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Reconstructed epidermis versus human and animal skin in skin absorption studies

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Abstract

European chemical policy in general and the REACH initiative in particular will increase the number of chemical substances submitted to toxicological evaluation by several orders of magnitude compared to the current status. To limit animal exposure the resulting enormous increase in testing, however, asks for validated in vitro test systems. While the OECD favours in vitro testing for cutaneous absorption using viable human and animal skin (Guideline 428) the availability of viable human skin is already limited today.

We present a comparison of various in vitro techniques suitable for routine skin absorption studies including commercially available reconstructed human epidermis which may be a reliable alternative to excised human and animal skin. In order to develop a protocol for the subsequent transfer to partner laboratories the experimental set-up was analysed stepwise using the OECD reference compounds caffeine and testosterone. Franz cell type, the donor and receptor media for hydrophilic/lipophilic substances, albumin and tensid addition, and storage conditions of the excised skins were systematically varied. A protocol has been developed which now allows to proceed to the pre-validation process.

Keywords: Alternative methods; Skin absorption; Skin; Reconstructed human epidermis; Skin models; Epidermis models; Regulatory toxicology; Replacement of animal experiments

Abbreviations: BSA, bovine serum albumin; ECVAM, European Centre for the Validation of Alternative Methods; K_p, permeability constant; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OECD, Organisation for Economic Co-operation and Development; PBS, Phosphate buffered saline; REACH, Registration, evaluation, and authorisation of chemicals; SCCNFP, Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers; SD, standard deviation.

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1. Introduction

Percutaneous uptake is of high relevance in the risk assessment of various types of chemicals. For the application of pesticides it was estimated that about 90% of the total exposure concerns the skin (Lundehn, 1992; Van Ravenzwaay and Leibold, 2004). The estimation of percutaneous absorption of compounds using excised human or animal skin is widely accepted (for review see: Simon and Maibach, 2000; Hadgraft, 2001) for the toxicological risk assessment (for review see: Mattie et al., 1994; Poet and McDougal, 2002). It is also relevant for the development of drugs (for review see: Moser et al., 2001; Bauerova et al., 2001) and safety assessment of cosmetics (for review see: Indans, 2002; Diembeck et al., 1999). Recently, the OECD adopted Test Guideline 428 (OECD, 2004a) and a corresponding Guidance Document 28 (OECD, 2003) for in vitro testing which is based on the use of viable human, rat and pig skin. Moreover, in parallel another Test Guideline 427 has been adopted for in vivo testing (OECD, 2004b). Considering the European Centre for the Validation of Alternative Methods (ECVAM) criteria these guidelines are based on retrospective validation of data sets generated before (Hartung et al., 2004). In about 1990 reconstructed epidermis became commercially available and the use of these tissues has been approved by the OECD for skin corrosivity testing (OECD, 2004c). Possibly, reconstructed epidermis can be used also for the determination of percutaneous uptake. However, this requires a thorough validation of their applicability which started with financial support of the 5th Framework Programme of the European Commission (Lotte et al., 2002; Dreher et al., 2002). Huggins additionally stresses the need for validation of alternative methodologies in general (Huggins, 2003).

Recent changes in the European chemicals policy (REACH) were the major stimulus to enter into a validation process. In the near future, within 15 years, all existing chemicals which have not been notified with documented toxicological profiles will have to be assessed. This will amount to a total number of about 30,000 substances (Höfer et al., 2004). Under REACH, more emphasis will be given to a tailor-made testing program taking into account human exposure. Therefore, assessment of skin absorption will become more important. In addition to that, in the Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP)'s Notes of Guidance skin absorption is of high relevance (SCCNFP, 2003). The 7th Amendment to the EC Cosmetic Directive prohibits the performance of animal testing of cosmetic products and their ingredients, making validated in vitro skin absorption tests inevitable (EC, 2003). The present sources for excised human skin from surgery, however, are not at all sufficient to cover this need. Therefore, animal skin mainly from rat and pig is used instead. The OECD Guidance Document 28 (OECD, 2003) states that reconstructed epidermis may be used, too, given equivalency is proven. Given so, not only penetration and permeation studies can be run in human skin models, but also the data can be directly compared to the results of other approved tests based on reconstructed epidermis.

