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T. Hahn, U.F. Schaefer, C.M. Lehr:

Measuring Skin Absorption in Vitro

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Measuring Skin Absorption In Vitro

Introduction

To measure skin absortion *in vitro*, different setups and skin from different species (*e.g.* human or animal skin) preparations may be used, depending on the scientific question under investigation. Moreover, there are different ways to prepare the tissue, to conduct the actual experiment, and also to present and to analyse the obtained data, *e.g.* either to provide information on skin permeation or penetration.
While human skin is obviously still regarded as a »gold standard«, animal skin or even biotechnologically reconstructed skin equivalents may be used as well. As setups, the Franz diffusion cell as a static cell and the flow-through cell as a dynamic diffusion cell are both commonly known and applied in many groups. For penetration experiments, the specially designed Saarbrücken model is also discussed. Treatment of the obtained data is summarized for permeation and for penetration experiments to elucidate the potential of both methods.

Introduction

Quantitative data on the bioavailability of compounds applied to the skin are of increasing interest. This does not hold only for the pharmaceutical, but also for the cosmetic industry. Apart from studying efficacy, the rate and extent of dermal and transdermal absorption is most relevant also for the safety assessment for all kinds of xenobiotics, such as *e.g.* sun blocker, pesticides etc. In fact, such information is explicitely requested by the EU initiative »REACh - Registration, Evaluation, Authorisation and Restriction of Chemicals For ethical reasons, skin absorption data can not easily be obtained by conducting *in vivo* studies on humans or on animals. Besides, since 2009 *in vivo* animal testing of cosmetical products is banned by EU regulation 76/768/EEC throughout the European Union (1). Therefore, other techniques must be used to obtain the desired information. One possibility is the use of *in vitro* penetration and permeation models. General instructions concerning skin absorption studies are provided by the OECD Guidline 428 (2) combined with Guidance 28 (3), Scientific Committee on Cosmetic Products and Non-Food Products (4), by the European Commision (5), and by the Food and Drug Adminstration of the United States of America (6). However, in all of these documents detailed information on practically suitable methods is missing.
To study and describe skin absorption, two different cases have to be distinguished: *penetration* and *permeation*. Penetration refers to the absorption of a substance by the different layers of the skin itself, which is important *e.g.* for locally acting drugs and most cosmetic products. Permeation on the other hand is defined as the amount of substance, which is transported across the skin and thus may become systemically bioavail-

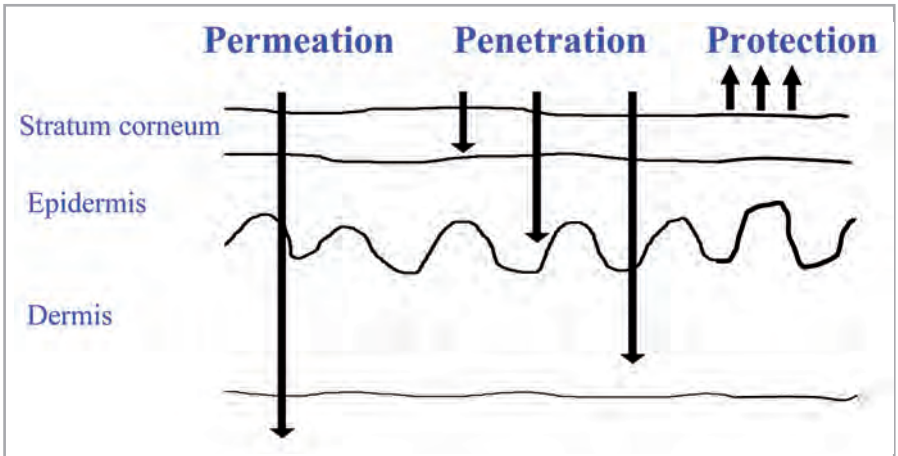

Fig. 1 Different cases of skin absorption: permeation, penetration, or just protection

able. This aspect is most relevant in the context of transdermal therapeutic systems, but also to assess systemic toxicity of compounds applied to the skin. Apart from that, there are also substances, which are intended not to penetrate or permeate, but to solely stay on the skin's surface in order to provide for its *protection*, such as *e.g.* sunscreens. **Fig. 1** illustrates the differences between these three terms.

This review focuses on tissue based *in vitro* models for skin absorption, neglecting skin flap models and perfused skin models. The different skin layers suitable for absorption experiments, ranging from full-thickness skin over dermatomed skin, heat-separated epidermis and *stratum corneum* are discussed. Intentionally, release studies with artificial membranes are omitted, because normally no information about the absorption of a substance into the skin is provided by those experiments. Moreover it is the intention of the authors to carefully distinguish between permeation and penetration models. Among the permeation models the differences of static diffusion cells, *e.g.* the Franz diffusion cell and the flow-through diffusion cell, *e.g.* the Bronaugh cell, will be discussed. Furthermore, as a suitable model for penetration experiments the so-called Saarbrücken penetration model is described and compared to the permeation models. Besides, a short introduction in data analysis of permeation and penetration experiments is provided. The information presented here should help the reader in identifying the model best suited to gather the needed information.

