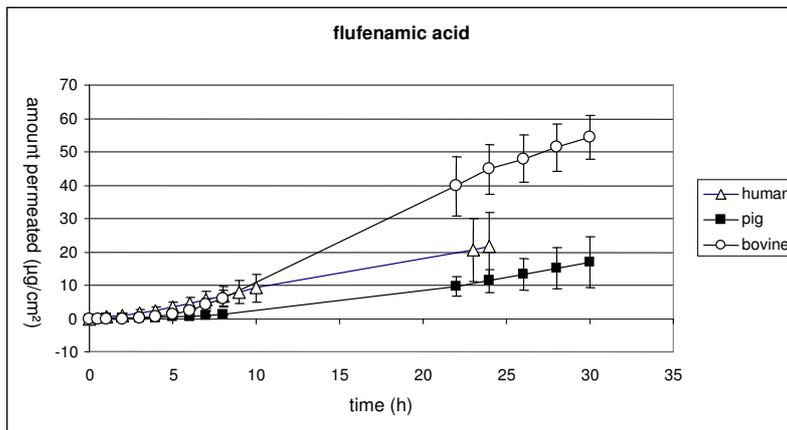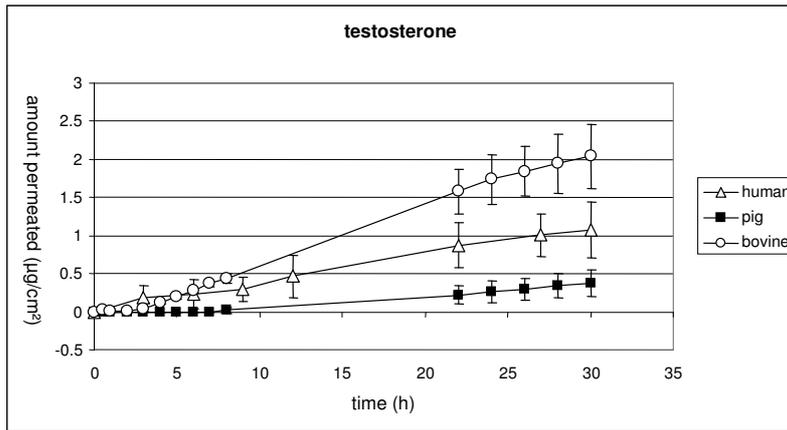
testosterone

flufenamic acid

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Figure 2: P$_{app}$ values of skin from all the individual sources (Values are cm/s)

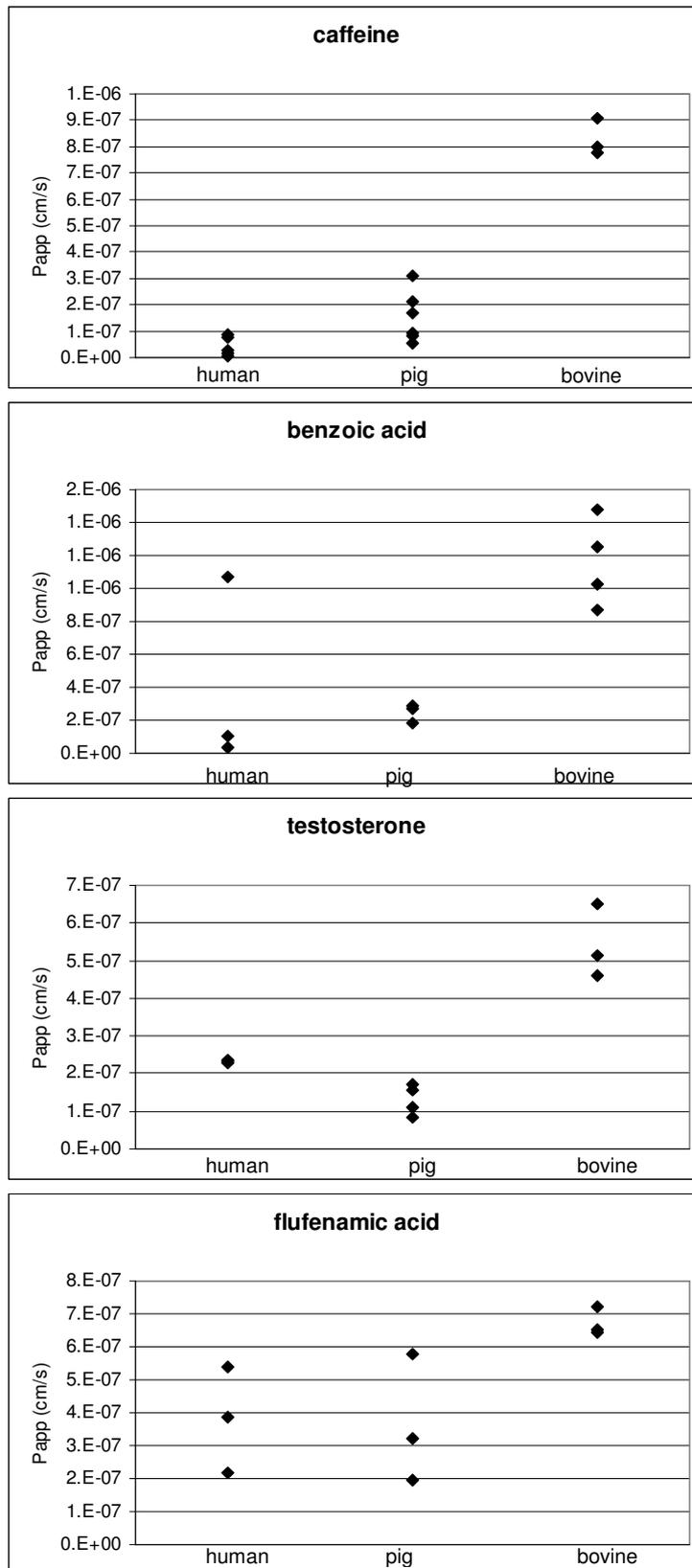caffeine

benzoic acid
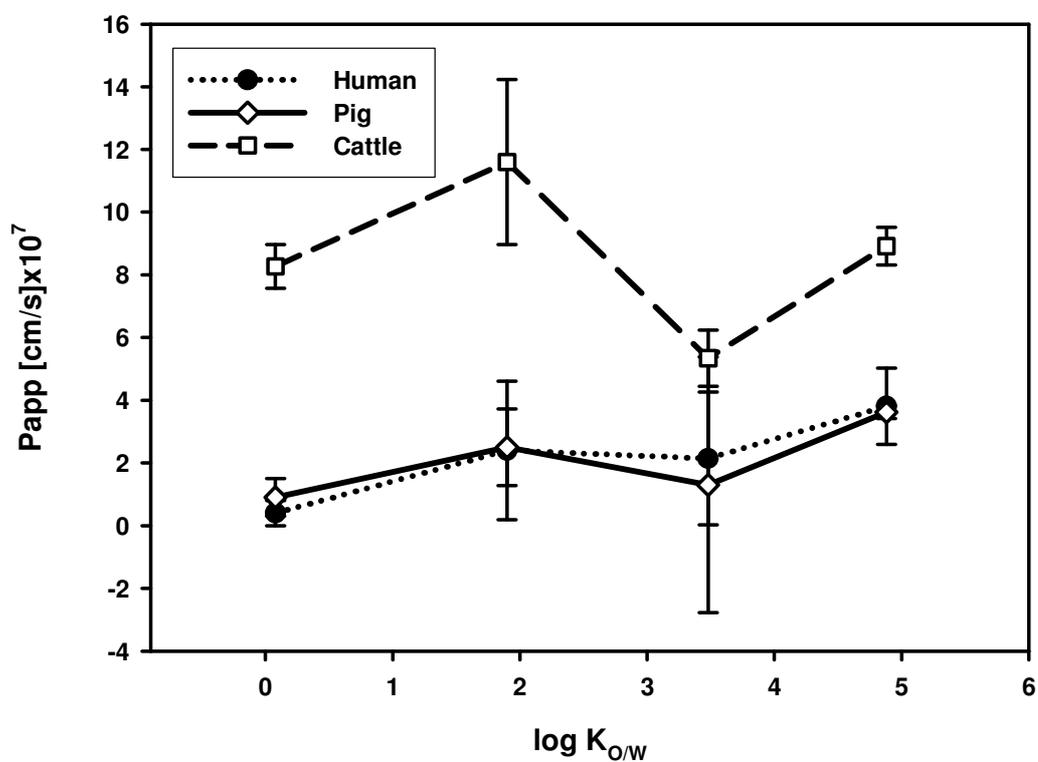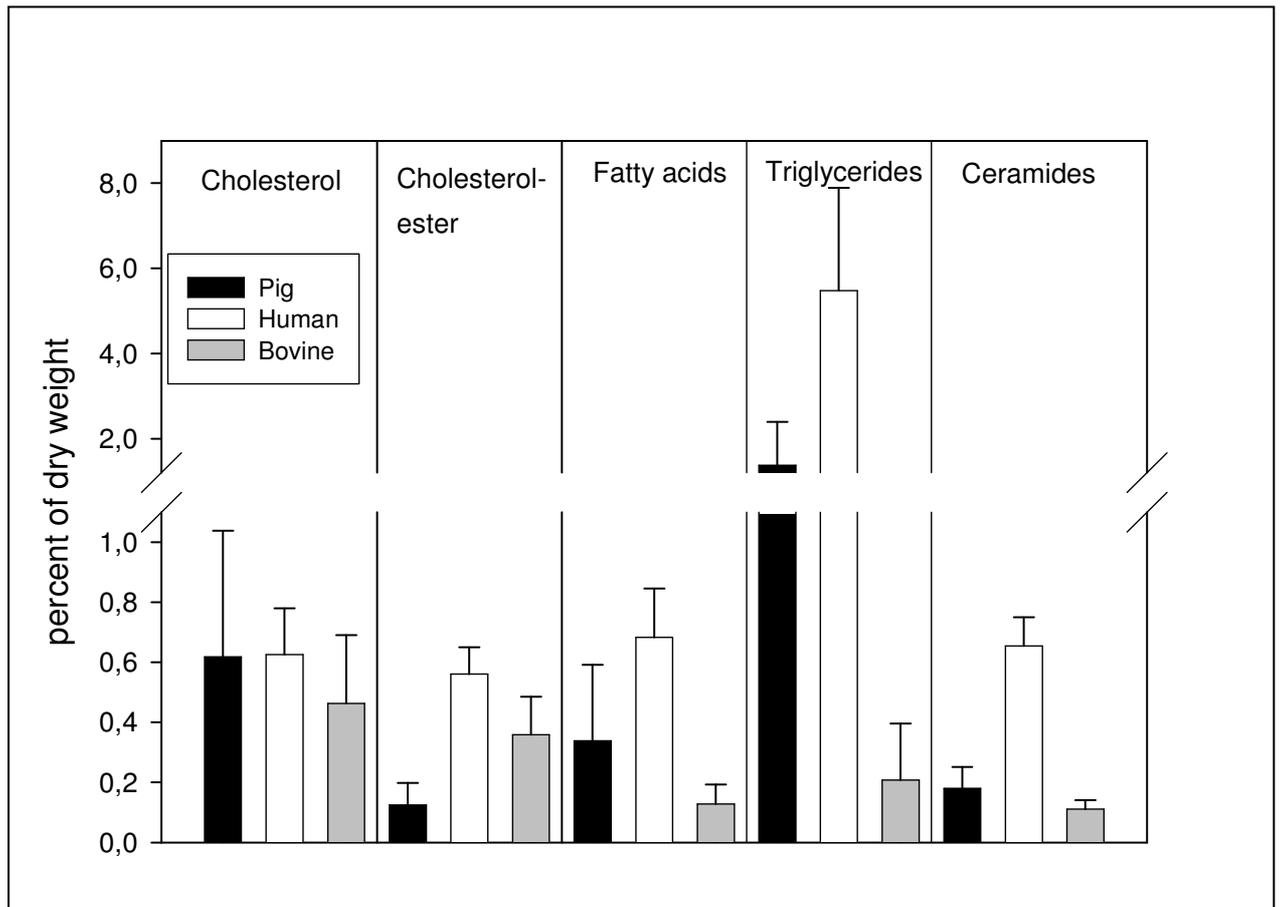

testosterone

flufenamic acid

Figure 3: P_{app} values plotted against log $K_{O/W}$ for the three species investigated
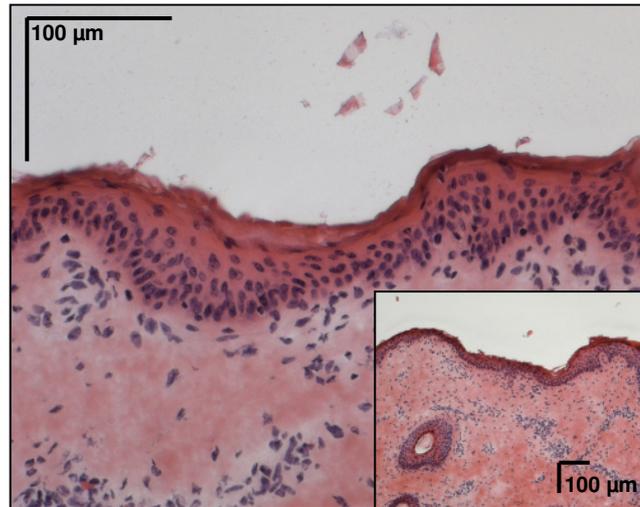

The following $K_{O/W}$ values were used for the four test compounds: caffeine = 0.08; benzoic acid = 1.9; testosterone = 3.48; flufenamic acid = 4.88 (all data show mean ± standard deviation).

Figure 4: Skin lipid profiles of pig, human and bovine udder skin

Figure 5: Histology of bovine udder skin, pig skin and human skin
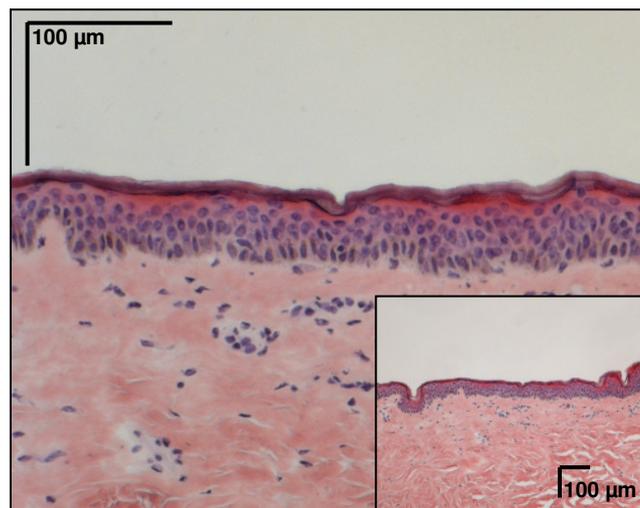
a)

b)

c)

a = bovine udder skin; b = pig skin; c = human skin.
The three skin layers, stratum corneum, epidermis and dermis, are clearly visible in the large images (magnification 20 x). The small images (magnification 10 x) show an overview.