A lot of effort has lately been put into the evaluation of skin models for absorption studies. A formal validation, however, is lacking. Dreher et al. (2002) compared the bioavailability of caffeine and α-tocopherol from different vehicles in skin models (EpiDerm™ and EPISKIN®) and excised human skin. Except for alcohol-containing vehicles, the rank order of solute permeability was similar. Uptake of estradiol via another commercially available skin model (SkinEthic®) was compared with uptake from porcine skin and the perfused porcine forelimb (Mahmoud et al., 2005), while cream and a lipid-based drug carrier system were compared with respect to glucocorticoid and antiandrogen uptake and targeting (Santos Maia et al., 2002; Sivaramakrishnan et al., 2004; Münster et al., 2005). Furthermore, reconstructed skin models have been well characterized and compared to human skin ex vivo with respect to their morphological characteristics and epidermal lipid composition (Ponec et al., 2002). The less developed barrier function of the skin models may explain differences observed in penetration data using alcohol-containing vehicles (Dreher et al., 2002; Mahmoud et al., 2005). For percutaneous permeation and skin absorption, the above mentioned epidermis models EpiDerm™, EPISKIN® and SkinEthic® showed greater reproducibility within one lot than between different lots. The reproducibility was dependent on the lipophilicity ($\log P$) and other physicochemical parameters of the investigated substances (Lotte et al., 2002). In another project, intralaboratory variation of uptake via the SkinEthic® model was compared by seven independent laboratories using benzoic acid, caffeine and testosterone (Heylings et al., 1998).

Van de Sandt et al. performed an international ring trial in 10 independent laboratories with the OECD test substances benzoic acid, caffeine and testosterone in human and rat skin defining inter- and intralaboratory variation. They came to the conclusion that the in vitro methodology for assessing skin absorption is relatively robust and therefore independent of the experimental conditions. They focused on human skin and did not evaluate reconstructed epidermis (Van de Sandt et al., 2004). Suhonen et al. (2003) reported a good correlation of the permeability characteristics of an organotypic epidermal culture model derived from a rat epidermal keratinocyte cell line and isolated human cadaver epidermis by testing 18 compounds covering a large range of physicochemical parameters.

In order to prepare for a formal validation study comparing the uptake of chemicals by reconstructed epidermis to uptake in human and animal skin, experiments were performed to set up a standard protocol for the subsequent transfer to the partner laboratories. To allow a broad comparison of our data to those established previously, experiments were run using the Franz cell technique and the OECD reference compounds caffeine and testosterone (OECD, 2003).

2. Materials and methods

2.1. Materials

Caffeine [58-08-2], testosterone [58-22-0], bovine serum albumin fraction V (BSA) [9048-46-8], 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [298-93-1], sodium chloride [7647-14-5], potassium dihydrogen phosphate [7778-77-0], disodium hydrogen phosphate [7558-79-4], potassium chloride [7447-40-7], ethanol [64-17-5], glycerol [56-81-5], Igepal® CA-630 ([octylphenoxy]polyethoxyethanol) [9043-52-1], MIGLYOL® 812 (medium chain triglycerides) [9043-52-1] and propylene glycol [57-55-6] were obtained from Sigma (St. Louis, MO), 1,2,6,7-^{3}H-testosterone (100 Ci/mmol) [6384-79-8] from Amersham (Freiburg, Germany) and 1-methyl-^{14}C-caffeine (51.2 mCi/mmol) [77196-81-7] from Perkin Elmer Life Sciences (Boston, MA). The radiochemical purity of caffeine and testosterone was checked by thin layer chromatography before use and was found to be greater than 95%. Stock solutions of testosterone (10 mg/ml) in ethanol kept at 4 °C were stable for at least 4 weeks. Scintillation cocktail (Optiphase Supermix) was purchased by Wallac (Turku, Finland). Donor solutions of caffeine (1% or 0.1%) and testosterone (1% or 0.004%) were prepared by dissolving caffeine in purified water or PBS (phosphate buffered saline, pH 7.4) and testosterone in 96% ethanol, an ethanol/MIGLYOL® 812 (10/90 v/v) mixture or in 2% Igepal® CA-630 in PBS, respectively. These solutions were spiked with labelled caffeine/testosterone, respectively, to achieve a total radioactivity of 37 kBq (=20.9 kBq/cm^2) per Franz cell. The dialysis membrane (mw-cutoff 10,000, very high permeability) was obtained from Diachema (München, Germany), biopsy punches (diameter: 4 mm and 8 mm) from Stiefel (Offenbach, Germany). All other reagents were obtained from Merck (Darmstadt, Germany) and were of the highest quality available.