■ Absorption Pathways of the Skin

When a substance is applied to intact skin different absorption pathways are available.

Along the skin appendages

The appendages route consists of the glandular and the follicular pathways, of which the latter one appears to be more important. Recent studies report that follicular route may be of particular relevance to liposomes and nanoparticles (7, 8).

Across the intact *stratum corneum*

Intercellular route

The intercellular route is considered as the predominant one for most compounds. In this case, substance transport occurs within the bilayer-structured, continuous intercellular lipid domain of the *stratum corneum*. Although this pathway is very tortuous and, therefore much longer in distance than the overall thickness of the *stratum corneum*, the intercellular route is considered to yield much faster absorption due to the higher diffusion coefficient of most drugs within the lipid bilayer compared to the hydrophilic corneocytes.

Transcellular route

Under normal conditions, the transcellular route is considered as of minor importance for dermal absorption. The reason is the very low permeability through the corneocytes and the obligation to partition several times from the more hydrophilic corneocytes into the intercellular lipid layers of the *stratum corneum* and vice versa. The transcellular pathway may, however, gain importance when some penetration enhancer, *e.g.* urea, is present, which increases the permeability of the corneocytes by altering the keratin structure.

■ *In Vitro* Skin Absorption Experiments vs *In Vivo*

Clinical studies, i.e. *in vivo* experiments on humans, will always remain the »gold standard« to proof the safety and efficacy of drugs in (trans)dermal therapy. However, considering ethic aspects as well as high costs of clinical stuides, pre-clinical *in vitro* skin absorption models are still very valuable as development tools and frequently used for such purposes. Moreover, *in vitro* experiments also have certain advantages over *in vivo* studies (3, 9):

- conditions can be controlled more easily;
- normally easier to perform;
- also radiolabelled or highly toxic chemicals can be investigated;
- a large number of experiments can be run simultaneously;
- single parameters (*e.g.* release of the drug from different vehicles, influence of moisture, temperature, pre-treatment) can be studied more readily;
- the amount of penetrating substance can be measured directly in the skin or in an acceptor medium beneath the skin without dilution in tissue fluids or organs.

However, there is also a risk to observe some artifacts which has to be born in mind when carrying out *in vitro* studies:

- the acceptor medium may influence the transfer of substance through the skin or the condition of the skin itself;
- the absence of blood flow may lead to changes of the barrier properties of the different skin layers;
- exploring excised skin specimens, the tissue can only be maintained unchanged for a relatively short period of time;
- *in vivo* conditions cannot be fully simulated *in vitro* (*e.g.* the shedding of the *stratum corneum*, muscular tonus);
- metabolic effects are difficult to address.

■ Tissues Used for *In Vitro* Skin Absorption Experiments (Membranes)

As recommended by the ECVAM (10), the physiological hierarchy of methods as shown in **Fig. 2** for measuring percutaneous absorption is generally accepted. Obviously, human skin is preferred for all *in vitro* skin experiments, as animal skin often leads to false prediction of skin absorption. However, sources of human skin are limited and therefore alternatives have to be discussed. Due to its high similarity to human skin, both in morphology and permeability, pig skin is a practical alternative. Skin of other animal species should only be used when the method is fully validated. For comparison of relative permeabilities of different formulations, animal skin, preferably from hairless strains, might be useful.

However, the results have to be confirmed by relevant additional experiments. Human skin is usually provided from plastic surgery, amputations or cadavers. While for *in vivo* studies the volar forearm is often used due to its good accessibility, *in vitro* skin sources are mostly abdominal or breast skin. The skin can be used either directly after excision or after storage in a freezer. The advantage of fresh skin is that a certain metabolic activity can be maintained during the experiments, which is essential when skin absorption is influenced by active metabolic processes. However, it is generally assumed that skin absorption is a passive diffusion process and the absorption barrier is situated in the *stratum corneum*, which is no longer metabolically active. Therefore, for most skin absorption experiments formerly frozen skin can be used.

After excision, the skin should quickly be freed from subcutaneous fatty tissue and stored deep frozen in tightly sealed plastic bags at -20 °C to -30 °C until use. Storage for up to several months without impairing the barrier function is possible, assuming that repeated thawing and freezing is avoided (12, 13). Furthermore, the absence of dermal blood flow *in vitro* may build up a significant hindrance to diffusion (14). Reducing the membrane thickness will generally reduce the run time of an experiment and thus minimize the risk of microbial contamination.

Full-thickness skin **(Fig. 3a)** is often used for penetration experiments, as information about all skin layers can be gained. However, full-thickness skin is often not appropriate for permeation experiments, as the permeated amount is quite low and diffusion resistance of the dermis over predicted (15).