Table 1: Permeability coefficients of the four different test compounds in human HSE, porcine skin, and bovine udder skin Permeability coefficients measured in cm/s e–7 W.b. precision = within-batch precision; B.b. precision = between-batch precision

		Caffeine	Benzoic acid	Testosterone	Flufenamic acid
Human	Individual 1	0.85	1.01	2.29	3.86
	Individual 2	0.15	10.70	2.34	5.37
	Individual 3	0.29	0.14	2.30	2.19
	Individual 4	0.06	–	–	–
	Individual 5	0.76	–	–	–
	Individual 6	–	–	–	–
	Mean	*0.42*	*4.03*	*2.31*	*3.81*
	B.b. precision	*0.36 (86%)*	*5.79 (152%)*	*0.03 (1%)*	*1.59 (42%)*
	W.b. precision	*0.36 (85%)*	*2.38 (63%)*	*0.56 (24%)*	*1.43 (38%)*
Pig	Individual 1	1.69	1.82	0.84	1.97
	Individual 2	0.55	2.88	1.09	3.23
	Individual 3	0.84	2.69	1.54	5.80
	Individual 4	3.10	–	1.70	–
	Individual 5	2.14	–	–	–
	Individual 6	0.91	–	–	–
	Mean	*1.54*	*2.46*	*1.29*	*3.67*
	B.b. precision	*0.97 (65%)*	*0.56 (22%)*	*0.40 (30%)*	*1.96 (54%)*
	W.b. precision	*0.67 (45%)*	*1.37 (55%)*	*0.36 (26%)*	*0.69 (19%)*
Bovine	Individual 1	7.75	8.71	6.52	6.44
	Individual 2	8.00	14.80	4.61	7.21
	Individual 3	9.05	10.22	5.14	6.51
	Individual 4	–	12.54	–	–
	Individual 5	–	–	–	–
	Individual 6	–	–	–	–
	Mean	*8.27*	*11.57*	*5.42*	*6.72*
	B.b. precision	*0.69 (8%)*	*2.67 (23%)*	*0.99 (19%)*	*0.43 (16%)*
	W.b. precision	*2.72 (33%)*	*2.83 (24%)*	*1.23 (23%)*	*1.35 (20%)*

Table 2: Results of the lipid analysis of the skin from the three species (Percent of dry weight)

Human	Total lipids	15.90 ± 4.00
	Cholesterol	0.63 ± 0.15
	Cholesterol ester	0.56 ± 0.09
	Fatty acids	0.68 ± 0.16
	Triglycerides	5.48 ± 2.42
	Ceramides	0.65 ± 0.10
	Sum of quantified lipids	***8.00 ± 1.20***
Pig	Total lipids	6.35 ± 2.50
	Cholesterol	0.62 ± 0.42
	Cholesterol ester	0.12 ± 0.07
	Fatty acids	0.34 ± 0.25
	Triglycerides	1.37 ± 1.02
	Ceramides	0.18 ± 0.07
	Sum of quantified lipids	***2.63 ± 0.80***
Bovine	Total lipids	6.50 ± 2.20
	Cholesterol	0.46 ± 0.23
	Cholesterol ester	0.36 ± 0.13
	Fatty acids	0.13 ± 0.06
	Triglycerides	0.21 ± 0.19
	Ceramides	0.11 ± 0.03
	Sum of quantified lipids	***1.27 ± 0.70***

n = 3 for each species; the values given are the mean ± standard deviation

Table 3: Morphological parameters of skin from different species

	Human	Pig
Bovine udder		
Number of hair follicles (/cm^2) 207–338	6 (48)	30–36
Hair follicle penetration 1.5	1.8–2.5 ([a])	1.3–3.5 (47)
Epidermal thickness (µm) 54–92	57–82 (46)	40 (47)

[a]*J. Lademann, Department of Dermatology and Allergy, Center of Experimental and Applied Cutaneous Physiology, Charité, Berlin, Germany, personal communication. The other esults are our own, unless otherwise indicated by reference numbers in brackets.*

4. Discussion

The predictability of data from animal skin studies for the permeability of human skin has been the subject of many investigations (2, 41–43). The suitability of porcine skin for the *in vitro* replacement of human skin is especially well established (2, 5, 11, 13). This is confirmed by our study, where, except for the slightly higher permeation of caffeine, porcine skin was found to have permeability coefficients of the same order of magnitude and variability as human skin (Figure 2). On the other hand, bovine udder skin was found to have a higher permeability than human and porcine abdominal skin for all four test compounds, while both the relative within-batch and between-batch variations were considerably lower. This might reflect a general higher permeability of bovine skin. Nevertheless, the human skin barrier is known to exhibit variations of the same magnitude, depending on race, sex, age and body region (11). Thus, differences in permeation coefficients over this range may not indicate that a replacement models have limited suitability. The substance selectivity of replacements as compared to human skin must therefore be considered to be a more suitable estimate of their predictability.

Potts *et al.* (44) reported that the P_{app} values of different substances increase with higher lipophilicities. Figure 3 shows that this trend is not clearly reflected by our data. This might be linked to the presence of the solubiliser (Igepal®) in our testosterone donor medium. Nevertheless, the three curves clearly have a similar shape, indicating an equal influence of substance properties on the selectivity of the skin barrier in the different species. Judging from this, and given the nearly constant factor between the P_{app} values from pig and cattle, bovine udder skin is a promising alternative to pig skin in permeation studies. Its low variability is an especially attractive feature from a practical viewpoint.

In order to elucidate the possible role of species differences in skin morphology and biochemistry in the different transcutaneous absorption rates through bovine udder skin as compared to human and pig skin, we conducted further studies. In the analysis of skin lipids, we focused on lipid classes which are thought to contribute to the formation of the skin barrier. Such a correlation has been attempted several times (22–25), and different classes of lipids have been described. Lampe *et al.* (22) and Grubauer *et al.* (25) suspected that the total amount of lipids was crucial for the barrier function of the skin. Judging from our data, a simple relation between skin permeability and total lipid content must be ruled out, as there was no difference between porcine and bovine skin.

However, this simplified approach has already been extended by Elias and Menon (26), who stated that, beyond the total lipid content, the permeability barrier was a function of the lipid distribution and the supramolecular organisation of its constituent lipids. The lipid profiles of human skin and pig skin support previous findings, as triglycerides and free fatty acids constitute the biggest lipid fraction in both species (22), whereas considerably lower amounts were found in bovine skin. Mao-Qiang *et al.* (27) established that fatty acids are required in the homeostasis of the skin barrier, and it may be expected that permeation of hydrophilic substances is particularly impaired by high amounts of free fatty acids and neutral lipids. The comparison of skin from the different species showed this to be the case for caffeine, which is the most hydrophilic substance we examined. Though very variable in human skin and pig skin, its permeability coefficient appeared to be highest in bovine skin followed by pig skin and human skin, thus reflecting the inverse order of fatty acid and triglyceride contents in the different species. The hypothesis of a high impact of the contents of these lipids on skin permeability is further supported by the fact that the permeability of hydrophilic substances in bovine udder skin was far higher than that of the more lipophilic substances (Figure 2). However, the transfer of these results to the *in vivo* situation is complicated, as it is a well-known problem that it is almost impossible to avoid contaminating excised skin with subcutaneous fat, which results in increased levels of both triglycerides and fatty acids (28). Thus, the low amount of neutral lipids in bovine udder skin may simply reflect the fact that the subcutis of this organ is essentially free of fat cells (14).

Cholesterol levels were found to be about the same in all three species, whereas cholesterol ester varied approximately five times between pig and human. A relationship between the contents of these lipids and permeation coefficients can therefore be ruled out.

Ceramides are a complex group of lipids, comprising at least nine different classes, with numerous individual lipid species. Up to now, the function of each individual class remains unclear (45). Nevertheless, ceramides have been shown to play a role in normal skin barrier function (46). For example, this lipid fraction is decreased in humans suffering from atopic dermatitis (29) and in aged skin (30), resulting in a disturbance of the skin barrier function. Seven ceramide classes can be separated by thin layer chromatography, but, unfortunately, we were able to quantify only ceramide III and ceramide IV, as standards were only available for these classes. Our examinations showed that human skin contained significantly higher amounts of these two classes than skin from the other two species. However, an impaired barrier function of the animal skin as compared to human skin, due to

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the lesser ceramide content, is unlikely, as the HPTLC of porcine and bovine skin revealed strong additional bands in the ceramide region. Therefore, crucial ceramide functions might be linked to other ceramide classes contained in pig and bovine skin, but this has to be confirmed in future studies.

The results of our histological studies concur with those of Ludewig (14), who described bovine udder skin as showing the principal morphological features of mammalian skin. In our examinations, the epidermal thickness of bovine udder skin varied between 54 and 92µm due to a distinct folding of the underlying dermis. Thus, bovine skin is not markedly different from human and pig skin, which are reportedly about 70µm (47) and 40µm (48) thick, respectively (for detailed data, see Table 3). The penetration depth of follicles in udder skin was found to be about 1.5mm, which is much smaller than that of porcine abdominal skin, where primary and secondary follicles have been found to penetrate about 3.5mm and 1.3mm, respectively (48). An impaired barrier of bovine udder skin due to deeper hair follicles can thus be ruled out. However, the number of hair follicles was much higher in bovine udder skin than in human skin (about $6/cm^2$, 49) and pig skin (30–36 follicles/cm²). This confirms the data of Meyer (48), who reported 26 ± 6 follicles/cm² for this body region of pigs. Hair follicles have been described as shunt routes, especially for hydrophilic substances, whereas the transport of lipophilic substances is enhanced to a lesser extent (50, 51). Therefore, the overall higher permeability of bovine udder skin might be linked to a higher follicular transport. Furthermore, the hypothesis of hydrophilic shunt routes is supported by the striking similiarity between the number of hair follicles and the transport rates of the most hydrophilic test substance, caffeine, in the different species.

Follicular transport might explain the differences noted by Pitman and Rostas (16), who have described bovine skin as exhibiting a barrier factor 400 times weaker than that of human skin. They used the far more densely-haired bovine back skin, which is reported to contain about 900 follicles/cm² (52), whereas the udder skin used in our examinations contained only about 300 follicles/cm², which may be the reason for the higher barrier strength of this body region. Furthermore, the factor of 400 reported by these authors was based on experiments using an organic solvent as the donor medium, which may have affected the stratum corneum barrier function by lipid derangement and extraction (53). Based on the epidermal lipid profiles, such functional alterations would be expected to differ quantitatively in different species, and should therefore be avoided.

5. Conclusions

Both the barrier strength and variability of permeation parameters of pig skin closely resemble those of human skin. Bovine udder skin is a promising alternative as a replacement model for examinations on human skin permeation. Its excellent reproducibility, substance selectivity comparable to human skin, and ready availability without the need to sacrifice animals specifically for this purpose, are attractive features. Although its barrier strength was found to be weaker than those of human skin and pig skin, the differences are moderate as compared to those found in other alternative preparartions, such as reconstructed epidermis models and rat skin (1, 10).

Differences in the barrier strength of the three species studied might be linked to differences in skin morphology and biochemistry. In particular, the amounts of free fatty acids and triglycerides and the density of hair follicles should be taken into account. The impact of these findings with regard to skin permeability should be evaluated in future studies in order to further characterise the skin of various animal species as a substitute for human skin.

Acknowledgments

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Reconstructed human epidermis for skin absorption testing: results of the German prevalidation study (Summary)

This publication presents the results of a prevalidation study financed by the german ministry of education (BMBF) conducted by companies and university institutes all over Germany. The purpose of this investigation was to define a test protocol for performing comparative permeation experiments with heat separated human epidermis, porcine skin, bovine udder skin and the reconstructed human epidermis models Skinethic, Episkin and Epiderm. Huge effort went into harmonizing the experimental protocol. The OECD standard test compounds testosterone and caffeine were used.

In comparison to human epidermis, the permeation of the chemicals was overestimated when using RHE. The following ranking of the permeation coefficients for testosterone was obtained: SkinEthic > EpiDerm, EPISKIN > human epidermis, bovine udder skin, pig skin. The ranking for caffeine was: SkinEthic, EPISKIN > bovine udder skin, EpiDerm, pig skin, human epidermis. The inter-laboratory and intra-laboratory reproducibility was good. Long and variable lag times, which are a matter of concern when using human and pig skin, did not occur with RHE.

My contribution to this work

- Taking part in the development of the experimental protocol (sampling points human skin, development of adaptor for RHE in Franz cells)

- Performing all experiments marked with US

The results presented in this chapter have been published as:

Reconstructed human epidermis for skin absorption testing: results of the German prevalidation study

1. Introduction

To protect human health and the environment, substances and products are evaluated by toxicological testing, hazard analysis and risk assessment. During the next few decades, the REACH [Registration, Evaluation and Authorisation of Chemicals] initiative of the European Union [EU] will lead to an increase in the toxicological testing of chemicals. Yet animal welfare is of ethical concern to Western society, so alternatives to animal experiments are necessary. Non-animal testing procedures for regulatory purposes must be independently validated, to ensure that they provide relevant and reliable data for hazard prediction and risk assessment in humans. The principles to ensure test validity and the prerequisites for experimental validation have been published by the European Centre for the Validation of Alternative Methods [ECVAM; 1] and the Organisation for Economic Cooperation and Development [OECD; 2]. Prevalidation involves performing a small-scale inter-laboratory study with a few test samples. In the first steps, an experimental procedure is established, transferred to another laboratory, and refined. The refined protocol is subsequently evaluated with respect to reliability and relevance by testing a limited number of compounds in at least three laboratories. If the procedure is deemed suitable with respect to transferability and performance, it is then tested in a larger-scale validation study. In addition, a prediction model is developed, which is then tested to assess the predictive capacity of the test system. Indeed, the EU [2000] and the OECD have already accepted the scientifically validated *in vitro* tests for phototoxicity, skin corrosion and embryotoxicity testing [for reviews see 3, 4]. In 2003–2004, the OECD released Test Guidelines [TGs] 427 and 428, which are used to study the percutaneous uptake of chemicals *in vivo* [5] and *in vitro* [6], and are accompanied by Technical Guidance Document [TGD] 28 [7]. This is of key importance, since the skin is the third major absorption organ — after the gastrointestinal tract and the lung — and is the primary exposure route for chemicals such as pesticides [8, 9]. Moreover, several groups of professionals are exposed to dangerous chemicals in an occupational setting via dermal absorption; for example, the poisoning of tobacco farmers due to the percutaneous absorption of nicotine when handling wet tobacco leaves [10]. Since *in vitro* studies are aimed at predicting skin absorption in man, viable human skin should be used for testing in preference to animal skin, which is generally more permeable — except for porcine skin [11–14]. Nevertheless, due to their ready availability, *in vitro* studies are also conducted with pig and rat skin. Rat skin is often used for *in vitro* testing, since this species is also used for the estimation of toxicity following single and repeated dose administration, as well as to study the percutaneous absorption of chemicals and formulations *in vivo* [5]. In the rat, an *in vitro–in vivo* comparison of the skin absorption of eight pesticides showed that absorption *in vitro* was 2–3-fold higher than *in vivo* [15]. While the OECD proposals will accelerate the spread of the diffusion [Franz] cell technique for penetration studies, other *in vitro* approaches, such as the isolated perfused porcine skin flap [16, 17], perfused pig forelimb [18, 19] and perfused bovine udder [20, 21], can be used to study specific problems. Due to the limited availability of excised human skin for experimental purposes, human-based alternatives for uptake testing are being investigated. In recent years, organotypic models have become more useful for investigators, and today, reconstructed human epidermis [RHE] models are commercially available. They are well described with respect to tissue architecture and lipid composition [22, 23], and have already proved to be of value for corrosivity testing [24, 25]. In a project funded by the 5th Framework Programme of the EU and the German Research Foundation, the uptake of reference standard compounds by animal skin was compared to that by human skin [26, 27]. These studies also included investigations on formulations [28–31]. In another investigation, the penetration properties of four dermatological drugs with human, pig and rat skin were compared with results with the SkinEthic® RHE and the Graftskin™ LSE models [32]. In fact, the OECD TGD 28 states that reconstructed human skin models can be used for hazard assessment, if the data obtained with reference chemicals are consistent with those in the published literature [7]. Therefore, RHE may become another favoured test matrix. Scientists in the field of skin absorption testing have decided to define what relevant procedures are, and to evaluate them as the basis for validation studies. The results of initial experiments, in which the skin permeation of caffeine, testosterone and a dye was compared, permitted the identification of highly relevant parameters for ensuring reproducible permeation. Aspects related to protocol development will be, or have been, published separately [33–35]. Based on the outcome of an initial comparison of a small number of comparative experiments, the authors agreed on a test protocol which was then tested thoroughly by five toxicological laboratories in Germany [both in academia and in industry] and at ZEBET. This prevalidation study, funded by the German Ministry of Education and Research [BMBF], was carried out to qualify RHE for uptake studies. The test protocols focused on skin permeation and the results of the inter-laboratory comparison are reported here.

2. Materials and Methods

The study was performed according to the modular approach for validation, a stepwise procedure proposed by ECVAM [1]. Uptake tests using human, pig and rat skin can be regarded as retrospectively validated due to the approved OECD *in vitro* TG 428 [6], and data generated with RHE can therefore be compared to those for uptake with human and animal [pig] skin. The experiments were conducted from March to August 2004, in general accordance with the principles of Good Laboratory Practice.

2.1 Study management and organisation

An initial test protocol was developed, based on the principles of TGD 28 [7]. Specific questions concerning test substance preparation, analytics [techniques used for quantification], and composition and histopathological structure of the skin models used, as well as the handling of the different models, were each evaluated by one of the participating laboratories. Based on the outcome of these experiments, refined and detailed standard operation procedures [SOPs] were defined for conducting the skin penetration tests.

2.2 Test chemicals and preparation of solutions

For cutaneous uptake studies, the OECD proposes the use of caffeine [logP = 0.01; MW = 194; 58-08-2] and testosterone [logP = 3.32; MW = 288; 58-22- 0] as reference standard substances with a low or high lipophilicity, respectively. Therefore, these agents were included in this study. The chemicals were obtained from Sigma [St. Louis, MO, USA], as were ethanol [64-17-5], Igepal® CA-630 [[octylphenoxy] polyethoxyethanol, 9043-52-1] and phosphate buffered saline [PBS], pH 7.4. All the laboratories used donor solutions of caffeine [0.1%, 284.1μg/cm²] and testosterone [0.004%, 11.36μg/cm²], which were freshly prepared by dissolving caffeine in PBS and diluting the stock solution of testosterone [10mg/ml 96% ethanol] in PBS containing 2% [v/v] of the solubiliser, Igepal® CA-630. The stock solutions were stable for at least four weeks when stored at 4°C. Two laboratories assessed penetration by using radiochemical detection and therefore spiked the samples with 1-methyl-14C-caffeine [51.2mCi/mmol; 77196-81-7; Perkin Elmer Life Sciences, Boston, MA, USA] and 1,2,6,7-³H-testosterone [100Ci/mmol; 6384-79-8; Amersham, Freiburg, Germany], both at purities of higher than 97%, to achieve a total radioactivity of 1μCi per Franz cell.

