Reconstructed Skin Equivalents for Assessing Percutaneous Drug Absorption from Pharmaceutical Formulations

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Summary
Excised human skin has so far been considered to be one of the most suitable in vitro methods to evaluate the penetration of dermatologically applied substances. The limited supply and the relatively high donor variability stimulated many research groups to use animal skin as a substitute for human skin. Since nowadays reconstructed skin equivalents are commercially available, we examined these cultures for their suitability as a percutaneous absorption model for different pharmaceutical formulations.
One such equivalent is EpiDerm™ (EPI-606, MatTek corporation, Ashland Massachusetts) which was investigated using the lipophilic model drug flufenamic acid. Permeation studies with the Franz diffusion cell were undertaken to evaluate the model for the establishment of a new in vitro method to study the percutaneous absorption of different dosage forms. The drug was applied in two pharmaceutical formulations to the intact surface of the skin disk: dissolved in wool alcohol ointment (0.1125%), and dissolved in Soerensen phosphate buffer pH 7.4 (0.1125% solution). HPLC was used for the analysis of drug content. It was shown that the model forms a barrier towards diffusion by comparing the permeation across the tissue-free inserts to the equivalents. Flux values were calculated and the permeation across the skin equivalent from the solution was noted to be almost forty times higher than from the ointment. Two different batches of the skin equivalent showed no statistically significant difference. Finally the permeability of the reconstructed skin was compared to human epidermis, and a five times higher flux value was found for the skin equivalent model.
Our results suggest that reconstructed skin equivalents based on human keratinocytes have potential as a pharmaceutical test system to study dermal drug transport from topical formulations.

Keywords: reconstructed skin equivalent, EpiDerm™, percutaneous absorption, Franz diffusion cell

Zusammenfassung: Rekonstruierte Hautäquivalente zur Ermittlung der perkutanen Resorption aus pharmazeutischen Arzneizubereitungen
Zur Erfassung der perkutanen Resorption wird für in vitro Untersuchungen exzidierte Humanhaut als am besten geeignet angesehen. Da sie jedoch nur in einem sehr beschränkten Umfang zur Verfügung steht und je nach Spender eine hohe Variabilität gegeben ist, wird statt dessen Tierhaut als Alternative eingesetzt. Neuerdings sind jedoch rekonstruierte humane Hautäquivalente käuflich erhältlich. Aus diesem Grunde führten wir mit einem der möglichen rekonstruierten Hautäquivalente, EpiDerm™ (Epi-606; Fa. MatTek, Ashland, MA, USA) für die lipophile Modellsubstanz Flufenaminsäure in zwei verschiedenen pharmazeutischen Zubereitungen in vitro Permeationsstudien in Franz-Diffusionszellen durch, wobei Sörensen Phosphat-Puffer pH 7,4 als Akzeptormedium diente. Als Testzubereitungen wurde Flufenaminsäure, die in einer Konzentration von 0,1125% entweder im Akzeptormedium oder in Wollwachsalkoholsalbe (DAB) gelöst war, auf das Stratum corneum aufgebracht. Die Arzneistoffbestimmung erfolgte mittels HPLC-Analyse.
Durch Vergleich der Permeation über die Stützmembran mit und ohne Zellauflage konnte gezeigt werden, daß EpiDerm™ eine Diffusionsbarriere darstellt. Des weiteren wurde für den Diffusionsfluß aus der Lösung heraus ein um das 40-fache höherer Wert erhalten als für die Salbe gleicher Konzentration. Bei Untersuchungen an zwei verschiedenen Lieferungen von EpiDerm™ konnte kein statistisch signifikanter Unterschied für die Permeation festgestellt werden. Der Vergleich der Permeabilität der rekonstruierten Hautäquivalente zu Werten von humaner Epidermis ergibt einen 5-fach höheren Diffusionsfluß für die Hautäquivalente.
Unsere Resultate deuten darauf hin, daß rekonstruierte humane Hautäquivalente, die auf Keratinozytenkulturen basieren, in Zukunft dazu dienen könnten, die dermale Resorption aus topisch applizierten Arzneizubereitungen in vitro zu ermitteln.