In that case, *full-thickness skin* can be treated by a dermatome, which cuts a layer of the skin of a certain thickness. *Dermatomed skin or split skin* **(Fig. 3b)**, which comprises of epidermis including *stratum corneum* and parts of the dermis. Dermatomed skin is often used in a thickness between 200 to 500 µm (3, 15). Although the dermatome will cut through hair follicles, the holes will readily close during incubation in aqueous media, due to swelling of the tissue.

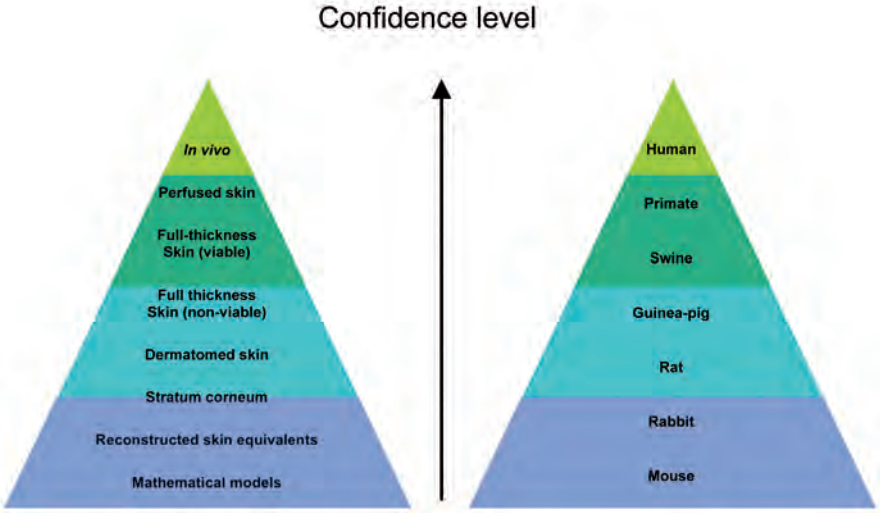

Fig. 2 Membrane hierarchy and skin donors recommended by ECVAM (10), according to (11)

Heat-separated epidermis **(Fig. 3c)** can be prepared by complete removal of the dermis by several mechanical, thermal and chemical techniques. Most commonly, the epidermal-dermal junction is split by heating to 60 °C for 30-120 seconds (16). *Pitman* et al. could show that such a treatment does not impair the barrier function (17).

Finally, the *stratum corneum* **(Fig. 3d)** may be isolated by enzymatic digestion of the connective epidermal tissue. This may be achieved by complete immersion of full-thickness skin in a trypsin soution buffered at pH 7.4 or by placing heat-separated epidermis on a filter paper soaked with the enzyme preparation for 24 h at 37 °C (16, 18).

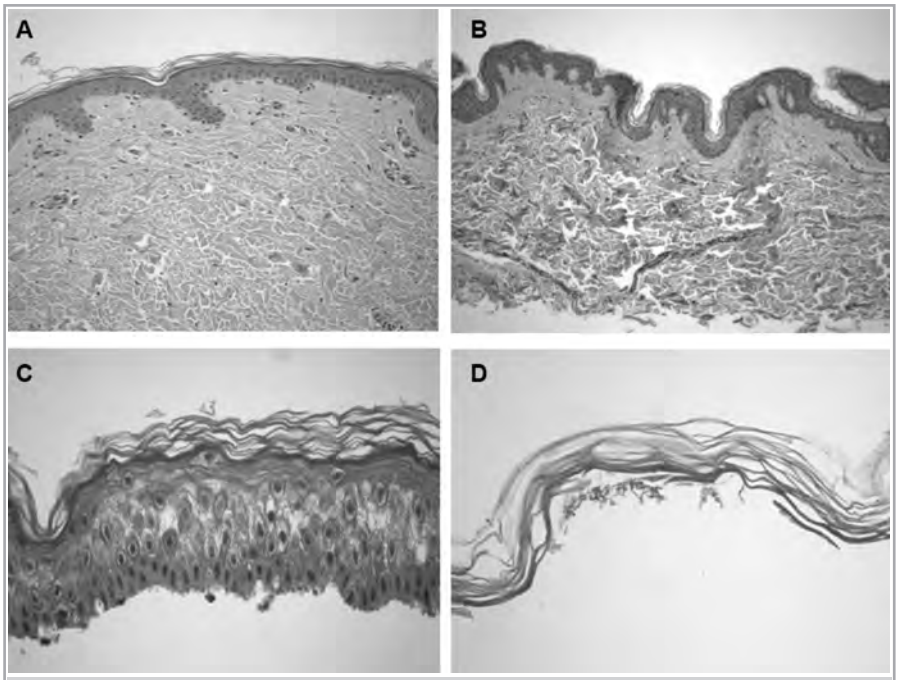

Fig. 3 Different human skin membranes for absorption studies. **A** full-thickness skin, **B** dermatomed skin, **C** heat separated epidermis, **D** enzyme split *stratum corneum* according to (11)

Due to the limited availability of human skin, there has been an increasing demand for bioengineered human skin equivalents, of which several have become commercially available and been evaluated for their use as *in vitro* dermal absorption models. For more information on this topic, the reader is refered to *e.g. Netzlaff* et al. (19). However, a first validation study has revealed that bioengineered human skin equivalents have a significantly lower barrier function than natural human skin and thus a much higher permeability for the compounds tested (20). Nevertheless, bioengineered skin epidermis equivalents have been shown to be suitable for corrosivity and phototoxicity testing (19).