2.3 Reconstructed human epidermis models and skin

RHE was purchased from three manufacturers: EpiDerm™ Skin Model [EPI-606X] from MatTek Corporation [Ashland, MA, USA]; Reconstructed Human Epidermis Kit EPISKIN® [J13, 1.07cm²] from L'Oréal [Paris, France]; and SkinEthic® Skin Model [RHE/L/17: Reconstituted Human Epidermis, large, age day 17, 4.00cm²] from Laboratoire SkinEthic [Nice, France]. All the RHEs were shipped for delivery on a Tuesday or Wednesday morning, and were used for the experiments within 24 hours after delivery, according to the recommendation of the manufacturers. The storage period was documented for each experiment. All the handling before incubation was performed under a sterile air flow. Before opening the EPISKIN kit, the integrity of the kit was verified by the colour of the agar medium and the temperature indicator. The EPISKIN and SkinEthic tissues were removed from nutrient agar immediately after delivery, transferred into six-well plates [SkinEthic] or twelve-well plates [EPISKIN], filled with the manufacturer's maintenance media, then kept overnight in an incubator at 37°C and 5% CO2. The EpiDerm™ tissue was stored overnight at 4°C. The next morning, the tissues were transferred into six-well plates and kept in the incubator at 37°C and 5% CO2, for at least 1 hour. Human skin [abdomen or breast] was obtained from females aged 20–75 years, who had been subjected to cosmetic surgery. Pig [Deutsche Landrasse breed; no soaking of the cadaver in boiling water] and bovine udder skin [Schwarzbunte breed] were obtained from local abattoirs. The skin was placed in an ice-cold cloth and immediately transferred to the laboratories. Great care was taken to avoid contamination of the skin surface by subcutaneous lipids. In the laboratories, subcutaneous fat and connective tissue were removed from the skin, and the tissue then subjected to cryopreservation at −25°C for at least 1 day, up to a maximum of 6 months. The skin was thawed immediately before performing the experiments. Human epidermis sheets [HES] were prepared from human skin by heat separation [34, 36], whereas skin with a thickness of 1000 ± 100μm was prepared from pig and udder skin by using a Dermatome™ [Aesculap, Tuttlingen, Germany]. Alternatively, the upper side of the skin was frozen to obtain split skin of identical thickness by a microtome [Leica 1325CM, Nussloch,

Germany]. A detailed comparison based on pig skin from the same donor did not indicate any differences in the permeation of testosterone when these different approaches were used for skin preparation.

2.4 Refined test protocol and SOPs

The refined test protocols were transformed into SOPs and used for this study. Except for the inspection of tissue integrity, the protocols used were more detailed than TGD 28 [7] but followed the procedures described therein. Briefly, following a visual check of tissue integrity by using a magnifying glass, human epidermis sheets, animal skin [both rehydrated in PBS for 30 minutes] and RHE were mounted in Franz cells [15mm in diameter, with a receptor chamber of 12cm³; PermeGear, Bethlehem, PA, USA]. Tissues were discarded if there was a wet skin surface due to the appearance of receptor medium. Since the size of the available EPISKIN models was too small to mount directly into the Franz cells used, a special EPISKIN insert was made, in order to accommodate the reduced surface area of 0.357cm2. The stratum corneum was placed facing the air and the dermis was in contact with the supporting membrane and the receptor medium PBS. The receptor medium was kept at a constant temperature of 33.5 ± 0.5°C by using an incubator or a water bath, and stirred with a magnetic bar at 500rpm. The absence of air bubbles was monitored throughout the experiment. After equilibration for 30 minutes, 500μl of the donor solution was applied to the skin surface of the tissues. When using EPISKIN, 110μl was used to adapt the applied amount to the smaller surface area. The opening of the Franz cell was kept covered by Parafilm®. In all the experiments, 281.4μg/cm² caffeine or 11.36μg/cm² testosterone was applied and left in place for the entire experiment. The concentrations of the donor solutions chosen ensured that the concentration in the receptor fluid was clearly below the solubility limits of the compounds. The uptake of caffeine and testosterone was measured by high performance liquid chromatography [HPLC; 3 laboratories] or radio-chemical detection [2 laboratories] of the amounts that had permeated into the receptor medium. The receptor medium was sampled at 6 hours and 24 hours, as well as at additional time points selected by the individual centres, to estimate skin permeation from a regression line based on six valid samples. Thus, the individual sampling times varied between the types of test skin and the laboratories. To assure the free diffusibility of test compounds, the saturation of solubility was determined by preparing saturated solutions of caffeine and testosterone in PBS [pH 7.4], which were kept at 37°C. After 24 hours, an aliquot was taken and filtered immediately. Then the solution was diluted and the concentrations of the compounds were quantified by HPLC.

2.5 Analysis

Laboratories using HPLC analysis to quantitate the permeated substances in the receptor medium used a validated method. Briefly, a Waters Alliance HT 2695 equipped with a Waters 996 Photo diode array detector [Waters, Milford, MA, USA] was used for detection. The HPLC column used [XTerra MS C18; 50 × 2.1mm i.d. 5μm; Waters] was maintained at 40°C. The mobile phase consisted of a 10mM phosphate buffer, adjusted to pH 3.5 with *o*-phosphoric acid; the organic modifier was acetonitrile [MeCN]. Chromatography was performed by using a gradient [5–95% MeCN from 0–5 min, 5% MeCN from 5.1–7 min]. The flow rate was set to 0.6ml/minute, and 10μl of the solution was injected. For caffeine, concentrations ranging from 0.25 to 10μg/ml, corresponding to the linear portion of the regression line, with a correlation coefficient of 0.999, were used for calibration; the limit of detection [LOD] was 0.25μg/ml. For the calibration of testosterone, concentrations from the linear portion of the curve ranging from 0.025 to 10μg/ml and exhibiting a correlation coefficient of 0.999 were used; the LOD was 0.025μg/ml. In addition, an isocratic HPLC procedure was used after testing for validity. Radiolabelled caffeine and testosterone were quantified in the receptor medium by scintillation counting [Microbeta Plus, Wallac, Turku, Finland] employing a scintillation cocktail [Optiphase Supermix, Wallac], as previously described [34]. The results were comparable, independently of the method chosen — HPLC or radiochemical quantification.

2.6 Data analysis procedure/biostatistical methods

Human epidermis sheets were tested in three laboratories, and reconstructed epidermis and animal skin in two laboratories. Animal skin was obtained from at least two donors, RHE from at least two batches was used, and the human epidermis sheets originated from three donors. In general, experiments with human epidermis sheets were performed in triplicate for each donor, and experiments involving the other matrices were conducted in quadruplicate for each donor or batch. Therefore, in general, eight tests for each test agent and skin type [nine for human epidermis sheets] were run by the laboratory in charge, resulting in the performance of about 25 parallel experiments for

each substance [Table 1]. Permeation values were given as caffeine or testosterone amounts in the receptor medium at 6 hours [$\mu g/cm^2$], normalised to the exposed skin surface, as well as by the apparent permeability coefficient Papp [= $[V/A*C_i]*dC_A/dt$], which takes the exposed surface area [A] into account [EpiDerm, SkinEthic: $1.768cm^2$; EPISKIN: $0.385cm^2$]. The volume [V] was $12cm^3$ in all the experiments, with the exception of experiments with EPISKIN due to the different size of the EPISKIN insert [$11.4cm^3$]. C_i gives the initial concentration of the applied substance in $\mu g/cm^3$, and dC_A/dt is the increasing concentration of the substances in the receptor medium with increasing time. The Papp value and lag time [intersection of the linear part of the regression line with the x-axis] are derived from the skin of each donor or batch. Data are presented as either the arithmetic mean value, standard deviation [sd] and the coefficient of variation [CV] of the experiments performed with the skin of each donor/batch, or as the mean of the respective laboratory, as indicated. For each test agent, differences of Papp were analysed by a two-factor analysis of variance [ANOVA] with the factor "skin" [6 types] and the factor "laboratory" [5 types]. Intra-laboratory and inter-laboratory variations were calculated according to DIN ISO 5725-2. The parameter sr% estimates the intra-laboratory CV, and sL% estimates the inter-laboratory CV, to describe the repeatability [37].

3. Results

The experimental procedure was first tested to ensure that the concentrations of the test compounds in the receptor medium did not exceed the permitted range of 10% saturation solubility. The maximum caffeine concentration was 10.8µg/ml, corresponding to 0.04% saturation solubility [cs: 31.08mg/ml, pH 7.4]. With respect to testosterone, 0.6556µg/ml was found, corresponding to 2.09% saturation solubility [cs: 31.31µg/ml, pH 7.4]. Thus, diffusion was not restricted. The partner laboratories performed the experiments according to the refined SOPs. There were only very few variations of the protocol procedures. Since testosterone concentrations in the receptor fluid were below the detection limit when using pig skin with 1000µm thickness, one laboratory used pig skin with a thickness of 700µm. These experiments were repeated with skin of the correct dimension. These latter data were included in the further processing. Figure 1 depicts mean caffeine concentrations and Figure 2 mean testosterone concentrations in the receptor media, as determined by the partner laboratories. When evaluating the concentrations of both substances, permeation of human epidermis sheets showed pronounced variability of the Papp value. The CVs for caffeine and testosterone were 62% and 93%, respectively. The CV of the Papp values was less with RHE [20–55%]. Significant differences between the two laboratories studying the same tissue were not found, except for caffeine permeation when using the SkinEthic model [Figure1e; $p \leq 0.05$]. A more detailed analysis of differences between RHE models is based on Papp values, total permeation within 6 hours, and lag times, as summarised in Table 2. In fact, the amounts of testosterone and caffeine permeated after 6 hours are in good accordance with the Papp values.

3.1 Testosterone permeation

Testosterone permeation through the EpiDerm and EpiSkin model [Papp: $2.89 \pm 1.09 \times 10$–6cm/s and $2.11 \pm 0.63 \times 10$–6cm/s, respectively] was very similar, while the SkinEthic model appeared to be more permeable [$6.00 \pm 1.17 \times 10$–6cm/s; $p \leq 0.05$]. Compared to human epidermis [$0.42 \pm 0.39 \times 10$–6cm/s], pig skin [$0.08 \pm 0.01 \times 10$–6cm/s] and bovine udder skin [$0.32 \pm 0.28 \times 10$-6 cm/s], the Papp values suggested that the barrier functions of the reconstructed tissues were clearly less developed [$p \leq 0.05$]. The ANOVA grouped skin permeability, as measured by Papp, for testosterone as follows: SkinEthic > EpiDerm, EPISKIN > human epidermis, bovine udder skin, pig skin. Total permeation within 6 hours ranged from 0.6% of the applied testosterone when using pig skin, to 39.5% when using the SkinEthic model. The grouping of the tissues was the same as that of the Papp values except for an additional significant difference for EpiDerm and EPISKIN [Table 2; $p \leq 0.05$].

3.2 Caffeine permeation

The EpiDerm model appeared to be less permeable [Papp: $0.24 \pm 0.14 \times 10$–6cm/s; $p \leq 0.05$] with respect to caffeine, compared to the EPISKIN and SkinEthic models [Papp: $2.77 \pm 0.78 \times 10$–6cm/s and $3.63 \pm 1.91 \times 10$–6cm/s, respectively]. In fact, according to the ANOVA, the Papp value of caffeine permeating EpiDerm was in the range of the Papp values of human epidermis [$0.06 \pm 0.04 \times 10$–6cm/s], pig skin [$0.07 \pm 0.05 \times 10$–6cm/s], and bovine udder skin [$0.63 \pm 0.23 \times 10$–6cm/s]. The following rank order of the preparations was found: SkinEthic, EPISKIN > bovine udder skin, EpiDerm, pig skin, human epidermis. The order changed slightly [pig skin and human epidermis] when comparing the amounts of permeated caffeine at 6 hours, which was 0.17–26% of the amount applied

— the extreme values in this case were obtained with human skin and SkinEthic. ANOVA again discriminated between SkinEthic and EPISKIN.

3.3 Lag times

Another difference between RHE and human epidermis sheets or animal skin is the delay of permeation [Table 2]. The lag time of both test substances was low to absent, depending on the RHE model used. For example, with caffeine the lag time for EPISKIN was approximately 1 hour, and that for EpiDerm less than 0.5 hours, while there was no lag time with the SkinEthic model. In contrast, when testing caffeine, a mean lag time of 4 hours was observed with pig skin, and enormous fluctuations of lag time were found with human epidermis, sometimes making test periods of 24 hours or more necessary. The lag times found when using bovine udder skin were in between those of the RHE and human epidermis or pig skin.

3.4 Inter-laboratory variability

Importantly, while the ANOVA revealed clear differences between the test skins, inter-laboratory variability [sL%] between the five laboratories was low. Even when studying human epidermis sheets, the sL% of the Papp values varied by 9.3 for caffeine and was almost zero when testosterone was tested [inhomogenities excluded a precise quantification].Thus, the protocol was successfully transferred between the partner laboratories.

Table 1: Donors of skin and batches of RHE as used in the partner laboratories.
Tissues from at least 2 donors or batches and experiments were run in quadruplicate per each batch or donor [br: breast; ab: abdomen, T: testosterone, C: caffeine]
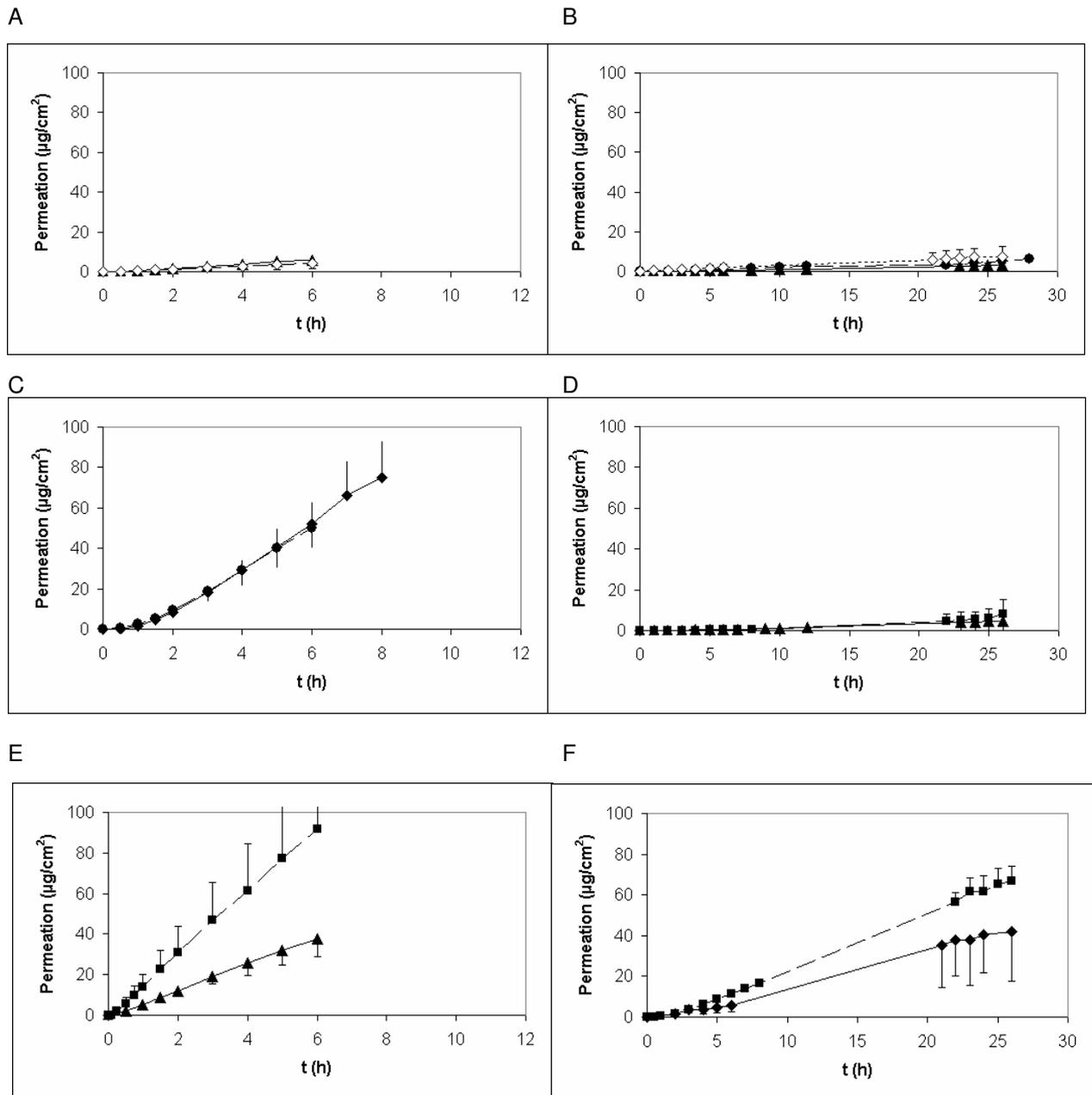
skin	FU	US	TiHo	LMU	ACB
HES/ experiments in triplicate per donor	br, 39^C br, 50^T br, 55^C br, 58^C br, 72^T	ab, 39^{T,C} ab, 49^C ab, 51^{T,C}		br, 30^{T,C} br, 55^{T,C} br, 52^{T,C}	
pig skin	S030^C S032^C S033^T		S027^C S028^C S034^C S037^T S040^T S041^T		
bovine udder skin			R023^C R031^C R034^C R044^T R045^T R047^T		126-0503^C 178-0904^C 179-0904^C 180-0904^C 181-0904^T 183-1104^T 184-1104^T
SkinEthic [04/05 022A]	0414^{T,C} 0501^{T,C}		0402^C 0405^T 0406^T 0501^T 0503^C 0702^C 0704^C		
EpiDerm	5025^C 5034^T 5036^T 5412^C			5025^{T,C} 5412^{T,C} 5619^{T,C}	
EPISKIN [04 EPIS-] J13		021^{T,C} 022^{T,C} 031^{T,C}			014^{T,C} 015^{T,C} 018^{T,C} 031^{T,C}

Table 2: Summary of permeation data: P_{app} values, lag time and drug permeated into the acceptor medium after 6 h for caffeine 0.1% and testosterone 0.004% solution applied to human epidermis sheets [HES], reconstructed epidermis as well as pig and bovine udder skin.