1 Introduction

The skin has been considered a favorable route of drug administration for a long time now. Patient compliance and the ease of administration make the transdermal approach an appealing choice for drug delivery. Among the advantages are the avoidance of intestinal and hepatic first-pass metabolism inherent with oral administration and avoidance of inconvenience associated with parenteral administration.

There is no general guideline until now to study the penetration of foreign substances into the skin and therefore several different *in vitro* models are used for these studies. Concerning the penetration behavior of topical dermatological dosage forms and cosmetics laboratories either utilise animal skin or excised human skin to study the transport across the skin to a

fluid receptor compartment. Increased awareness of animal welfare within the scientific community has lead to drastic reduction in the use of animals for scientific purposes. Excised human skin has proven to be one of the most appropriate methods to assess percutaneous absorption of topically applied substances, but the limited number of skin specimens and the relatively vast donor variability are limiting factors for its use. In response to these difficulties, advances have been made towards the use of commercial and non commercial human skin equivalents as *in vitro* models for dermal drug transport testing.

The technology of reconstructing human skin equivalents has been derived predominantly from research into the treatment of burns. Reconstructed skin has been used widely for cutaneous metabolic studies (Gysler et al., 1999) and skin corrosivity testing (Liebsch et al., 2000). The purpose of this study was to determine the suitability of a commercially available epidermal human skin equivalent (EpiDerm™ , EPI-606, MatTek corporation, Ashland, Massachusetts, USA) in testing the drug permeation of different pharmaceutical formulations. The intrinsic permeability barrier function was examined using flufenamic acid as a model drug. The skin equivalent was characterised in terms of flux values and batch variation. Finally, EpiDerm™ was compared to heat-separated human epidermis. To investigate the architecture of the culture system, morphologic studies using light microscopy and scanning electron microscopy were performed.

2 Material and methods

2.1 Drug formulations applied

Flufenamic acid, a non steroidal anti-inflammatory drug was used as a lipophilic model drug. This drug was chosen as sink conditions could easily be maintained through pH adjustment and it has a relatively low detection limit of 25 ng/ml.

Flufenamic acid was applied in two pharmaceutical formulations :

i) 0.1125% dissolved in wool alcohol ointment (German Pharmacopoeia, D-Beiersdorf) stored at 32°C until use.

ii) 0.1125% dissolved in Soerensen phosphate buffer pH 7.4 as solution, (Ingredients purchased from Merck, D-Darmstadt).

2.2 Permeation barriers (membranes)

2.2.1 Reconstructed skin equivalent

EpiDerm™ (EPI-606, ∅ 22mm) was purchased from MatTek corporation (Ashland, MA 01721, USA). This three dimensional system is composed of normal, human derived epidermal keratinocytes which have been cultured to form a multilayered, highly differentiated model of the epidermis.

Upon arrival, the wells were removed from the shipping agar and placed in the maintenance medium (2000 µl). After one hour incubation, the skin sample was punched out from the plastic part of the Millicell using a 21 mm punch.

2.2.2 Tissue-free membranes

Tissue-free inserts (microporous teflon based filters), which are normally incorporated in the culture of the reconstructed skin equivalent, were provided by MatTek corporation.

2.2.3 Heat-separated human epidermis

Excised human skin samples were obtained from abdominal cosmetic surgery, wrapped in aluminum foil and stored in polyethylene bags at –26°C until use.

Human epidermal membranes were prepared by a heat separation technique. After thawing, the skin specimen was immersed in hot water (60°C) for 90 seconds (Kligman et al., 1963). The epidermis was peeled off from the underlying dermis and floated on phosphate buffer for one hour to allow hydration of the epidermis. Previous experiments have shown that minimal flux variations were obtained upon standardised hydration conditions of all epidermal sheets used. Skin samples were used from one donor to avoid interindividual variation.

2.3 Permeation studies

All permeation experiments were performed using a glass Franz diffusion cell apparatus (orifice diameter: 15mm and cell volume: 12 ml, Permegear, PA 18077, USA). The diffusional area of the skin was 1.767 cm^2.