In Vitro Skin Permeation Models

For years, researchers have used variations of two-compartment *in vitro* test systems to measure the diffusion of a compound from one side of a membrane/ skin to the other. Both static, as well as dynamic diffusion cells are approved by the authorities (3, 10), and data is available on their relevance for predicting the *in vivo* situation (18, 21-23). Basically, a donor and an acceptor compartment are separated by a membrane of either native skin, specific skin layers or some artificial equivalents. Sampling from the acceptor compartment, usually a physiological buffer solution of pH 7.4, is performed either continuously or at pre-determined time intervals.

Dosing is possible in infinite (typically > 10 µl/cm² or 10 mg/cm²) or finite manner (< 10 µl/cm² or 10 mg/cm²). Using infinite dosing, a maximum amount of drug is offered to the skin. Such conditions ensure a very reproducible exposure and steady state flux in the skin layers is expected after a certain period of time. Therefore permeability can normally be calculated based on first Fickian's law.

In the finite dose regime, only a limited amount of the drug preparation is applied to the skin's surface so that some special effects, like the influence of evaporation of excipients, can be observed. The application of a finite dose best resembles the *in vivo* situation when applying *e.g.* an ointment. Moreover, a mass balance by determination of drug amount on top of the skin, inside the skin and permeated through the skin can be determined at the end of the experiment.

Control of temperature may be provided by a water jacket around each permeation cell, an external water bath or by warm air in a drying oven. Experiments should be carried out in a way that surface temperature is 32 °C mimicking the *in vivo* situation.

Whether a static or flow-through diffusion cell is more suitable for the aimed information has to be decided beforehand. In the following section, one static diffusion cell, the Franz diffusion cell, and one dynamic diffusion cell, the flow-through diffusion cell, are introduced.

Franz Diffusion Cell

Today, the most widely accepted permeation test system is the Franz diffusion cell (FD-C) (24, 25). The FD-C consists of two compartments **(Fig. 4)**: a donor compartment and an acceptor compartment with the appropriate membrane sandwiched between these two compartments. Suitable membranes are described before under 'Tissues Used for *In Vitro* Skin Absorption Experiments'. The donor compartment holds the drug preparation. An ointment as well as a solution or even a patch can be investigated. The chamber beneath the membrane/skin holds an acceptor fluid, which is continuously mixed with a magnetic stirrer. To simplify data analysis, often experiments are run under sink conditions. These are generally maintained as long as not more than 10% of the compound has been transported to the acceptor compartment or, vice versa, more than 90% of the compound are still in the donor compartment (10).

Fig. 4 Franz Diffusion Cell. Images courtesy of Mr. *Pütz*, Saarland University

The FD-C combines many advantages: It is an inert, robust instrument, which is easy to handle. It is maintainable at a constant temperature and has precisely calibrated volumes and diffusion areas, and it facilitates easy sampling and replenishment of the acceptor medium.

The permeation behavior of a substance through the membrane can be determined by sampling at defined time points. Aliquots of the receptor fluid are removed through a side arm for analysis to determine rate and extent of transdermal absorption. After removing a probe from the sampling arm, the volume is replaced by fresh medium.

The donor chamber may either be left open or be occluded. Non-occluded conditions permit an exchange with the environment, such as evaporation of volatile substances and drying of the skin surface. In contrast, a tight occlusion of the skin surface may lead to excessive skin hydration.

An often encountered problem of this setup is to realize sink conditions for low soluble substances in aqueous media. For such purposes organic solvents, non-ionic surfactants, proteins, *e.g.* cyclodextrine or BSA are sometimes being added as solubility enhancers (3). It is, however, important to keep in mind that these additives may have a severe impact on skin integrity and barrier function and therefore the interpretation of such data has to be done very cautiously.

Typical data of a permeation experiment with infinite dosing are shown in **Fig. 5**. Based on these data, an apparent permeation coefficient and lag time can be calculated based on first Fickian's law. Further information is provided in (26).

In addition to the determination of the permeation through a particular biological membrane, a mass balance can be made at the end of the experiments by

determining the drug amount on top of the skin, inside the skin and permeated through the skin. Furthermore, skin segmentation comparable to that described under '*In Vitro* Skin Penetration Model' can be conducted after taking the last sample from the receptor compartment (18).