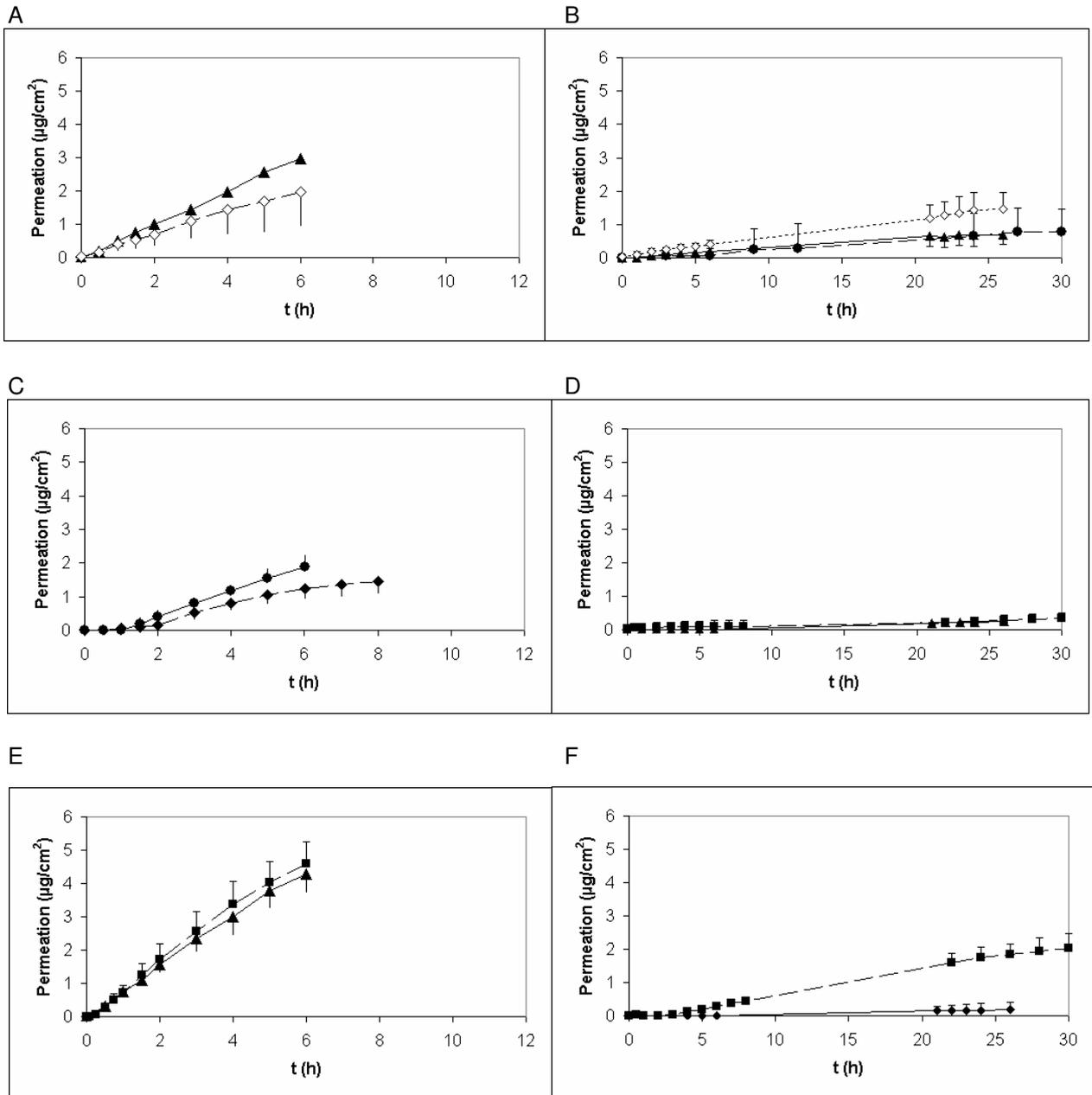| Skin type	Permeation 6 h [µg/cm^2]	P_{app} [10^{-6} cm/s]		lag time [h]	
	$\bar{x} \pm s$	$\bar{x} \pm s$	CV	$\bar{x} \pm s$	n
Caffeine					
HES	1.12±1.18	0.06±0.04	62,29	1.73±1.48	8
Pig skin	0.48±0.41	0.07±0.05	74,82	3.92±0.87	6
Udder skin	8.24±3.86	0.63±0.23	37,23	1.88±0.42	7
EpiDerm	4.87±2.67	0.24±0.14	55,59	0.33±0.06	5
SkinEthic	73.65±36.58	3.63±1.91	52,74	0.14±0.05	6
EPISKIN	51.25±9.84	2.77±0.78	24,37	1.04±0.26	7
Testosterone					
HES	0.32±0.27	0.42±0.39	93,18	1.03±2.52	8
Pig skin	0.07±0.15	0.08±0.01	14,91	-0.13±11.92	4
Udder skin	0.14±0.15	0.32±0.28	89,89	1.19±1.30	6
EpiDerm	2.36±0.90	2.89±1.09	37,82	0.00±0.09	5
SkinEthic	4.47±0.57	6.00±1.17	19,55	0.14±0.09	5
EPISKIN	1.53±0.47	2.11±0.63	29,89	0.93±0.33	7

Figure 1:

Caffeine permeation [µg/cm²] into phosphate buffered saline following the application of 500 µL or 110 µl [EPISKIN] of a 0.1% solution in PBS for at least 6 h to reconstructed epidermis [A: EpiDerm, C: EPISKIN, E: SkinEthic] and 26-30 h to human epidermis sheets [B] or split skin from pigs [D] or bovine udder [F]. Each line depicts the results obtained with the skin of a single donor or batch respectively; the symbols indicate the laboratory performing the experiment.   ACB◆ FU ▲ LMU ◇

TiHo ■ US ●

A

B

C

D

E

F

Figure 2:

Testosterone permeation [µg/cm²] into phosphate buffered saline following the application of 500 µL or 110µl [EPISKIN] of 0.004% in PBS solution for 6-8 h to reconstructed epidermis [A: EpiDerm, C: EPISKIN, E: SkinEthic] and 26-36 h to human epidermis sheets [B] or split skin from pigs [D] or bovine udder [F]. Each line gives the results obtained with the skin of a single donor or batch respectively, the symbols indicate the laboratory performing the experiment. ACB◆ FU ▲LMU ◇TiHo ■US ●

A

B

C

D

E

F

4. Discussion

During the last decade, major progress was made in the replacement of animal experiments, including the approved *in vitro* approaches for regulatory phototoxicity [38] and skin corrosivity testing [24, 39]. In addition, non-animal tests for skin irritation [40–42] and skin sensitisation [43] are being developed, but their final validation and regulatory acceptance have not yet been achieved. These *in vitro* tests are either based on monolayer cultures of skin cells [phototoxicity, sensitisation] or make use of RHE [corrosivity, irritation] or human skin explant cultures [44]. RHE is often favoured for skin toxicity testing, because of its close resemblance to human skin [for a review, see 42]. The cutaneous uptake of a chemical is the first step in the induction of skin damage in contact irritant and allergic dermatitis, as well as for systemic toxicity. Therefore, a validated *in vitro* approach involving the use of RHE for skin uptake testing would be welcome. The method should permit the determination of the amounts penetrating [e.g. for sensitisation] and permeating [e.g. for systemic toxicity] the skin. In safety assessment, the sum of the amounts of a substance permeating and penetrating the skin is used to determine absorption. If no data are available, the worst-case-scenario absorption of 100% is normally used. Approval of methods by the OECD, in this case the use of human and animal skin in diffusion cells in skin absorption studies [6, 7], results in an almost world-wide acceptance of the data generated according to the appropriate OECD TGs.

There are, however, two major drawbacks. First of all, the enormous number of tests expected as a result of the REACH initiative will result in a shortage of human skin for *in vitro* experiments. Testing might then be delayed, or animal skin will have to be substituted for human skin. Moreover, the rather generally defined test procedure laid down in OECD TGD 28 [7] can result in a large variability in the data generated when relying only on this document. In fact, the results of a recent ring trial, in which the OECD reference substances, benzoic acid, caffeine, and testosterone, were tested, varied widely between the ten laboratories of the EDETOX project. The published data [45] suggested a maximum flux variation of 50% with caffeine and 109% with testosterone, between the nine EDETOX laboratories using human skin as a test matrix. These differences may result from the variations in the thickness of the human skin obtained from surgical intervention and from human cadavers, which ranged from 300 to 1800µm. Additionally, the Franz cell surface areas employed varied by ten-fold and the Franz cell volumes by even more [45]. In order to be able to compare the results of permeation experiments performed in one laboratory with those of another laboratory, a higher degree of standardisation of testing procedures needs to be achieved than would follow from the use of the OECD TGD alone. In this study, five laboratories used human and animal skin [generally originating from different donors], in addition to different batches of various RHE models. The highly standardised test procedure clearly reduced the variability of the permeation profile. This applied to both the medium and highly lipophilic agents [caffeine, Figure 1; testosterone, Figure 2], as well as to the permeation parameters, Papp value, permeated amount and lag time [Table 2]. When studying human epidermis sheets, the inter-laboratory variability [sL%] of the Papp values was only 9.3 for caffeine and almost zero for testosterone, although inhomogeneities did not allow precise quantification. Taking into account the fluctuation seen when using human skin with a thickness of 1000µm [34], heat isolation of the epidermis does not increase variability to a relevant extent. Therefore, any variability is probably caused by the individual skin structure of the donors. The variability observed in this study was in the upper range of scatter found in the permeation of 18 compounds [mostly beta-blockers] tested on human cadaver epidermis [variation coefficients 27–92%] in addition to a rat keratinocyte model [6–83%; 46]. However, the data from the latter model were generated only in a single laboratory. Recent studies comparing the permeability of chemicals tested on reconstructed epidermis of human and rat origin with that of human epidermal sheets [32, 34, 46], human skin [18, 27, 30, 32, 34], pig skin [18, 19, 32, 34] or rat skin [32], have already indicated a higher degree of permeability for the reconstructed tissues. The detailed study protocol adhered to by the five independent laboratories during testing in our study allows a more precise analysis of the differences, as well as a comparison of the three commercially available RHE models. The Papp values for caffeine and testosterone permeation exceeded those calculated for human epidermis sheets by 4.0-fold and 6.9-fold with EpiDerm, 46.0- fold and 5.0-fold with EPISKIN, and 60.6-fold and 14.3-fold with the SkinEthic model. Thus, hydrophilic agents seem to permeate RHE even better than lipophilic ones, and the lack of hair follicles that facilitate permeation of hydrophilic compounds is not relevant for caffeine. Whether, this holds true for more hydrophilic substances, e.g. mannitol, will be evaluated during the validation study. Suhonen and coworkers [46] reported that, on average, there was a two-fold to three-fold enhancement of the permeability coefficients of 18 test compounds, ranging from 0.3 [hydrophilic compounds] to 5.2 [lipophilic compounds], in the rat epidermis model compared to HES. In particular with testosterone, which was tested in both studies, the permeability of reconstructed rat epidermis [46] and the RHE models, EpiDerm and EPISKIN, exceeded HES permeability by about 5-fold. Low lag times were also seen with reconstructed epidermis built up from a rat keratinocyte cell line [46],

while long and variable lag times of several hours had already been reported in previous studies when using HES [11,12]. The higher permeation and thus overestimation of skin absorption when using reconstructed epidermis models is in accordance with the incomplete barrier found in these models. The deficiencies in the barrier are caused by lower concentrations of free fatty acids and hydrophilic ceramide fractions, and also by the expression of cytokines and growth factors leading to hyper-proliferation of the epidermal cells [23]. In our study, bovine udder skin also tended to be oversensitive for predicting the skin absorption of chemicals in comparison with human epidermis [Papp ratio: 10.5 for caffeine and 0.8 for testosterone]. Pig skin [Papp ratio: 0.2 for testosterone and 1.2 for caffeine] tended to be less permeable. A higher uptake by bovine skin as compared to pig skin was also observed when studying the veterinary drug, abamectin [47]. To permit correlation of the permeability of RHE to the permeability of human skin, a prediction model will be developed after finishing the next step of the formal validation process, which is described below. In the limited number of experiments conducted in this study, a tendency for lower variation of permeation data was observed when using RHE. Should this also be demonstrated in the validation phase, a reduction in the number of individual experiments necessary to be performed may be possible. From a practical point of view, the constant and short lag times found in permeation studies when using RHE [Table 2] also have various advantages. Uptake experiments can be performed within 6–8 hours, the sampling of receptor medium needed for valid experiments can be kept to a minimum, and, more importantly, a smaller number of batches are needed, in comparison with the numbers of donors needed for studies on human and animal skin. This will also facilitate the evaluation of the influences of donor vehicles, which is relevant for pesticides [48], actives of cosmetics, and drugs in human medicine [29–31] and veterinary medicine [47]. In addition, studies on interferences from the environment [14, 16, 17], the pre-treatment of skin, and the influence of dermal metabolism [13, 29, 49], which have only recently become a matter of concern, can also be simplified. The important effects of these factors have been reported; for example, the pre-treatment of skin with various ingredients of sunscreens and also N,Ndiethyl-m-toluamide [DEET] has been found to increase the absorption of 2,4-dichlorophenoxyacetic acid. This is relevant when agricultural workers use sunscreens and/or repellents, which may increase the risk of pesticide uptake [14]. OECD TG 428 [6] calls for integrity testing before permeation experiments are performed, and the TGD 28 [7] recommends the measurement of transepidermal water loss [TEWL] or transepidermal electrical resistance [TER]. Alternatively, tritiated water can be used as a permeation marker. When agreeing on the test protocol, however, the study group decided against the use of these methods. This decision was made because of the poor predictability of TEWL measurements for skin barrier integrity, as was recently observed by one partner laboratory when comparing intact, stripped and needle-punctured human epidermis sheets. TEWL measurements detected only very severe damage to the stratum corneum [35]. Furthermore, the application of solutions prior to each experiment limited the use of the TER, since a complete removal of the solution, especially of lipophilic preparations, could not be ensured. Finally, the use of tritiated water was not regarded as suitable, since its application for 5 hours prior to each experiment might affect the quality of the skin samples [50]. Moreover, the might result in an overestimation of the quantity of permeated test compound, due to radioactive contamination. Furthermore, the study group aimed to define and characterise methods that could easily be established by each laboratory. Based on these considerations, the study group decided to check for integrity by visual inspection only. According to the ECVAM principles for validation [1], the acceptance of a non-animal test does not only require transferability and acceptable variability both within and between laboratories, but also relevance based on predictive capacity, as determined experimentally, and on mechanistic relevance. The mechanistic principles for the uptake into and through human skin and RHE are quite similar. In both systems, the stratum corneum is the major barrier to the uptake of chemicals. In contrast to the skin of furry animals, in human skin the shunt pathway of absorption via the hair follicles is of little relevance. Since the prevalidation experiments reported here were run in five laboratories, each of them performing an overlapping subset of experiments, the results are in line with the ECVAM principles for demonstrating the predictive capacity of new testing procedures. The validation study will further improve the predictive capacity of the protocol, since the number of participating laboratories will be greater and the set of test chemicals will be expanded to cover a broader spectrum of physicochemical parameters. Substances with a molecular weight over 500 Daltons will also be included, since the skin penetration of these substances may be very low [51]. Moreover, a finitedose approach which reflects accidental intoxication will be included, to comply with the needs of regulatory toxicology. At the time when the project was designed, all the known commercially available RHE models were included. However, the situation, changed, when in 2002 to 2004, new commercially available reconstituted skin models were introduced into the market [for example: the full-thickness model AST-2000, CellSystems, St. Katharinen, Germany; the fullthickness models EpiDerm FT, Mattek, Ashland, MA, USA; SkinEthic RFT, Laboratoire Skinethic, Nice, France; and Phenion FT, Phenion, Frankfurt, Germany; as well as

the epidermal model, EST-1000, CellSystems]. In addition to the commercially available organotypic models, there are several inhouse models, including a rat keratinocyte cell line [46], which may also be suitable for uptake studies. According to the principles of the modular validation approach, new methods may be evaluated experimentally, without conducting a full validation study [1]. In this case, all the skin models available for testing may be used for skin absorption testing in the near future, without any restrictions.