The permeation barrier (membrane) was sandwiched between the upper (donor) and lower (acceptor) compartment of the Franz diffusion cell. An infinite dose of the ointment (3 mm layer) or 0.5 ml of the 0.1125% solution were applied to the intact surface of the permeation membrane mounted on the permeation apparatus. Soerensen Phosphate buffer pH 7.4 was used as an acceptor solution maintaining sink conditions throughout the experiment (maximum acceptor concentration 73 µg/ml compared to C_s = 2050 µg/ml at 32°C (Wild, 1988)). The acceptor solution was continuously stirred at 500 rpm and the temperature was kept at 32 ± 1°C by a water jacket.

The experiment was carried out for six hours and samples were drawn at specified time points, and the withdrawn volume was immediately replaced with fresh acceptor solution. Analysis of samples was corrected for all previous samples removed.

The barrier integrity of the skin equivalent was checked by measuring the permeation of a marker molecule (Na-fluoresceine, 20 µg/ml).

2.4 Drug analysis

Analysis of samples for flufenamic acid content was conducted using high performance liquid chromatography (HPLC, Merck-Hitachi, D-Darmstadt). The equipment consisted of an AS-2000A autosampling system (with an injection volume of 20 µl), an L-6220 pump and an L-4250 UV-VIS detector. The samples were analysed using a reversed phase LiChrosphere· 100 RP-18 (5 µm) column (LiChroCART· 125-4 HPLC-Cartridge). Flufenamic acid was detected at λ= 284 nm with a retention-time of approximately 3.5 ± 0.2 minutes. The mobile phase consisted of 80% methanol (Baker, NL-Deventer) and 20% McIlvaine citric acid phosphate buffer pH 2.2 (components purchased from Merck, D-Darmstadt) at a flow rate of 1.2 ml min^{-1}.

Unknown flufenamic acid concentrations were calculated against known standards using the area under the absorption time curves and a calibration curve was constructed.

Cumulative amounts of the test substance in the receptor fluid are plotted as a function of the exposure time. Depending on Fick's first law of diffusion the slope of the linear portion of the curve provided flux values (J, µg/cm^2 hour).

$J = P_{app}/C_i$

C_i is the initial concentration (µg/cm^3) of the drug in the donor chamber

P_{app} is the apparent permeability coefficient (cm/hour).

All experiments were done on two independent batches of reconstructed skin equivalents and data were expressed as mean ± standard deviation (n= 3 to 5).

2.5 Histological examination

Light Microscopy (LM) and Scanning Electron Microscopy (SEM) were used to study the morphology of the skin equivalents. The skin specimens were detached from the plastic insert with a scalpel and forceps. For LM they were fixed in Bouin solution (a mixture of formaldehyde, picric acid and glacial acetic acid) and then processed for embedding in paraffin. Vertical sections were cut and stained with Nuclear Fast Red, Eosine Y, Aniline Blue and Orange G. To further evaluate the skin equivalents scanning electron microscopy was performed. Skin samples were fixed in 2.5% glutaraldehyde phosphate buffer solution (pH 7.4) and then post fixed with 1% osmium tetroxide in phosphate buffer pH 7.4 according to Millonig (1961). The tissue was then dehydrated in graded ethanol and acetone, critical point dried and afterwards sputtered with gold. The observations were done using a SEM type CamScan2 (UK-Cambridge).

3 Results

3.1 Histological analysis

Our light microscopical examinations showed a compact stratum corneum and stratified epidermal like layers (A) with a morphology comparable to human skin (B). Scanning electron microscopy showed a topographical view of the skin model (C) and less resemblance to human skin (D) could be detected (Figure 1). The surface of the skin equivalent was missing the classical appearance of the flattened hexagonal cornified cells and instead a rather diffuse surface was observed. This could partly explain the higher permeability of EpiDerm™.