Flow-through diffusion cell

In the flow-through diffusion cell the **(Fig. 6)** fresh buffer is continuously pumped through the receptor chamber (27). Thus, sink conditions can be easier maintained throughout the whole experimental period. This is important when the absorption rate of the substance is high and/or solubility in the receptor phase is low. A minimum flow-rate is necessary to ensure thorough mixing and to remove the absorbed material rapidly and to minimize unstirred layers (28). For the majority of substances a higher flow-rate does not change the absorption characteristics, with the exception of very low soluble substances, such as *e.g.* testosterone. Furthermore, the skin viability is prolonged (27, 29) due to constant replacement of the physiological receptor solution. On the other hand, the constant flow of fresh donor below the skin leads to an increased partitioning of hydrophobic compounds from the skin to the acceptor solution. This may change skin properties und therefore influence the permeation process.

In summary, the flow-through diffusion cell allows experiments with a wider range of substances, but requires preliminary experiments to determine the correct flow-rate.

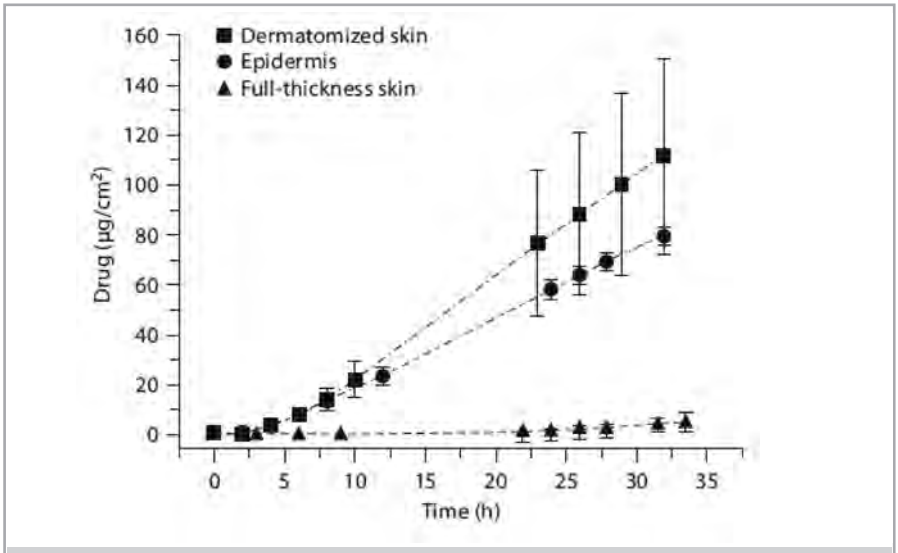

Fig. 5 Graphical representation of data from permeation experiments, according to (15). Cumulative drug amounts Q versus time t for three different barriers using a lipophilic donor

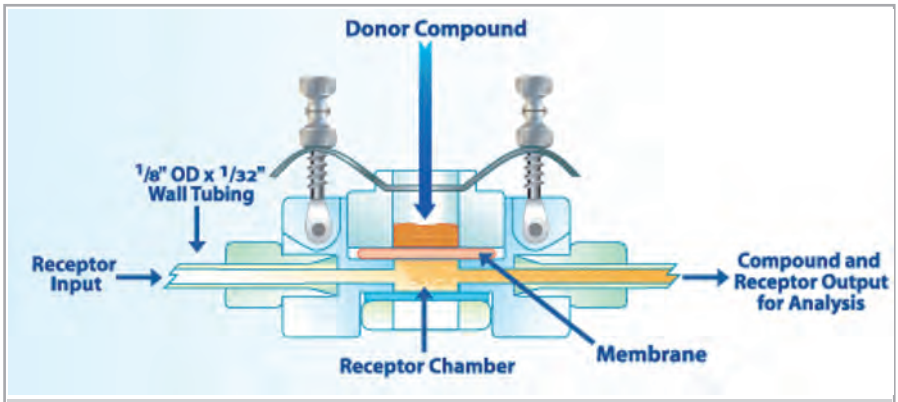

Fig. 6 Flow-through diffusion cell according to Perme Gear: In-line cell http://www.permegear.com/inline.htm

■ *In Vitro* Skin Penetration Model

Saarbrücken penetration model

The Saarbrücken penetration model (SB-M, **Fig. 7 a)** was developed by Prof. *Loth* and his coworkers at the Saarland University (30) to study the rate and the extent of drug penetration into the different skin layers. Here, in contrast to the Franz diffusion cell and the flow-through cell, the skin for itself is acting as the acceptor for the penetrating drug. The major advantage of this setup is that excessive hydration of the skin may be avoided. In addition, possible changes of the skin quality and barrier function caused by the acceptor medium are prevented. Depending on the drug, the incubation time should be limited until drug diffusion reaches the lowermost layer of the skin. Afterwards, sink conditions are not further maintained resulting in complicated kinetics which are difficult to interpret.