Acknowledgements

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Part 2: Unpublished results

1. Storage I: Influence of the time lapse between excision and preparation on the outcome of permeation experiments
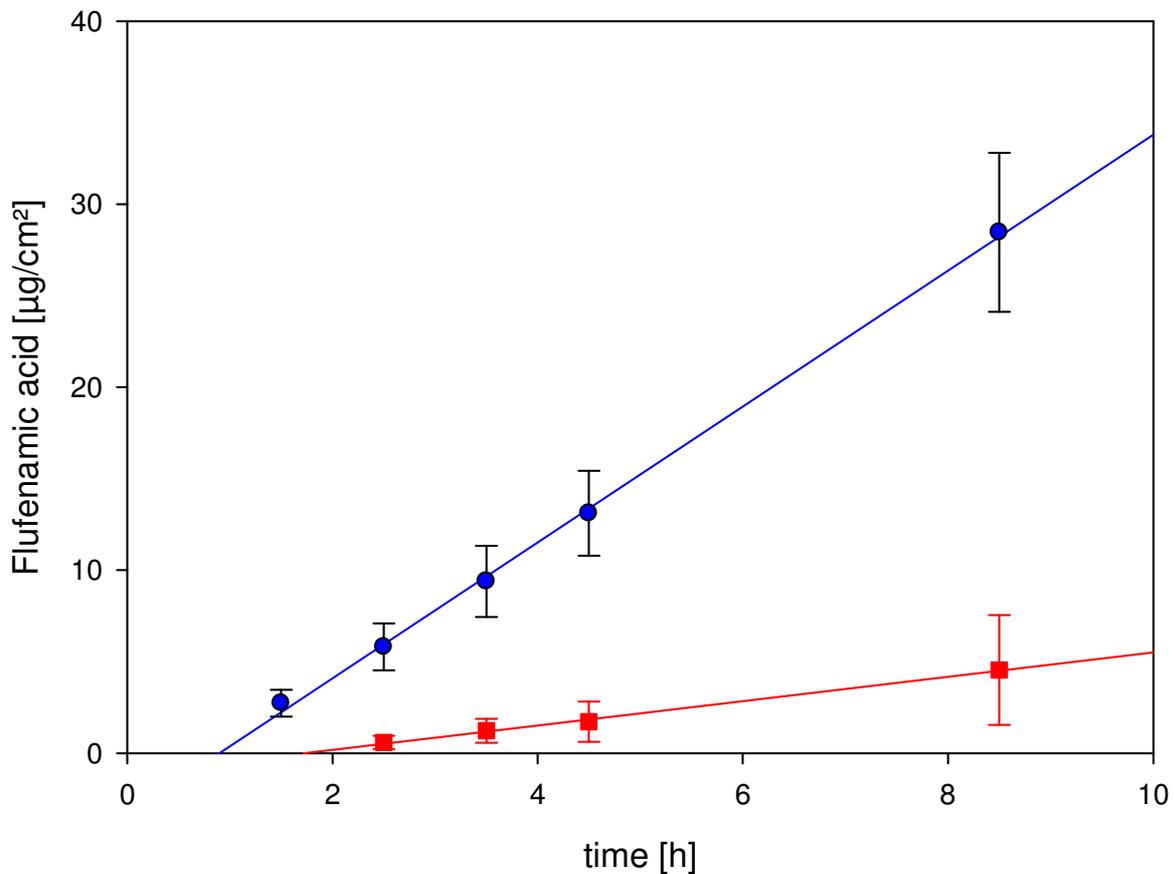
1.1 Introduction

A point so far neglected by other researchers is the influence of the time interval between excision and preparation before freezing of excised human skin on subsequent permeation experiments. In an effort to increase the interalaboratory comparability of results of diffusion experiments this question was investigated. A time interval of six hours was selected.
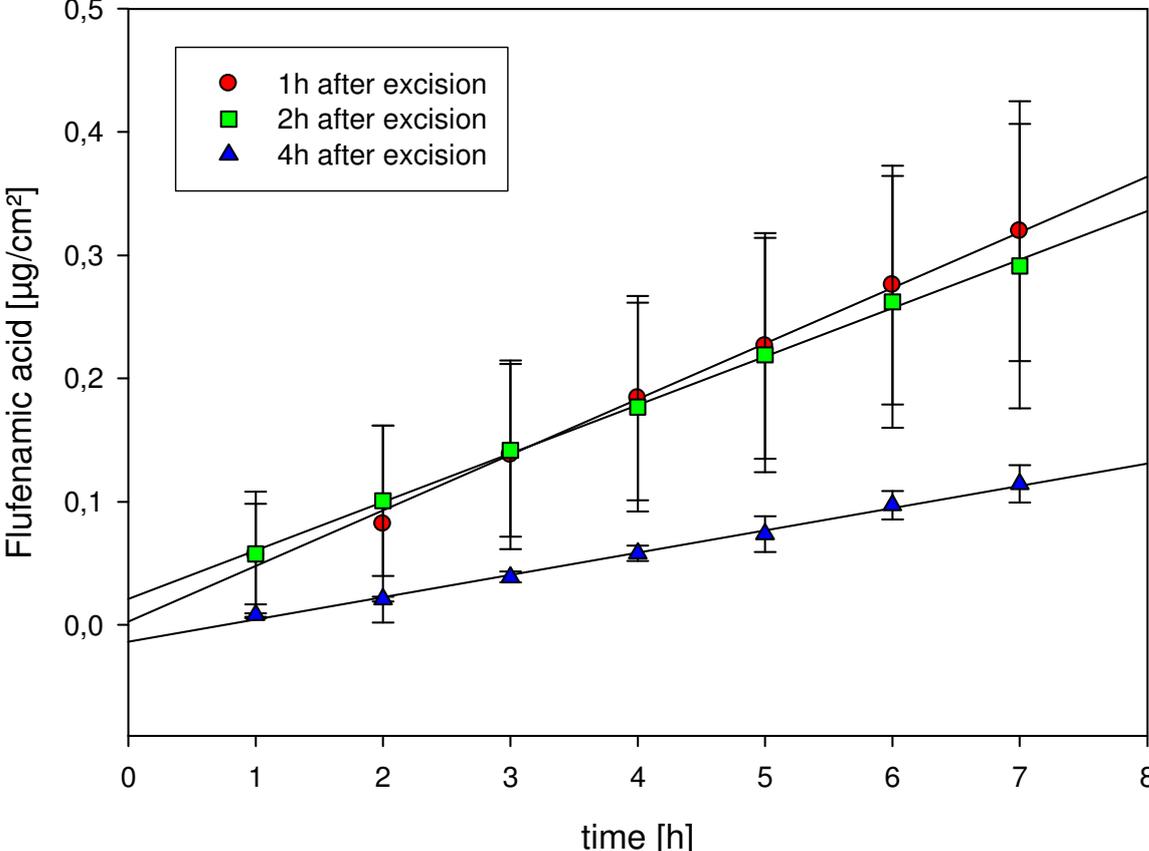
1.2 Results

In the first set of experiments one skin was investigated. The skin was divided in two pieces. The first piece was prepared and frozen one hour after excision and the second part after six hours. The following day permeation experiments (n = 4 in each group) with flufenamic acid were conducted following the protocol described by Wagner [1].

Figure 1: Permeation experiments with first skin piece

A clear difference (figure 5) was found between the 1h and the 6h sample was found. The experiment was repeated with a second piece of skin. This time it was divided into three pieces: 1h after excision, 2h after excision and 4h after excision (figure 6).

Figure 2: Experiments with second skin piece [µg/cm²] flufenamic acid versus time [h]

No difference was found between 1h and 2h samples however the 4h samples showed a clearly different behaviour. One fact which is especially intriguing is that in the first experiment the 6h values were cleary above the 1h value while in the second experiment the 4h value lay below the 1h and 2h value.

Table 1: Papp values of both skins

Time after excision [h]	First experiment $P_{app} \cdot 10^8 \pm$ SD [cm/s]	Second experiment $P_{app} \cdot 10^8 \pm$ SD [cm/s]
1	1,04 ± 0,001	1,04 ± 0,2 (difference not significant)
2	0,87 ± 0,3	n.a
4	0,40 ± 0,1	n.a
6	n.a.	0,7 ± 0,1

1.3 Discussion

A clear effect of the time interval between extraction and freezing on the results of permeation experiments can be seen. The reasons

1.4 Conclusion

Whether the effect is due to bacteria or prolongated exposure to topical contamination the conclusion remains the same: During the first two hours after surgical extraction the skin has to be cleaned, prepared and frozen.
Otherwise the quality of the skin as testing material for in vitro permeation experiments might be compromised.

2. Storage II: Influence of freezing time on human skin lipids

The investigation of the effect of prolonged cryoconservation on human skin goes a long way back. Theobald [2] and Schaefer [3] have shown that the skin remained stable upon freezing as the penetration of drugs and the thickness of the SC was not affected after a freezing period of 3 and 6 months, respectively. Bronaugh et al. [4] showed that over a time period of up to one year at –20 °C no difference could be seen. Human skin could usually be stored at -20 degrees C for up to a year with no change in water permeability. In some cases after 6 months deterioration of the barrier was observed.
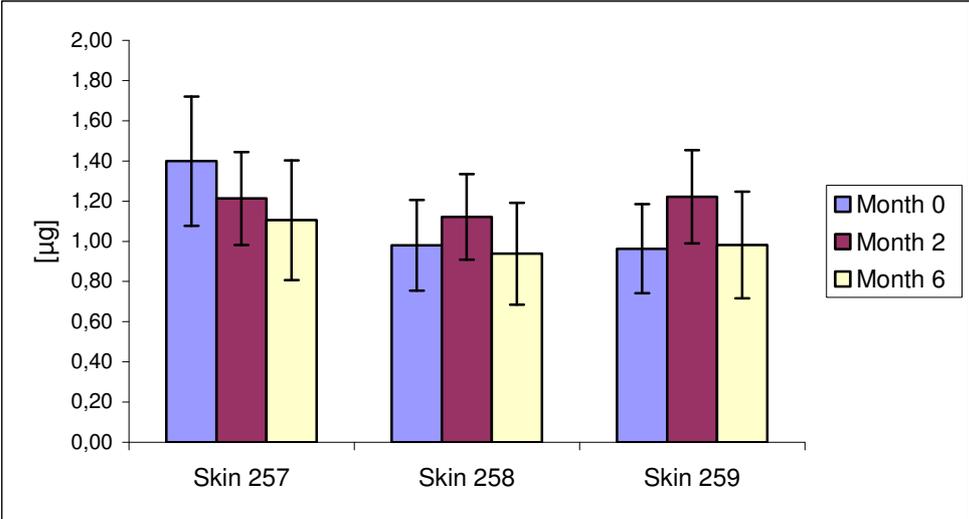The findings reported by Harrison et al. [5] supported the observations of Bronaugh.
The lipid content is critical for the barrier function of the skin. So far nobody has investigated an eventual influence of storage time in cryoconservation on the lipid content of excised human skin.
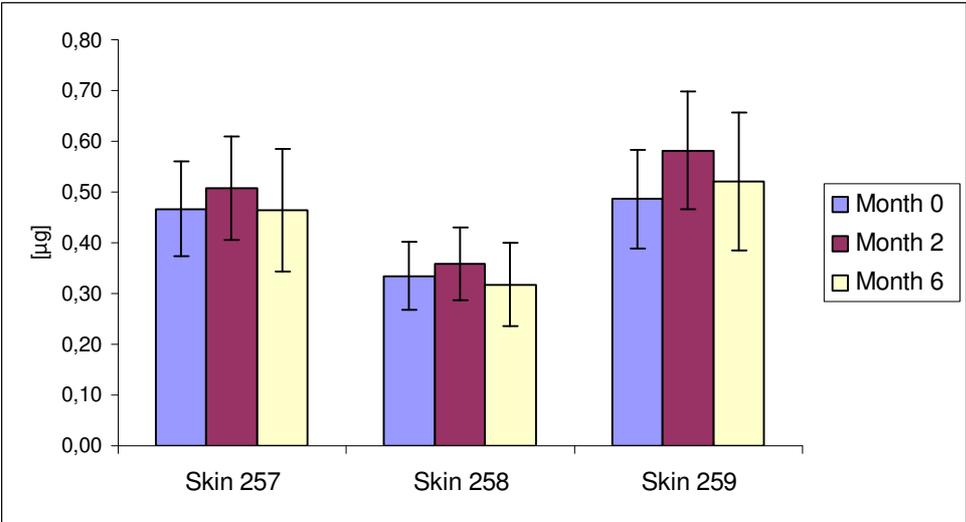Freshly excised human skin was prepared according to method described in the Material and Methods sections. After one day, two and six months three samples were drawn and the lipid content assayed.
The amounts of cholesterol, cholesterolester and ceramides remained stable over the whole experimental period. No increase or decrease can be observed aside from random variation (Figure 7-11). Since only one sample per timepoint was tested the standard error was added as error bar.
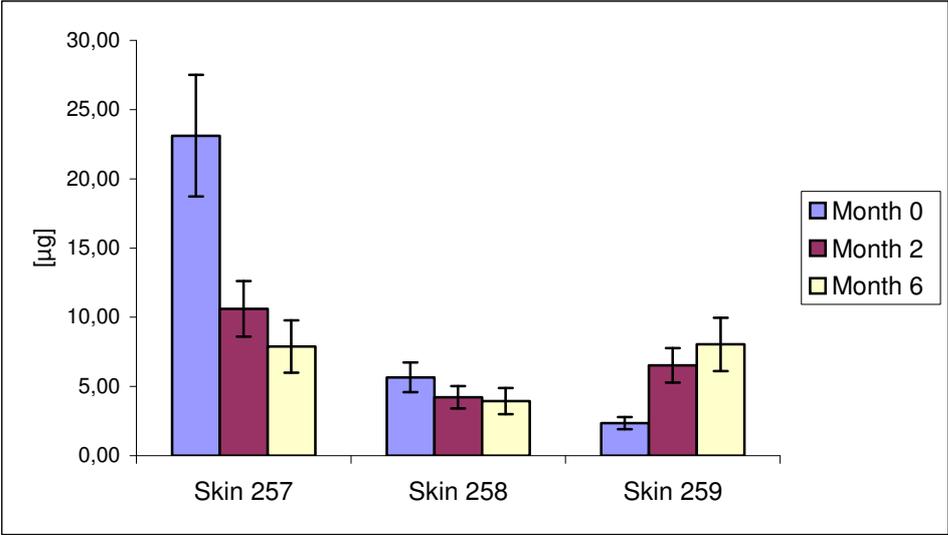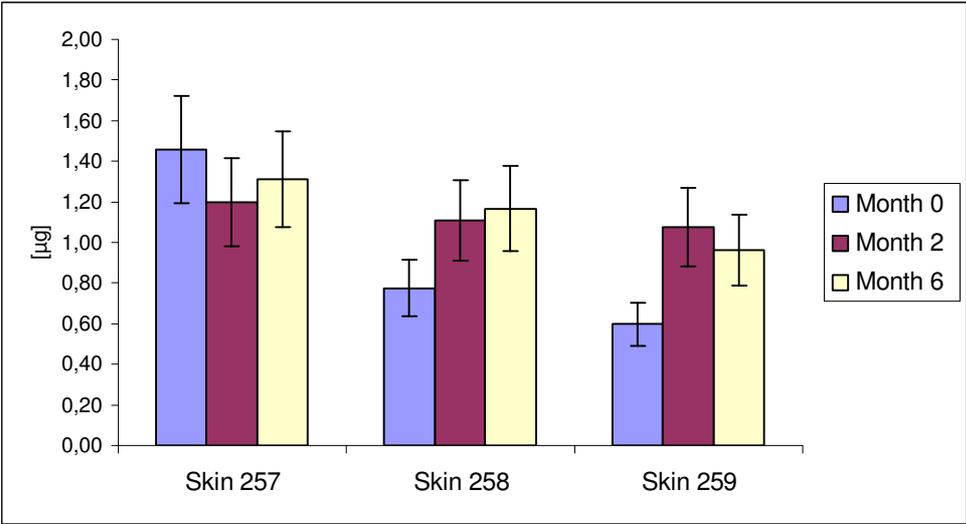
Figure 3: Cholesterol amounts

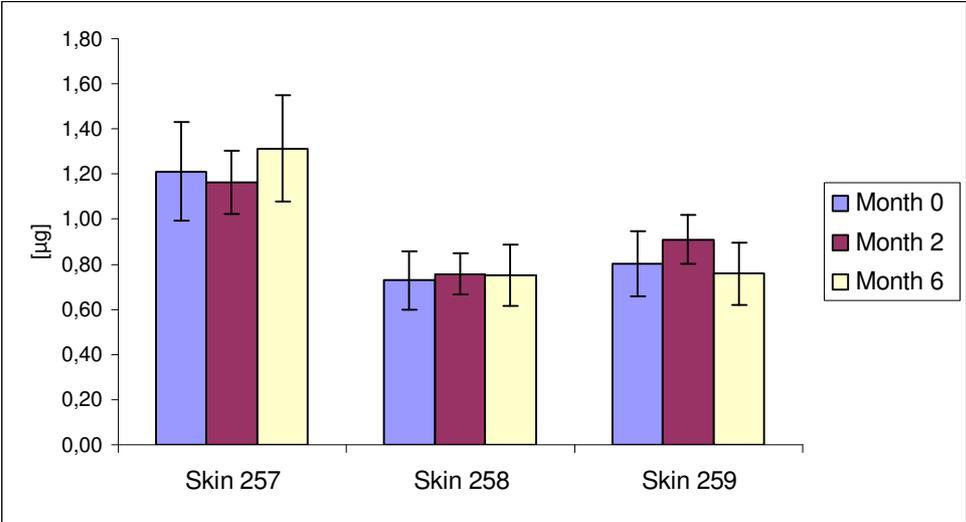**Figure 4: Cholesterolester amounts**

Figure 5: Triglyceride amounts

Figure 6: Fatty acids amounts

Figure 7: Ceramides amounts

Since the values for triglycerides are influenced by the contamination they have been excluded from the observation. An influence of cryoconservation on the content of the examined five lipid classes can not be shown. The variations seen in the bar charts are due to random sampling variation. These results reinforce the hypothesis of predecessor's work that cryoconserved human skin maintains a lipid profile comparable to fresh human skin for at least six months.