3.2 Permeation studies

The integrity of the skin equivalents was assessed using fluoresceine permeability (data not shown). The reconstructed skin equivalent is supposed to be not permeable to Na-fluoresceine. By performing the test at the end of the experiment, only non-leaky skin specimens were taken into consideration. This test for integrity could only be carried out in the case of liquid drug preparations.

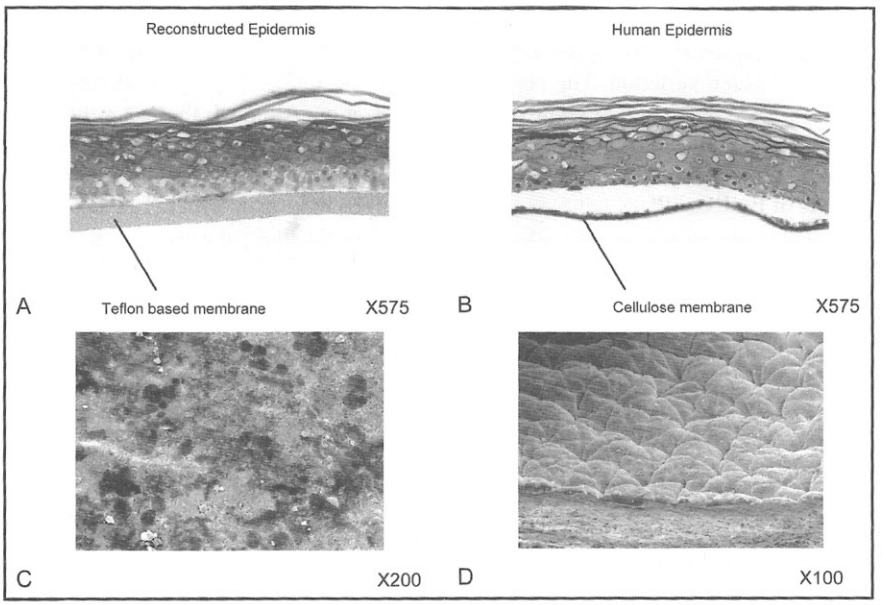

Figure 1: Light Microscopy of a cross section through the reconstructed human epidermis (A) and through heat-separated human epidermis (B). An arrow indicates the supporting membranes. Original magnification X575. Scanning Electron Microscopy of the skin equivalent (C) and human skin (D). Original magnification X200 and X100.

The barrier function of the reconstructed skin model was investigated by applying the lipophilic model drug flufenamic acid in the form of 0.1125% solution and, as can be seen in Figure 2, the barrier function of EpiDerm™ is clearly shown in contrast to that of the tissue- free filter membrane.

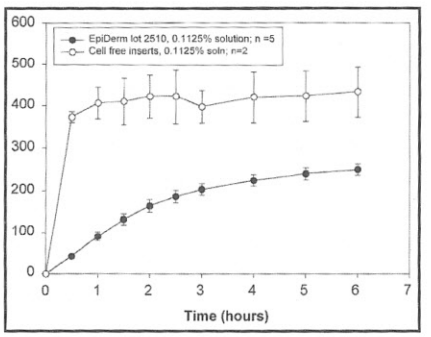

Figure 2: Diffusion of Flufenamic acid through tissue-free inserts compared to permeation across reconstructed skin equivalents. The cumulative amount permeated during 6 h is expressed.

Comparing the permeation of flufenamic acid from two different dosage forms it was found that the model drug permeated much faster when applied in solution (Figure 3). The flux of flufenamic acid was 40 times higher when using the 0.1125% solution as donor compared with the 0.1125% ointment. This means that the formulations solution and ointment could clearly be differentiated, which is essential for the use of this model in the optimisation of dermal drug preparations.