In the SB-M the skin is put on a filter paper soaked with Ringer solution and placed into the cavity of the teflon bloc **(Fig. 7b)**. The drug preparation is filled into the cavity of the upper punch resulting in an infinite dosing. Afterwards, the upper part of the SB-M is lowered onto the skin and a weight of 500 g is placed on the upper part of the model for two minutes. Subsequently the upper part is fixed in that position. The weight load is carried out to optimize the contact between the donor and the skin and to mediate reproducible experimental conditions. To avoid water loss from the skin the gap between the two teflon parts is sealed with Plastibase®, a petrolatum like preparation.

After a predetermined incubation period the formulation is removed and the skin surface cleaned with cotton balls. Subsequently the skin layers are separated and the amount of substance in each skin segment is determined by an appropriate method, *e.g.* HPLC.

Fig. 7a Saarbrücken penetration model from the front. Images courtesy of Mr. *Pütz*, Saarland University

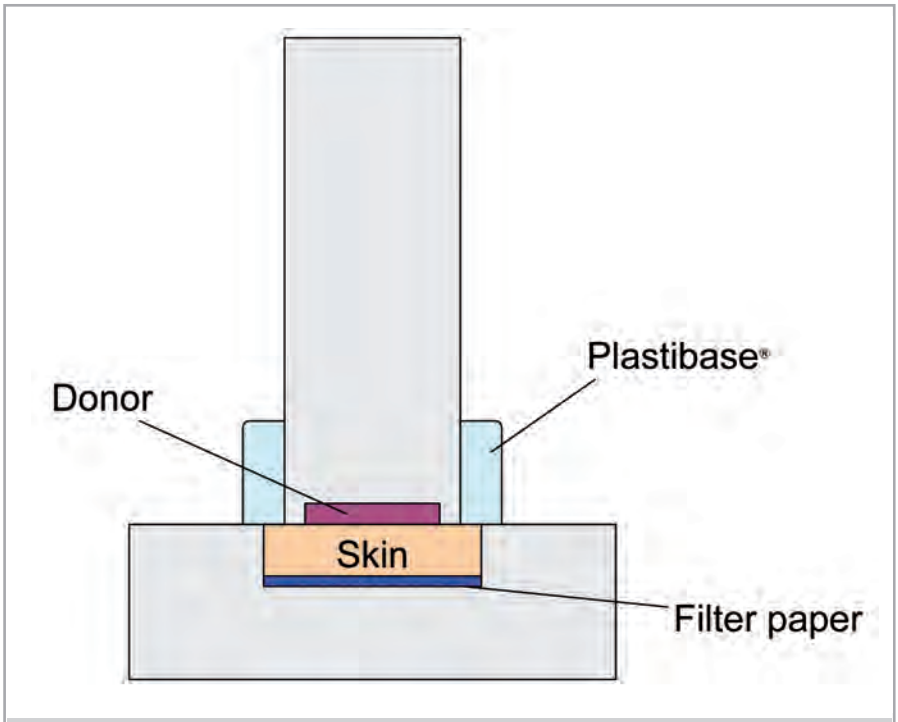

Fig. 7 b Saarbrücken penetration model in a schematic cross-section

Normally, skin segmentation is performed by two consecutive procedures: First by tape-stripping to remove the *stratum corneum* and afterwards by surface parallel cryo-sectioning of the deeper skin layers.

Segmentation of the *Stratum Corneum*

As an example for the segmentation of the *stratum corneum* the highly standardized tape-stripping method established by *Wagner* et al (18) is described. However, other methods are also available (31, 32).

For reproducible stripping the incubated skin specimen is transferred into a special stripping apparatus shown in **Fig. 8**. To avoid the influence of wrinkles the skin is moderately stretched and fixed with small pins on a cork disk. Afterwards a pre-cut tape is placed on the stripping area restricted by a stripping mask made from an inert material. To enhance the contact between tape and the skin surface the tape is charged by a weight of 2 kg for 10 seconds and subsequently the tape is removed in one swift move. For analytical reasons tapes from several strips may be advantageously pooled in corresponding fractions.