3. Influence of the heat separation technique on triglyceride contamination of human skin

Heat separation has first been described by Kligman 1963 [6]. The basic principle is simple: Full thickness skin is placed in water at a temperature of 60°C during 90 seconds. The stratum corneum and the viable epidermis are then removed gently with tweezers.
While performing heat separation in water one can see fatty substances swimming on the surface of the water. The question arose if the contamination encountered in the epidermis sheets could occur during the heat separation process. To investigate this question twelve samples of skin of the same donor were compared using two different heat separation techniques. The first one was Kligman's technique described earlier. The second method consisted in placing a punched out skin disk epidermis side down on a filter paper disk soaked in 60°C hot water. The paper and the skin were then transferred on a heating plate which was at a temperature of 60°C for 90 seconds and then peeled of with tweezers. The advantage of this technique is that topically adhering contamination on the dermis side of the skin can not reach the stratum corneum.

The lipid profiles of both groups were assayed and then compared using a t-test for indepent samples. Performing two different treatments on one subject is the typical prerequisite which allows using a paired sample t-test. This test offers the advantage of necessitating only half the number of observations a t-test for independent samples would need [7] reducing greatly the sampling error

Table 2: Paired samples statistic

		Mean [% of dry weight]	N	Std. Deviation	Std. Error Mean
Pair 1	Cholesterol1	2,1800	6	,84330	,34428
	Cholesterol2	1,8633	6	,52959	,21620
Pair 2	Cholesterolester1	,5900	6	,08485	,03464
	Cholesterolester2	,5767	6	,07367	,03007
Pair 3	Fattyacids1	1,4600	6	,37443	,15286
	Fattyacids2	1,5183	6	,38165	,15581
Pair 4	Triglycerides1	7,4517	6	1,98388	2,1800
	Triglycerides2	7,3750	6	2,69571	1,10052
Pair 5	Ceramides1	,5183	6	,09621	,03928
	Ceramides2	,4700	6	,07797	,03183

Table 3 : Results of paired t-test

		Paired Differences			95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
		Mean	SD	SE	Lower	Upper			
Pair 1	Cholesterol1 - Cholesterol2	,31667	1,27676	,52123	-1,02321	1,65654	,608	5	,570
Pair 2	Cholesterolester 1 - Cholesterolester 2	,01333	,13292	,05426	-,12615	,15282	,246	5	,816
Pair 3	Fattyacids1 - Fattyacids2	-,05833	,71435	,29163	-,80800	,69133	-,200	5	,849
Pair 4	Triglycerides1 - Triglycerides2	,07667	3,48629	1,42327	-3,58197	3,73530	,054	5	,959
Pair 5	Ceramides1 - Ceramides2	,04833	,12513	,05108	-,08298	,17965	,946	5	,388

The tables above show the result of the statistical analysis. There is no statistically significant difference between the means of both groups. The heat separation technique has no influence on the contamination level of human skin.

4. Influence of the sampling method

Two sampling methods are used currently for Franz cell experiments.
The first method is shown below.

The cell is turned for 90° and a 1 ml sample is taken with a Pasteur pipette. It had to be decided which method would be used for the diffusion experiments.
In the second method the cell is not moved. A sample size of 0,4 ml is take in the lateral branch of the Franz cell. It can be taken out without introducing an air bubble by using a syringue.
Both methods have their advantages and disadvantages:
The advantage of the first method is that a large sample can be taken. With a Pasteur pipette the content of the acceptor can be mixed thoroughly. However this method shows several disadvantages: The donor compartment of the cell must be closed in a perfectly tight manner. Otherwise donor liquid can be spilled during sampling. The second disadvantage is that the diffusion process is interrupted during sampling. Another problem is that the donor liquid comes into contact with the closing material. It has therefore to be shown for every substance investigated that the material used for closing the cell does not interact with the donor liquid - a tedious and time consuming work.Taking these reasons into consideration it seems safer to use a method which avoids moving the cell.
A diffusion experiment with methylene blue comparing both experiments was conducted.

Table 4: Results of methylene blue experiments

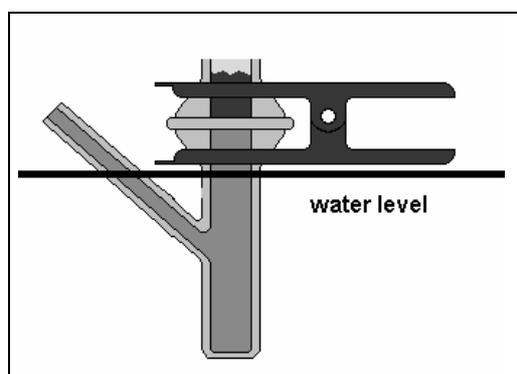|	P_{app} [cm/s] mean $\pm$ SD x 10^{-5}
Sampling (old method; n=3)	$5,90 \pm 0,12$
Sampling (new method; n=3)	$6,1 \pm 0,23$

No difference could be found between both sampling methods (t-test; $P < 0,05$). Since the sampling method with the syringue avoids many of the aforementioned problems and is much more convenient to use it was adopted for the experiment.

5. Influence of water bath vs cabinet drier

The passive diffusion of xenobiotics and therefore their skin absorbtion is affected by temperature. The diffusion chamber and skin should therefore be maintained at a constant temperature close to the normal skin temperature of 32 ± 1 °C [8]. Two possibilities were given in this laboratory for heating the experimental setup: The water bath and a cabinet drier. The problem here is to check the actual temperature of the membrane. A contactless method for checking the temperature was not suited for the Franz cell setup. The optical density of heat separated human skin was not high enough for the measuring beam. Further, the instrument needed a minimum measuring area which was superior to the area covered by the smallest skin model in this work, the Episkin model.

In the waterbath the Franz cell is placed in water which has a temperature of 32 ± 1 °C in the way indicated in the figure below. It is important that water level does not reach the junction donor acceptor. The problem here is that above the water level the cell is exposed to ambient conditions. The temperature in the acceptor was measured above water level since is the zone which through contact directly influences the skin temperature.

Figure 8: Franz cell in water bath

In the waterbath the donor is exposed to the ambient temperature which changes from season to season. Even if the effect is minimal it would be better for the sake of standardization to have everything at the same temperature.

For the measurements of donor temperature cells were mounted (n=6) with a dialysis membrane and filled with PBS. Donor volume was 1 ml.

For measurement of the acceptor only the donor of the cell was filled and no dyalisis membrane inserted.

Table 5: Mean temperature of donor and acceptor with different experimental setups

	Temperature [°C] $\pm$ SD
Donor waterbath	29 ± 1
Donor cabinet drier	32 ± 1
Acceptor waterbath	30 ± 1
Acceptor cabinet drier	32 ± 1

The results in table 17 show that neither the temperature of the donor nor the temperatur of the acceptor are at 32 ± 1 °C in the waterbath.

The results of the cabinet drier show that both donor and acceptor reach the required temperature. By carefully regulating the waterbath temperature it is also possible to reach the required temperatures in donor and acceptor. However this result can be achieved easier with the cabinet drier.

The experimental setup has therefore been moved to the cabinet drier. An eventual influence on the transport was not investigated.

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Summaries and conclusion

Summary and conclusion

Over the last twenty years reconstructed human skin models have undergone continous refinement. From an object of merely academic interest they have become reliable tools in many areas of skin research. As shown earlier in this work [1] there is a rich body of literature covering photoxicity, corrosivity and irritancy testing. However the question remained if these models could be used equally well for permeation testing. This question became the central question of this work:

Are skin models capable of replacing human and animal skin in in vitro permeation experiments?

In research answers are found by making tricky and time-consuming observations and then report what was seen and deduced from it. To be able to make these observations a sound and reliable experimental assay is of paramount importance. Therefore the first step of this work was to develop a flexible experimental assay which allows doing permeation experiments with human, animal and reconstructed skin [2].
A modification for the classical Franz diffusion cell setup first described by Franz [3] was developed which allowed direct comparison with the following human skin preparations: Full thickness skin (mentioned in OECD guidance documents [4, 5]), split thickness skin (mentioned in OECD guidance documents [4, 5]), human heat separated epidermis (mentioned in OECD guidance documents [4, 5]) and trypsin isolated stratum corneum. Experiments with these human skin preparations and the Episkin model were successful in showing on one side the suitability of the experimental assay and giving first experimental data with this model. This result was especially important since permeation data with the Episkin model is scarce. Only one paper has been published on this subject [6]. Judging from literature data of permeation experiments with other skin models of a similar type [6-9] higher permeation rates – in comparision to human skin - were expected. This expectation was confirmed with the Episkin model showing higher permeation rates than its human counterpart.

Some other aspects of the experimental setup were also investigated:
Part of the experimental setup is also the reference material used for these comparative studies which in this work was human skin. Several aspects were investigated:
The influence of cryoconservation on human skin lipids was one part of this work. Here it could be shown that the storage conditions described in the literature [10-13] did not impact negatively on the skin lipids – an aspect which so far nobody had investigated. These results confirmed the findings in the literature that skin conserves its barrier function in cryoconservation for at least six months [10-13]. One important finding was here that cleaning and conservation should take place in a time interval of two hours after surgical extraction. Contamination with triglycerides is a well known problem in skin research [14, 15]. On the matter of contamination the heat separation technique was examined if it had some impact on the contamination levels. Two techniques were compared: On one side the technique described by Kligman [16] which consists of putting punched out skin discs in to 60°C hot water for 90 sec and on the other side a variation of this technique with a heating plate. It could be shown that the hot water technique used so far had no negative impact on the contamination levels of the heat separated epidermis. Since Kligman's technique being faster and easier to use it was chosen as the heat separation technique for this work. With these matters closed the actual doing of the experiments became the focus of interest.

The question arose which was the best method for maintaining a constant temperature throughout the experiments. Two methods can be used to achieve the temperatures required by OECD guidelines [4, 5]: The cells can be placed in a water bath or the whole experiment can be performed in a drying cupboard set at constant temperature. It could be shown that basically both setups perform equally well. However the drying cupboard is more convenient to use and was therefore used in this work. The sampling method was also investigated. Sampling with syringes proved to be superior over the other method.

Another important part of the experimental setup is integrity testing as required by OECD guideline 428. Therefore the TEWAMETER which has shown its merits in in vivo integrity tested was a logical choice as a method for in vitro integrity testing.
A model system consisting of a Teflon membrane mounted in Franz diffusion cells filled with buffer was set up [17]. The membrane was used intact and punctured with a needle (up to five holes). After each puncturing the TEWL was measured. It was expected that the TEWL would increase with each additional hole. That hypothesis could not be confirmed: Only the TEWL of intact and punctured

membrane differed significantly regardless of the number of holes. The experiments were then repeated with human. From three donors intact human heat separated epidermis and punctured heat separated epidermis were compared and no significant difference of the TEWL was found. This was particularly annoying since permeation experiments with flufenamic acid showed a significantly higher diffusion rate through punctured HSE. The next step was to see if the TEWAMETER is capable of detecting large deterioration of the skin barrier. Similar experiments have already been reported by Pirot [18]. However Pirot did not correlate the decrease of the barrier function with permeation data. To achieve this objective the TEWL and drug permeation were compared for skin stripped three, seven and 15 times prior to heat separation to an intact control group. Only the TEWL values of intact HSE and HSE stripped 15 times differed significantly confirming the findings of Pirot. Once again no gradual increase could be observed. However, seven and 15 times stripping resulted in significantly higher diffusion rate. The conclusion was that TEWL measurements can detect severe damage of the stratum corneum but not small locally limited changes, which nevertheless may already influence drug diffusion. Therefore, the TEWAMETER is only of limited use as a barrier integrity test for human HSE in in vitro test systems and was no longer used for barrier integrity testing in this work.

The consequences of these findings are to be seen on a larger scale. With these findings one of the three methods suggested by the OECD guidelines [4, 5] has been proven to be unsuited. Unfortunately this was the easiest to use method of the three methods. The remaining methods, measurement of the transepithelial resistance and the permeation of tritium water are both not suited for validation studies.

In both techniques solutions must be added to perform the integrity testing which have to be removed quantitatively before starting the actual permeation experiment. From a regulatory point of view you it is then necessary to prove in a satisfying way that these solutions have no influence on the skin barrier and the outcome of the permeation experiment. This means a great deal of effort has to be invested. The TEWL seemed to be an elegant way for circumventing these problems.
Nevertheless these results will hopefully motivate other researcher to do further research in this area.

With this investigation the methodological part could be closed and the actual comparative permeation study undertaken. As said earlier this investigation was a multicentric effort. The large number of experiments could not be undertaken by one person. Therefore the permeation experiments have been distributed to many partners. The results were then pooled and compared. This is the reason why the results of this work are presented side by side with the results from the partners of the other institutions.

The first comparative permeation study [19] was the comparision of pig skin, bovine udder skin and heat separated human epidermis. This study was undertaken because human skin [20-24] and pig skin (see [25] for review) are already well investigated and plenty of permeation data has been published. Bovine udder skin on the other side has so far been used seldom for permeation experiments [26-31] even if it looks like another promising alternative to human skin. The first step was to compare the histology and the lipid profiles of the investigated skins. The skin of all three species contained the major lipid classes cholesterol, ceramides, cholesterol ester, fatty acids and triglycerides. There were no major morphological differences between the species. Permeation experiments with caffeine, testosterone, flufenamic acid and benzoic acid with all three skin types showed that pig and human skin were equally permeable, while bovine udder skin seemed to exhibit a weaker, but less variable barrier against caffeine testosterone and flufenamic acid. The conclusion was that bovine udder skin might be an alternative for studies of human skin permeation.

The insights gained about the experimental setup and the behaviour of reconstructed human epidermis, animal and human skin were then put to use for the prevalidation study [32]:
The prevalidation study comprised the transfer and practical testing of the experimental setup using reconstructed human epidermis. The permeation of the OECD standard compounds, caffeine and testosterone, through commercially available reconstructed human epidermis models was compared to that of human epidermis, animal skin – pig skin and cow udder skin - and reconstructed human epidermis. In comparison to human epidermis, the permeation of the chemicals was overestimated when using reconstructed human epidermis. A closer look to the results of the permeation experiments showed the following ranking for testosterone: Skinethic being the most permeable model followed by Epiderm, Episkin, human epidermis, bovine udder skin and finally pig skin. The rank order for caffeine was slightly different: SkinEthic being once again the most permeable model followed by Episkin, bovine udder skin, EpiDerm, pig skin, and human epidermis.

These findings confirmed the findings of the earlier mentioned experiments with the Episkin model [2] and literature data with the other models (see [1] for review). The reconstructed human epidermis models were much more permeable than human and animal skin used in this investigation.

The sobering conclusion is that reconstructed human epidermis models are still far away from being an alternative to human and animal skin in permeation studies. Their biggest limitation - their relatively weak barrier function - has so far not been improved. This is bad news for the defenders of animal rights who hoped that reconstructed human skin model might be the long sought alternative to animal experiments. However, as stated by the Research Defence Society (www.rds-online.org.uk) "by any common-sense definition, the word 'alternative' suggests a choice between two or more options" reconstructed human skin models can so far not be considered as a reliable option in permeation studies.

Human skin and animal skin will thus remain the most important tools of in vitro permeations studies for the next years.

Zusammenfassung

Das Ziel dieser Arbeit war es die Barrierefunktion (Durchlässigkeit) rekonstruierter humaner Hautmodelle mit menschlicher und tierischer Haut zu vergleichen, um zu sehen, ob sie als Ersatz für diese Häute im Rahmen von Permeationsuntersuchungen benutzt werden können. Das Vorgehen, um diese Frage zu beantworten lässt sich in zwei Abschnitte unterteilen. Im ersten Abschnitt wurden Arbeiten am experimentellen Ansatz durchgeführt während im zweiten Teil die eigentlichen vergleichenden Studien durchgeführt wurden, in deren Rahmen diese verschiedenen Häute miteinander verglichen werden.