3.3 Comparison to human epidermis

To further evaluate the suitability of the reconstructed human skin equivalent as a possible alternative method we compared

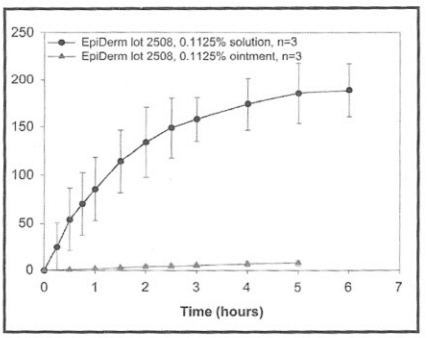

Figure 3: Permeation profiles of flufenamic acid through the reconstructed skin equivalents applied in two different pharmaceutical dosage forms.
Mean ±SD (µg/cm²), n=3.

the permeation of flufenamic acid from the 0.1125% ointment through heat separated human epidermis and the skin equivalent. The flux of flufenamic acid for the human epidermis was found to be 0.492 ± 0.027 µg cm^{-2} $hour^{-1}$ and for the skin equivalent (EpiDerm™ lot 2510) it was 2.505 ± 1.41 µg cm^{-2} $hour^{-1}$, which is almost 5 times higher than in human epidermis.

3.4 Batch to batch variation

In testing EpiDerm™ for reproducibility we studied batch variation. The preliminary results are illustrated in Figure 4; no statistically significant difference ($p > 0.05$) in permeation could be detected in two lots of EpiDerm™.

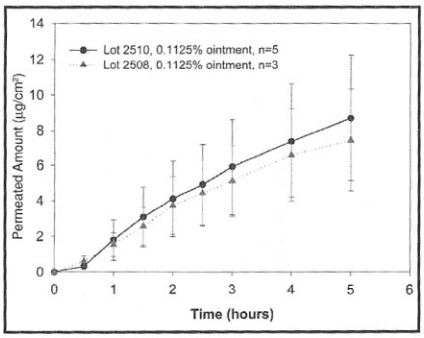

Figure 4: Comparison of the amount permeated (mean ± SD, µg/cm²) in two independent batches of reconstructed skin equivalents to test batch variation.

4 Discussion

Clinical studies until now are considered to be the most desirable method in testing the efficiency of dermally applied drug formulations. But due to ethical and economic reasons this will not be routinely applicable in the field of development and optimisation of different drug preparations. Therefore several permeation models were developed using artificial, animal and excised human skin to mimic the barrier function of the skin. Although human skin is currently considered the most suitable model, its limited availability and its high variability from donor to donor have limited its use. Often animal skin is used instead though it is well known that it is quite different from human skin with respect to the number of hair follicles, lipid composition, lipid content and morphological appearance. To date, no model is available which fully mimics human skin in terms of cell type, number of cells, blood vessels and appendages.

Since nowadays reconstructed skin models are available closely resembling human skin in terms of morphology and lipid composition (Fartasch et al., 1994), this study explored the usefulness of a cultured skin alternative consisting of human keratinocytes for drug permeation studies. Our results verify that the used skin equivalent builds up a permeation barrier, and it could differentiate the permeation of drug preparations like ointment and solution. This is a prerequisite for using it as an *in vitro* test model for percutaneous drug preparation. Although the mean flux was about five times higher comparing the skin equivalent to human epidermis, agreeing with reported data (Doucet et al., 1998; Asbill et al., 2000), the use of skin equivalents has several advantages. By testing different cell culture batches, these preliminary data suggest a satisfactory reproducibility in contrast to the high donor variability known in human skin. It also offers the advantage over excised human skin in its metabolic capacity (Slivka et al., 1993). Compared to animal skin, reconstructed skin models may serve as a better alternative since they are more similar to human skin, not only in terms of species, but also in the biochemistry and organisation, resulting in variable penetration rates in skins obtained from animal and man. Therefore, the replacement of animals in the testing of transdermal products appears to become possible in the future. Another advantage of the skin equivalents is their ready availability, allowing the quick and easy testing of drug permeation from topically applied preparations.

The purpose of this study was to examine the permeability of EpiDerm™ in order to assess its utility as an *in vitro* model for dermal formulation testing. The preliminary data presented here suggest that skin equivalents based on human keratinocyte cultures have potential as pharmaceutical test systems to study the penetration and permeation of drugs from topical formulations into or across the skin. Further refinements and validation of the system will be necessary so that in the future it will be considered a real alternative to human skin in drug delivery research.

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