Cryo-Segmentation of the Deeper Skin Layers

After removing the *stratum corneum* by tape-stripping and freezing of the skin,

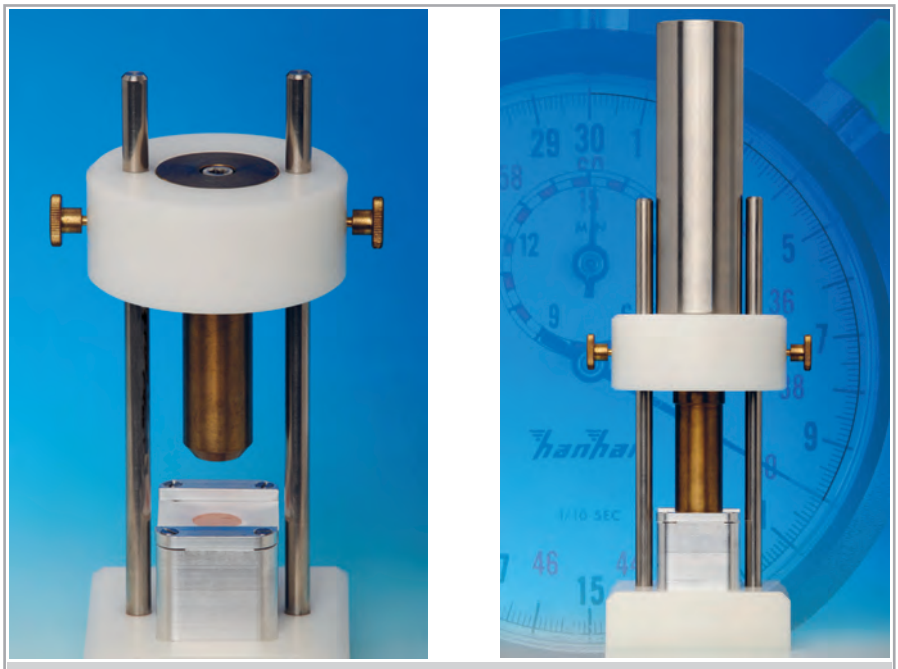

Fig. 8 *In vitro* tape-stripping procedure according to *Wagner* et al. (18). Images courtesy of Mr. *Pütz*, Saarland University

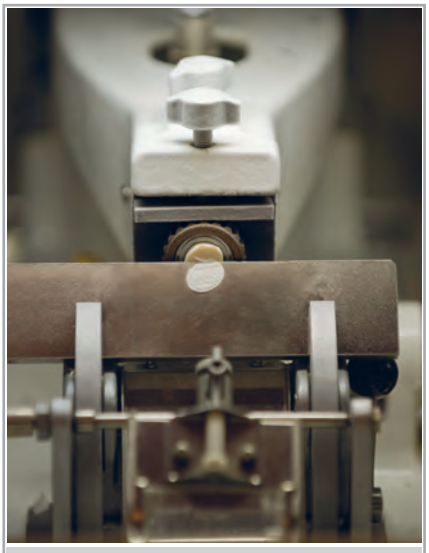

Fig. 9 Cryo-sectioning of the deeper skin layers according to *Wagner* et al. (18). *Step 1*: The knife moves down and cuts a slice of the skin biopsy. *Step 2*: The skin slice is transferred into a test-tube for extraction and quantification. Images courtesy of Mr. *Pütz*, Saarland University

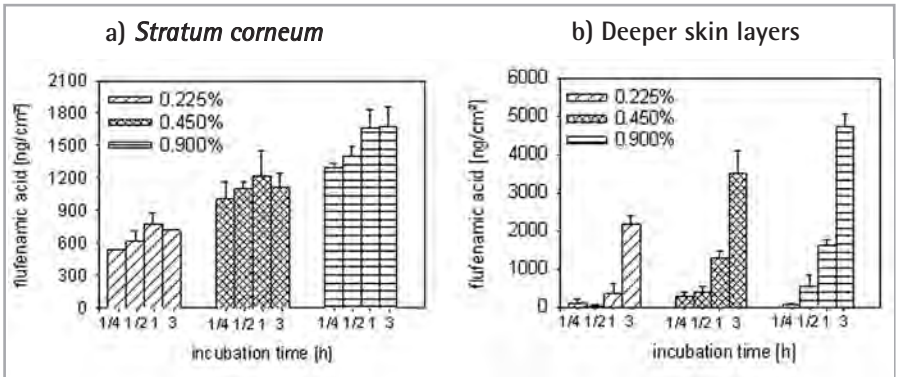

Fig. 10 a Flufenamic acid amount penetrated into the *stratum corneum* and the deeper skin layers. Flufenamic acid was incorporated into wool alcohole ointment in various concentrations according to (33)

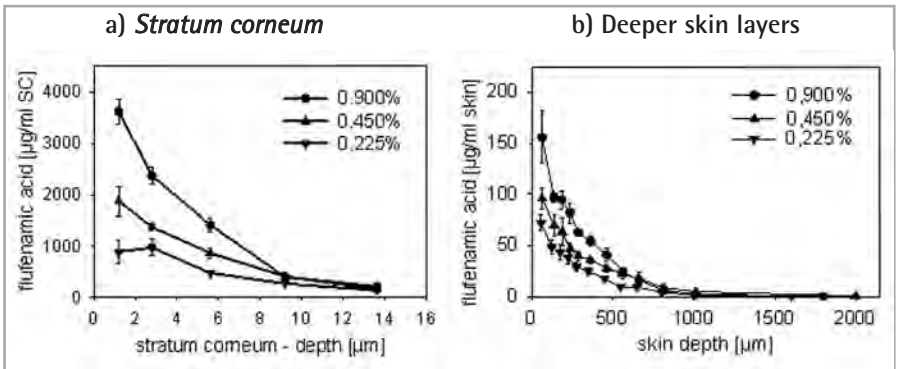

Fig. 10 b Drug concentration - skin depthprofiles of flufenamic acid in the *stratum corneum* and the deeper skin layers. Flufenamic acid was incorporated into wool alcohole ointment in various concentrations according to (33)