Die Arbeiten am experimentellen Ansatz umfassten unter anderem Einflüsse der Lagerzeit, Präparationstechnik , Temperierung und des Probenzugs. Ein besonders wichtiger Punkt bei der Entwicklung des experimentellen Ansatzes war die Erprobung einer Methode für die Durchführung von Integritätstests.

Hierfür wurde das TEWAMETER ausgewählt. Die durchgeführten Untersuchungen zeigten, dass das TEWAMETER zwar in der Lage ist grossflächige, schwere Beschädigungen aufzuspüren aber kleine Löcher in der Haut nicht entdecken konnte.

Aufgrund dieser Einschränkung wurde das TEWAMETER im Rahmen dieser Arbeit nicht mehr verwendet und es wurde ohne Integritätstest weitergearbeitet. Mit dieser Untersuchung konnte der methodologische Teil zur Entwicklung des experimentellen Ansatzes abgeschlossen werden.

Nach Klärung der den experimentellen Ansatz betreffenden Fragen konnten vergleichende Permeationsstudien mit humaner, tierischer und künstlicher Haut durchgeführt werden

In der ersten dieser Studien musste zuerst die Frage geklärt werden, welche Zubereitung menschlicher Haut am besten für Permeationsexperimente geeignet ist. Versuche mit menschlicher Vollhaut, dermatomisierter Haut, hitzeseparierter humaner Epidermis und trypsinisoliertem Stratum Corneum zeigten, dass hitzeseparierte Epidermis für diese Experimente am besten geeignet ist.

Im Anschluss daran wurden mit hitzesparierter Haut und dem künstlichen Hautmodell Episkin von L'Oréal vergleichende Experimente durchgeführt, bei denen sich Episkin als deutlich permeabler als die menschliche Haut herausstellte.

Porcine, bovine und humane Haut wurden in der nächsten Studie miteinander verglichen. Diese Untersuchung wurde durchgeführt, da für humane Haut und porcine Epidermis bereits umfangreiche Untersuchungen über ihre Eignung für Permeationsstudien durchgeführt worden waren. Die bovine Euterspalthaut allerdings war bisher nur selten untersucht worden.

Zuerst wurde der allgemeine Aufbau der Häute miteinander verglichen, wobei grosse Übereinstimmungen gefunden wurden. Im Anschluss daran durchgeführte Permeationsexperimente mit Koffein, Flufenaminsäure, Benzoesäure und Testosterone zeigten bei humaner und porciner Haut ähnliche Ergebnisse, während Kuheuterhaut durchwegs permeabler war. Kuheuterhaut zeigte allerdings eine geringere Variabilität als die beiden anderen Hauttypen. Da Kuheuterhaut zudem als Schlachtabfall leicht erhältlich ist, macht dies diese Haut zu einer interessanten Alternative zur humanen Haut in Permeationsstudien.

Die oben beschriebenen Erkenntnisse flossen in einer multizentrischen Prävalidierungsstudie zusammen. Unter standardisierten Bedingungen mit rekonstruierten Epidermen, humaner, boviner und porciner Haut durchgeführt. Die rekonstruierten Epidermen erwiesen sich wiederum als deutlich durchlässiger als humane und tierische Häute.

Die Schlussfolgerung aus diesen Untersuchungen ist, dass rekonstruierte humane Epidermen nur sehr eingeschränkt als Alternative zur humanen und tierischen Haut gesehen werden können.

Humane und tierische Haut werden deshalb in der nächsten Zukunft das Mittel der Wahl für Permeationsstudien bleiben.

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Materials and Methods

1. Skin preparations

1.1 Human skin

Excised human skin from female Caucasian patients, who had undergone abdominal plastic surgery, was used. The approvals from the ethic committee of the 'Caritas-Traegergesellschaft Trier e.V.' were available for the institute of biopharmaceutics pharmaceutical tecnology. For further details see Wagner [1, 2]

1.2 Full thickness skin

After excision surgical extraction the subcutaneous fatty tissue was removed and the remaining tissue stored at -26°C. Previous studies had shown that the skin is stable over 3 and 6 month [3]. This has been confirmed by other laboratories [4, 5]. For further details see Wagner et al. [6].

1.3 Human heat separated epidermis

Skin discs with a diameter of 25 mm were punched out and thawed prior to putting them in water at 60°C for 1 min. Afterwards the SC-epidermis layer of the skin was peeled off from the dermis using forceps [7]. The epidermis sheets were spread out in Petri dishes filled with PBS for at least 30 min. For further details see Wagner et al. [6].

1.4 Punctured human heat separated epidermis

Before heat separation the skin disks were punctured with a needle with a diameter of 1 mm in the center of the diffusion area in order to simulate damage sustained during surgical extraction or preparation. After heat separation the presence of the hole was checked with a magnifying glass.

1.5 Stripped human heat separated epidermis

Tape stripping has often been used to induce barrier disruption on human skin [8-10] and was therefore used in this investigation to inflict varying, clearly defined damage on the stratum corneum barrier. The skin was stripped prior to heat separation. The numbers of adhesive tapes used were fixed to three, seven and fifteen; the stripping procedure is described by Wagner [6]. Stripping more than fifteen times could not be performed due to the high fragility of the resulting HSE sheet.

1.6 Human split thickness skin

Skin sections with a thickness of 500 ± 100 µm were prepared from the thawed (full-thickness) skin samples using an Aesculap GA 630 dermatome (Aesculap AG, Tuttlingen, Germany).

1.7 Animal skins: bovine and porcine skin

As the presence of a dense pelage makes the preparation technique susceptible to produce leaks, it is uncommon to use the heat separation technique with animal skins. For this reason split thickness animal skin was used as recommended by the OECD [11]. Udder skin samples were obtained from healthy lactating Holstein Frisian cows that were slaughtered according to legal requirements. Only slightly pigmented organs were chosen. Skin flaps of about 10 x 20 cm were taken from the lateral udder wall 5 cm dorsal of the hairless teat skin. Porcine skin samples were taken from animals that were sacrificed for other purposes at the University of Medicine, Hanover. The sampling area was the lateral abdominal region. Each flap was cleaned with water and stored at –20°C until use. From the flaps, split skin samples with a thickness of 1000 µm were prepared using a dermatome (Aesculap) which was controlled using a pocket thickness gage (Model 7309, Mitutuyu Corp, Tokyo, Japan). Samples with deviations of more than 100 µm were omitted. For histological examinations, cross sections of bovine udder skin from 3 individuals were prepared from tissue samples, which were

embedded in TissueTec® (Miles Inc, Elkhart, IN, USA) and stained with hemotoxilin and eosin. By use of a microscope the epidermal thickness and the penetration depth of at least 24 follicles was measured for each skin. For both porcine and bovine skin, the number of follicles per cm² was counted using a grid and a magnifying glass after clipping of the hairs.

2. Buffers

All buffer salts were of analytical grade (Merck, Darmstadt, Germany).

Isotonic PBS buffer pH 7,4

KCl	0,2g
NaCl	8,0g
KH_2PO_4	0,2g
Na_2HPO_4 x $2H_2O$	1,44g
or Na_2HPO_4	1,1486g
in aqua bidest. ad	1l

Sörensen phosphate buffer solution (PBS) pH 7,4

K_2HPO_4	2,0g
Na_2HPO_4 x $2H_2O$	9,2g
in aqua bidest. ad	1l

McIlvaine buffer pH 2,2

Citric acid	20,8g
Na_2HPO_4 x $2H_2O$	0,4g
in aqua bidest. ad	1l

Buffer pH 2,6:

phosphoric acid	1,16 ml
KH_2PO_4	4,08 g
in aqua bidest. ad	1 l

3. Test compounds

Name	Manufacturer	Molecular weight [g/mol]	$logK_{ow}$	pKa	Reference
4-Androsten-17ß-ol-3-on (Testosterone)	Sigma-Aldrich, Deisenhofen, Germany	288,4	3,48	-	[12]
Benzoic acid	Sigma-Aldrich, Deisenhofen, Germany	122,1	1,9	4,2	[12]
Caffeine	Sigma-Aldrich, Deisenhofen, Germany	194,2	-0,08	1,4	[12]
Flufenamic acid	Sigma-Aldrich, Deisenhofen, Germany	281	4,88	7,5	[13]

4. Donor solutions

Name	Concentration [µg/ml]	solvent
Testosterone	40	Isotonic PBS buffer pH 7,4 with 2% Nonidet
Benzoic acid	1000	Isotonic PBS buffer pH 7,4
Caffeine	1000	Isotonic PBS buffer pH 7,4
Flufenamic acid	1000; 750; 1250	Isotonic PBS buffer pH 7,4 or Sörensen phosphate buffer

5. HPLC system

Pump	Dionex P580 Pump (Dionex GmbH, Idstein, Germany)
Autosampler	Dionex ASJ 100 automated sample injector (Dionex GmbH, Idstein, Germany)
Detector	UVD 170S detector (Dionex GmbH, Idstein, Germany)
Column oven	Dionex STH 585 column oven (Dionex GmbH, Idstein, Germany)
Software	chromeleon 6.50 SP2 build 9.68 (Dionex GmbH, Idstein, Germany)

5.1 Quantification of Flufenamic acid (SOP AL-HPLC_FLU_US02)

- Flufenamic acid (Sigma-Aldrich, Deisenhofen, Germany)

- Aqua bidest (Millipore Milli Q Synthesis)

- mobile phase flufenamic acid: 80: 20 (v/v) methanol : McIlvaine buffer pH 2,2

- Methanol (Lichrosolv, Merck)

5.1.1 HPLC Parameter

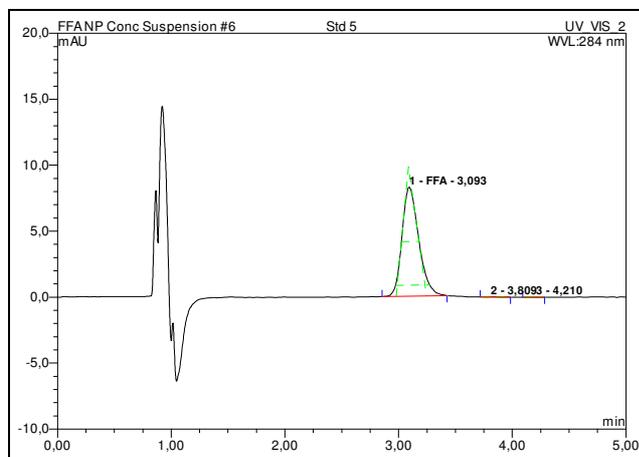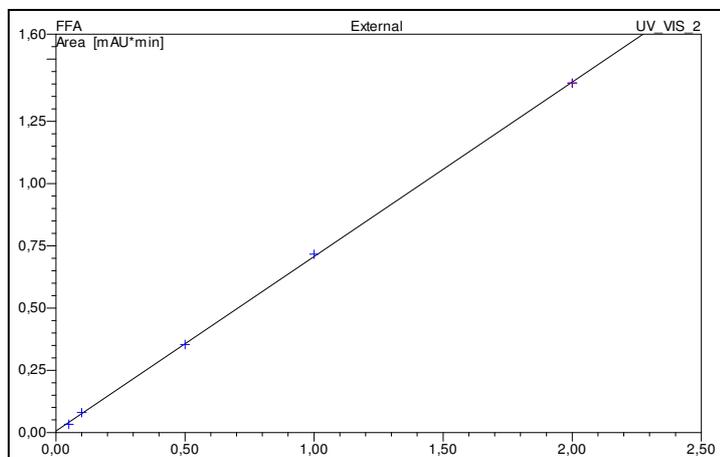| mobile phase	80:20 (v/v) Methanol : McIlvaine buffer pH 2,2
Flow rate	1,2 ml/min
Detection wave length	284 nm
Injection volumen	50 µl
Stationary phase	LiChrospher® 100/RP-18 column/12.5 cm×4 mm (Merck–Hitachi, Darmstadt,Germany)
Precolumn	Lichrocart 4 – 4; LiChrospher® 100/RP-18 column (5 µm) (Merck–Hitachi, Darmstadt,Germany)

5.1.2 Standards

The standards are prepared as follows:
Stock solution: 12,5 mg of flufenamic acid ad 100,0 ml (Sörensen buffer)

Standard	Vol. stock	ad (ml)
50 ng/ml	10 µl	25,0
125 ng/ml	25 µl	25,0
250 ng/ml	50 µl	25,0
500 ng/ml	100 µl	25,0
1000 ng/ml	200 µl	25,0

5.1.3 Regression

Retention time: 3,09 min +/- 0,2 min
Correlation coefficient r: min 0,995

Standard chromatogram shown above.

No.	Ret.Time min	Peak Name	Cal.Type	Points	Corr.Coeff %	Offset	Slope	Curve
1	3,09	FFA	LOff	5	99,9926	0,0051	0,7013	0,000
Aver.					99,9926	0,0017	0,9004	0,000

Quantification according to external standard method (y=ax+b)

5.1.4 Stability of samples

Samples are stable for 48 hours in at 6°C.

5.2 Quantification of Caffeine (SOP AL_HPLC_CO_US02)

5.2.1 Chemicals

• Caffeine (Sigma-Aldrich, Deisenhofen, Germany)

• Aqua bidest (Millipore Milli Q Synthesis, Heidelberg, Germany)

• Acetonitril HPLC grade (Merck, Darmstadt, Germany)

5.2.2 HPLC Parameter

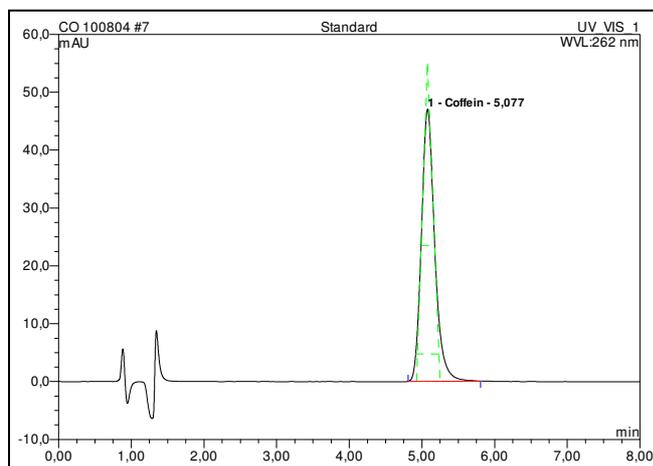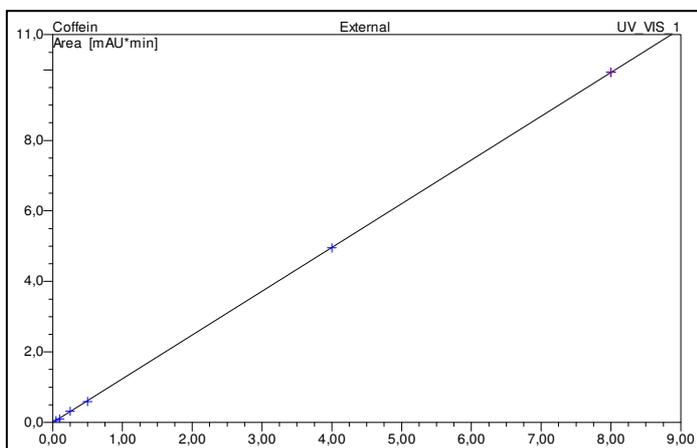| mobile phase	90 : 10 (v/v) buffer pH 2,6 : Acetonitrile
Flow rate	1,2 ml/min
Detection wave length	262 nm
Injection volumen	50 µl
Stationary phase	LiChrospher® 100/RP-18 column/12.5 cm×4 mm (Merck–Hitachi, Darmstadt, Germany)
Precolumn	Lichrocart 4 – 4; LiChrospher® 100/RP-18 column (5 µm)

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5.2.3 Standards

Stock solution: 12,5 mg Caffeine ad 100,0 ml Messkolben (PBS buffer)

Standard	Vol. Stammlösg.	ad (ml)
50 ng/ml	10 µl	25,0
100 ng/ml	20 µl	25,0
250 ng/ml	50 µl	25,0
500 ng/ml	100 µl	25,0
4000 ng/ml	800 µl	25,0
8000 ng/ml	1600 µl	25,0

5.2.4 Regression

Retention time: 5,08 +/- 0,2 min
Correlation coefficient r: min 0,995

Standard chromatogram shown above.

No.	Ret.Time min	Peak Name	Cal.Type	Points	Corr.Coeff. %	Offset	Slope	Curve
1	5,08	Caffeine	Lin	6	99,9995	0,0000	1,2405	0,0000
Aver.					99,9995	0,0000	1,2405	0,0000

Quantification according to external standard method (y=ax+b)

5.2.5 Stability of samples

Samples are stable for 48 hours in at 6°C.

5.3 Quantification of Testosterone (SOP AL_HPLC_TE_US02)

5.3.1 Chemicals

- 4-Androsten-17ß-ol-3-on (Testosteron) (Sigma-Aldrich, Deisenhofen, Germany)
- Igepal CA 630 (Sigma-Aldrich, Deisenhofen, Germany)
- aqua bidest (Millipore Milli Q synthesis)
- Acetonitril HPLC grade (Merck, Darmstadt, Germany)
- Methanol (Merck,Darmstadt, Germany)

5.3.2 HPLC Parameter

mobile phase	Methanol/ Wasser (v/v); 70:30
Flow rate	1,2 ml/min
Detection wave length	250 nm
Injection volumen	50 µl
Stationary phase	LiChrospher® 100/RP-18 column/12.5 cm×4 mm (Merck–Hitachi, Darmstadt)
Precolumn	Lichrocart 4 – 4; LiChrospher® 100/RP-18 column (5 µm)

5.3.3 Standards

The standards are prepared as follows:
Stock solution: 12,5 mg testosterone ad 100,0 ml Messkolben (Methanol water mixture 70:30).