a punch biopsy is taken. After thawing, the punch biopsy is placed on a metal holder, frozen onto it by a stream of expanding carbon dioxide, and the bloc is fixed in the cryotome **(Fig. 9)**. Subsequently the punch biopsy is cut into segments parallel to the skin surface. Then, the skin segments are transferred into suitable containers for drug extraction. For analytical reasons the cuts may be pooled in several fractions.

The SB-M is suitable to investigate semisolid preparations, patches and liquids, which have to be soaked into supporting materials, *e.g.* a paper filter. Mostly the experiments are carried out with varying incubation times and infinite dosing to examine the influence of incubation time on drug distribution or the formation of depots.

A typical example of such data is depicted in **Fig. 10 a**, where the cumulative drug amounts in the *stratum corneum* and the deeper skin layers, consisting of the viable epidermis layer and the dermis, are shown for different incubation times. Such data analysis illustrates how long it may take to saturate the *stratum corneum* and to what extent drug invasion into the deeper skin layers is occuring.

In addition, so called drug concentration skin depth profiles can be calculated. Examples are given in **Fig. 10 b**.

This presentation of the data reveals the different absorption rates of a given compound in the different skin layers; eventually accumulation of the drug in certain skin layers may be detected.

More details regarding the the calculation procedures is referred to (26).

■ Permeation Versus Penetration

In a typical Franz-cell experiment only the amount of substance passing the membrane is investigated (*i.e.* skin permeation) and no consideration is given to its temporal and spatial disposition within the tissue (*i.e.* the skin penetration). The advantage of such permeation experiments is the possibility for repeated sampling over a longer time-course. The samples are taken from the acceptor phase without further investigation of the different skin layers. This type of experiment is adequate for many questions, especially the flux of a substance through the skin can be determined. However, no membrane thicker than 1 mm should be used (34). Thus, full-thickness skin is not suitable, but it can be used after dermatomisation or heat-separation. Moreover, at the end of finite dose experiment a mass balance is feasible by determining the drug amounts on top of the skin, inside the skin and permeated through the skin.

In case the disposition of a substance within the skin becomes of interest, concentrations in the different skin layers have to be determined. For this purpose,

segmentation of the skin and analysis of the drug amount is necessary after incubation. Such penetration experiments in general give a broader picture of the substance in the different skin layers, *e.g.* the mass of substance in the *stratum corneum* or in the deeper layers of the skin. Furthermore, concentration-depth profiles can only be created from penetration experiments, after segmenting the different skin layers, *i.e.* by tape-stripping the *stratum corneum* and by cryocutting the deeper skin layers. Thereby, more detailed information is gained aside from the total amount in the complete skin or the permeated drug amount through the membrane. However, penetration experiments are time-consuming and require extensive laboratory work to acquire the separate different skin layers and to extract the substance from each segment for every time point. As for each time point, a different skin specimen has to be used, this approach also needs more skin material.

Whether a permeation or penetration study is better suited mainly depends on the parameters of interest and on the target. References which method and model are most appropriate for favored parameter are provided in **Table 1**.

Assignable parameter	Permeation models	Penetration models
Total substance mass balance	Possible	Possible
Permeated drug amount through the skin	Possible	Not possible
Substance amount in the distinctive skin layers	Normally not addressed	Possible
Concentration-depth profiles	Normally not addressed	Possible
Flux	Possible	Normally not addressed
Lag-time	Possible	Normally not addressed

Table 1 Table of the suitable models for the determination of different absorption parameters

■ Conclusion and Outlook

Detailed information on the skin absorption process, such as *e.g.* drug distribution to the deeper skin layers or enrichment in a distinct skin layer is not readily available from *in vivo* experiments. Although *in vitro*/*in vivo* correlations for the skin are not often presented, experiments have shown that *in vitro* experiments are predictive for *in vivo* skin absorption. *In vitro* permeation and penetration experiments with excised human skin are therefore useful tools to obtain information on drug transport across or into the skin, respectively.

Animal skin can only be used to predict the relative absorption of different formulations, but due to different skin morphology and absorption kinetics, it is not always suitable to predict absorption across human skin. The most suitable substitute might be porcine skin, which both in morphology and absorption characteristics is closest to human skin. Even when using human skin, care has to be taken to choose the appropriate setup and preparation of the tissue, depending on the objectives of the study. Provided these critical points are correctly addressed, however, skin absorption studies *in vitro* may provide a lot of valuable information.

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