Standard	Vol. Stammlösg.	ad (ml)
50 ng/ml	10 µl	25,0
125 ng/ml	25 µl	25,0
250 ng/ml	50 µl	25,0
500 ng/ml	100 µl	25,0
1000 ng/ml	200 µl	25,0
5000 ng/ml	1000 µl	25,0

5.3.4 Regression

Retention time: 4,8 min +/- 0,2 min
Correlation coefficien r: min 0,995

Coefficients:
b[0] 26,6102061509
b[1] 591,8620861004
r² 1

No.	Ret.Time min	Peak Name	Cal.Type	Points	Corr.Coeff. %	Offset	Slope	Curve
1	4,79	Testosterone	Lin	6	99,9995	0,0000	1,15	0,0000
Aver.:					99,9995	0,0000	1,15	0,0000

Quantification according to external standard method (y=ax+b)

5.3.5 Stability of samples

Samples are stable for 24 h at 6°C.

5.4 Quantification of benzoic acid (SOP AL_HPLC_BA_US01)

5.4.1 Materials

- Benzoic acid (Sigma, Deisenhofen,Germany)
- Aqua bidest (Millipore Milli Q Synthesis)
- Acetonitrile HPLC grade (Merck, Darmstadt,Germany)
- mobile Phase: 72: 28 (v/v) buffer pH 2,6 : acetonitrile

5.4.2 HPLC Parameter

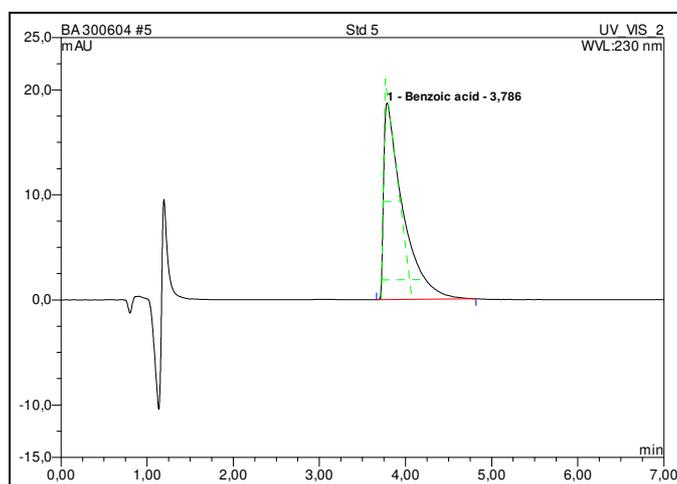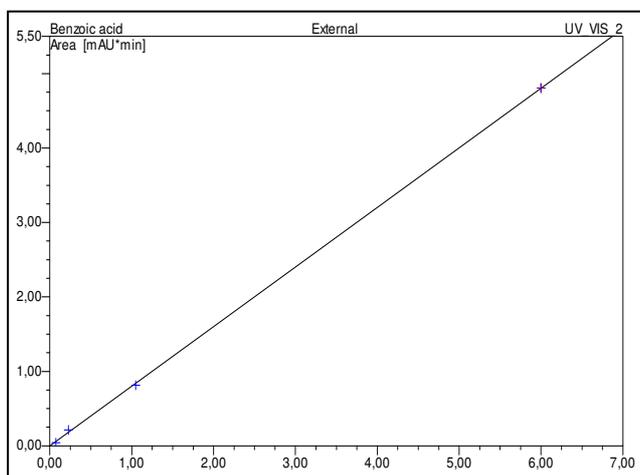| mobile phase	Acetonitrile/ buffer pH 2,6 (v/v); 72:28
Flow rate	1,2 ml/min
Detection wave length	228 nm
Injection volumen	50 µl
Stationary phase	LiChrospher® 100/RP-18 column/12.5 cm×4 mm (Merck–Hitachi, Darmstadt,Germany)
Precolumn	Lichrocart 4 – 4; LiChrospher® 100/RP-18 column (5 µm) (Merck–Hitachi, Darmstadt,Germany)

5.4.3 Standards

The standards are prepared as follows:
Stock solutions: 12,5 mg benzoic acid ad 100,0 (aqua bidest).

Standard	Vol. stock sol.	ad (ml)
50 ng/ml	10 µl	25,0
150 ng/ml	30 µl	25,0
500 ng/ml	100 µl	25,0
1000 ng/ml	200 µl	25,0
4000 ng/ml	800 µl	25,0

5.4.4 Regression

Retention time: 3,79 min +/- 0,2 min
Correlation coefficient r: min 0,995

No.	Ret.Time min	Peak Name	Cal.Type	Points	Corr.Coeff. %	Offset	Slope	Curve
1	3,79	Benzoic acid	Lin	4	99,9933	0,0000	0,8004	0,0000
Average:					99,9933	0,0000	0,8004	0,0000

Quantification according to external standard method (y=ax+b)

5.4.5 Stability of samples

Samples containing benzoic acid are to be analysed immediately.

6. Improved method for analysing skin lipids (SOP AL_HPTLC_SL_US02)

The method for the analysis of skin lipids presented here is based on the work of Hauck and Theobald [14-16] and includes improvement added by Phil Wertz [17]. The method was further improved to be able to analyse cow udder skin.

6.1 Materials

6.1.1 Chemicals

Diethylether	p.a.		Merck/Darmstadt,Germany
n-Hexan	p.a.		Merck/Darmstadt,Germany
acetic acid 100%	p.a.		Merck/Darmstadt,Germany
Methanol		Lichrosolv	Merck/Darmstadt,Germany
Chloroforme		Lichrosolv	Merck/Darmstadt,Germany
Petrolether	p.a.		Merck/Darmstadt,Germany
Isopropanol	p.a.		Merck/Darmstadt,Germany
Ceramide III			Sigma/Deisenhofen,Germany
Ceramide IV			Sigma/Deisenhofen,Germany
Triolein			Sigma/Deisenhofen,Germany
Oleic acid			Sigma/Deisenhofen,Germany
Cholesterol			Sigma/Deisenhofen,Germany
Cholesterol ester			Sigma/Deisenhofen,Germany
HPTLC plates, silicate 60 Merck Nr. 105641			Merck/Darmstadt,Germany
Fat free cotton			

6.1.2 Mobile phases

Mobile phase 1
Chloroforme / methanol / acetic acid (100%) (190 : 9 : 1, V:V:V)

Mobile phase 2
n-Hexane / diethylether / acetic acid (100%) (80 : 20 : 10, V:V:V)

Mobile phase 3
a.) n-Hexane / Diethylether (80 : 20, V:V)   (10 cm)
b.) Petrolether   (15 cm)

6.1.3 Derivatising agent

Copper sulfate pentahydrate	10,0 g
Phosphoric acid (85%)	10,0 g
dem. Water	80,0 g

6.2 General

The plates are to be processed with chloroforme/methanol (2:1) overnight in order to remove contamination from the plates. Then, the plates are heated at 110°C in a cabinet drier for 30 min. The plates can then be stored in an exsiccator.

6.3 Samples

- Heat separated human epidermis
- Reconstructed human epidermis
- Pig skin
- Cow udder skin

These types of samples have usually an overall lipid content of 5 – 10 %. Thus, the minimum sample size should be 15 – 20 mg (dry weight).

6.3.1 Preparation of samples

The samples are freeze dried, weighed and transferred to tight sealing containers. 5,0 ml chloroforme-methanol mixture (2:1 v/v) are added and left for extraction overnight with occasional stirring. The following day the solution is filtered through fat free cotton into a preweighed container. The solvent is removed with a stream of liquid nitrogen. The remaining lipids are to be weighed.
The remaining extract is dissolved in a chloroforme-methanol mixture (2:1 v/v) (100 µl for approximately 5 mg extract).

6.3.2 Standards

Cholesterol, oleic acid, cholesterol ester, triolein and the ceramides can be processed on one plate pairwise. Therefore it is possible to store them as one solution.
There are 6 standard stock solutions with following concentrations:

(A)   Three container with each 15 mg substance in 5 ml CHCl$_3$/MeOH (2:1, V:V; 3 µg/µl):

      - Cholesterol + oleic acid   (15 mg + 15 mg)
      - Cholesterol ester + Triolein   (15 mg + 15 mg)
      - Ceramide-III + Ceramide-IV   (15 mg + 15 mg)

(B)   Three container with each 70 mg substance in 5 ml CHCl$_3$/MeOH (2:1, V:V; 14 µg/µl):

      - Cholesterol + oleic acid   (70 mg + 70 mg)
      - Cholesterol ester + Triolein   (70 mg + 70 mg)
      - Ceramide-III + Ceramide-IV   (70 mg + 70 mg)

Starting with these three solutions (A) additional dilution are to be prepared (1:2 (1,5 µg/µl) and 1:4 (0,75 µg/µl)).
With the remaining solutions (B) and additional dilution of 1:2 (7 µg/µl) is prepared.

6.4 Analysis

Five standard dilutions are put on the HPTLC plate (1µl each) together with 1 µl of up to 4 samples totalling nine lanes. Ceramide III and V (MP 1), cholesterol and oleic acid (MP 2), and triolein and cholesterol ester (MP 3) can eache be processed on one plate. After processing the plates have to be dried for 15 min at room temperature.

6.5 Charring the plates

The dried plates are then sprayed with the coppersulfate/phosphoric acid mixture described earlier and placed on a heating plate. The temperature of the heating plate is gradually increased starting from 110°C to 160°C.
Then plates are removed from the heating and left for 20 min in a dark place.

6.6 Scanning the plates

As soon as the plates reach room temperature the plates are scanned with an flatbed desktop scanner (150 dpi).

6.7 Quantification

The resulting TIFF file is uploaded to the TNIMAGE (GNU public license, Thomas J. Nelson) program. There it is evaluated according to the external standard method. It is preferable to use the UNIX version of the program which is more powerful. The method strip densitometry is to be used. With this method all spots with the same rf-value can be quantified.
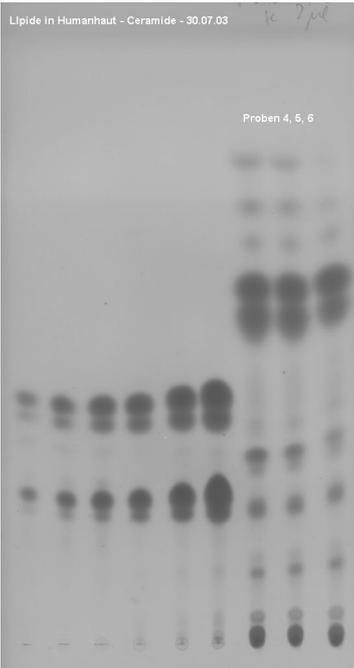Final correction (baseline adjustement) can be added manually. After that the peak area can be calculated manually.
Using the areas of the standard an exponential regression is calculated ($y = a * e^{b*x}$). With the resulting equation the lipid contents of the samples are calculated.
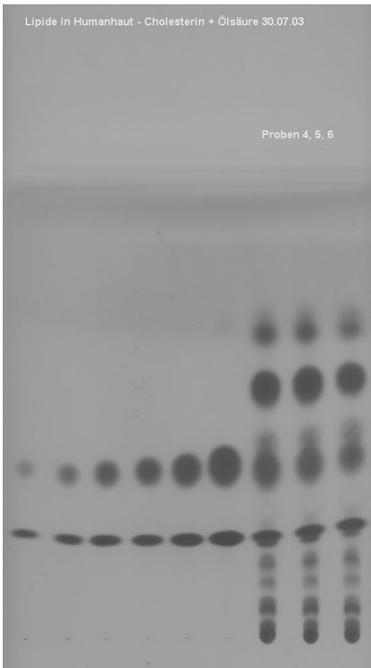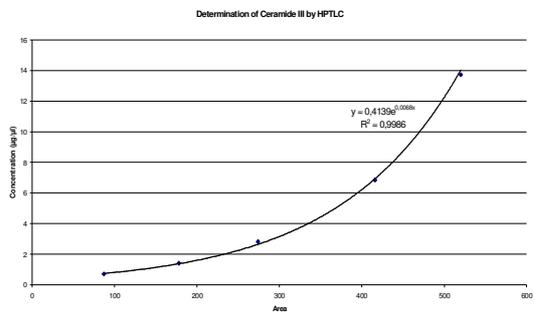
7. Examples

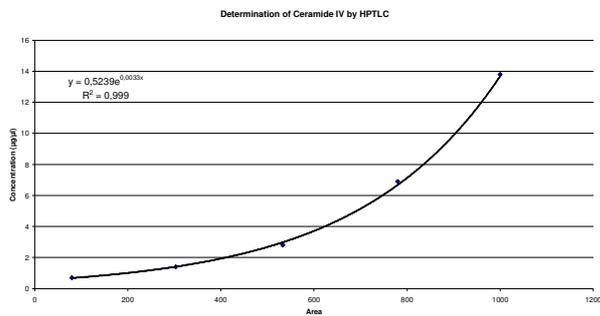Quantification of triglycerides (lower spots) and cholesterol ester (top)

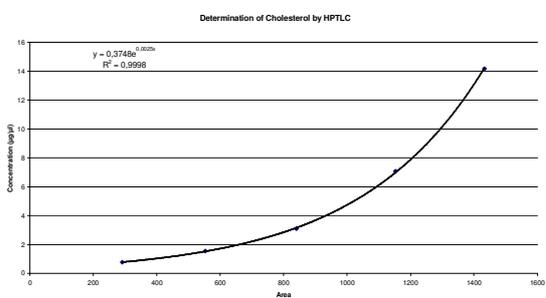Quantification of ceramide III (top) and ceramide IV (lower spots)

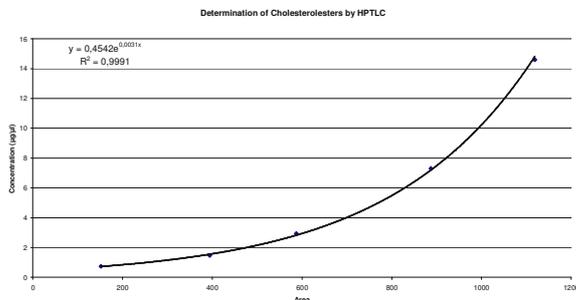Quantification of cholesterol (lower spots) and oleic acid (top)

Determination of Ceramide III by HPTLC
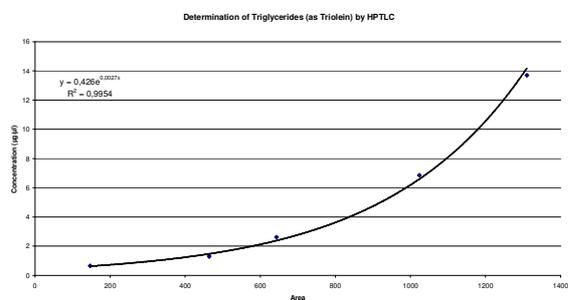
$y = 0{,}4139e^{0{,}0068x}$
$R^2 = 0{,}9986$

example: regression ceramide-III

Determination of Ceramide IV by HPTLC

$y = 0{,}5239e^{0{,}0033x}$
$R^2 = 0{,}999$

example:regression ceramide-IV

Determination of Cholesterol by HPTLC

$y = 0{,}3748e^{0{,}0025x}$
$R^2 = 0{,}9998$

example: regression cholesterol

Determination of Cholesterolesters by HPTLC

$y = 0{,}4542e^{0{,}0031x}$
$R^2 = 0{,}9991$

example: regression chol.l ester

Determination of Triglycerides (as Triolein) by HPTLC

$y = 0{,}426e^{0{,}0027x}$
$R^2 = 0{,}9954$

example: regression triolein

Determination of Oleic Acid by HPTLC

$y = 0{,}3498e^{0{,}0033x}$
$R^2 = 0{,}9998$

example: regression ol. acid

Abbreviations

Abbreviation	Explanation
HPTLC	**High performance thin layer chromatography**
IL	**Interleukin**
LDH	**lactate dehydrogenase**
mRNA	**Messenger RNA**
MTT	**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide**
SC	**Stratum corneum**
SG	**Stratum granulosum**
SKALP	**Skin derived anti-leukoproteinase**
SLS	**Sodium laurylsulfate**
SPRR	**small proline rich-protein**
TLC	**Thin layer chromatography**
UVA	**Ultraviolett light class A**
o/w	**Oil/water**
w/o/w	**Water/oil/water**
HSE	**heat separated epidermis**
FDC	**Franz type diffusion cell**
FFA	**Flufenamic acid**
PBS	**Phosphate buffer solution**
SC	**Stratum corneum**
TEWL	**Transepidermal water loss**
TISC	**Trypsin isolated stratum corneum**
FTS	**Full Thickness Skin**

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Acknowledgements