The reconstructed epidermis (SkinEthic® and EpiDerm™) was purchased by Laboratoire SkinEthic (Nice, France) and MatTek Corp. (Ashland, MA), respectively. Porcine skin was excised from the abdomen and the back of pigs of the breed "Deutsche Landrasse Hybride" at a local abattoir being able to avoid the procedure of soaking the cadaver in boiling water. Human skin (abdomen or breast) was obtained from females aged 20 to 62 years subjected to cosmetic surgery. Placed in ice-cold cloth skin was transferred to the laboratory immediately taking care to avoid contamination of the surface by subcutaneous lipids. After trimming for fat and connective tissues cryoconserved pig and human skin was obtained by freezing at −25 °C for at least 24 h, not exceeding 6 months of storage.

2.2. Methods

2.2.1. Permeation experiments

Full thickness skin (1000 ± 100 μm) was prepared from fresh and cryoconserved skin using a Dermatome™ (Aesculab, Tuttlingen, Germany) and circles of 25 mm diameter were punched out. Epidermis was separated from cryoconserved human skin by heating the punched skin to 60 °C for 60–90 s in a water bath. The human epidermis sheet was carefully removed by forceps and transferred to a supporting dialysis membrane (previously soaked in water for 24 h, then in the receptor medium for 1 h). The reconstructed epidermis was used for the experiments within 24 h after delivery according to the recommendations of the manufacturers: after delivery the EpiDerm™ model was stored at 4 °C overnight. The next morning it was removed from nutrient agar and placed into a six well-plate previously filled with maintenance medium. The plates were kept at 37 °C and 5% CO_2 for 1 h before starting the experiment. The SkinEthic® model was removed from nutrient agar directly after the delivery and placed into six-well-plates previously filled with maintenance medium for overnight storage in an incubator at 37 °C and 5% CO_2.

Thousand micrometer human and porcine skin, heat separated as well as reconstructed epidermis were mounted into Franz cells of 15 mm in diameter and of 12 cm^3 volume of the receiver chamber (PermeGear, Bethlehem, PA, USA), the stratum corneum facing the air and the dermis or the supporting membrane of the skin model or isolated epidermis in contact with the receptor medium. All skin types were visually inspected for integrity after equilibration before starting the experiment. Skin surface temperature was maintained at 32 ± 1 °C by a circulating water bath. PBS +/− 5% BSA or 0.5% Igepal® CA-630, respectively, served as receptor media in a dynamic (flow rate 8 ml/h) or static setting. With the latter 4 or 0.4 ml medium were withdrawn for analysis and replaced by fresh medium each hour. Receptor medium was magnetically stirred (magnetic stirrer Ikamag® RO 5 power, IKA, Germany) at a rate of 500 rpm. After equilibration for 30 min and sampling the appropriate amount of receptor medium as blank value, 282.5 μl/cm^2 of the 1% or 0.1% donor solution of caffeine or 1% or 0.004% donor solution of testosterone was applied to the skin surface

for 6 h or 24 h. The 1% concentrations were chosen as a first approach for future HPLC analytics. Due to solubility problems of testosterone in an aqueous medium suitable for all substances the concentration was then reduced and a lower caffeine concentration was studied, too. Receptor fluid was collected repeatedly for up to 24 h for analysis of the substances. For the viability assay and histological examination, 4 mm or 8 mm punch biopsies of the skin samples were taken immediately after the experiment.

2.2.2. Viability assays

In parallel to permeation of the substances viability of punch biopsies of the reconstructed human epidermis was measured by MTT-test as described (Mosmann, 1983; Liebsch et al., 2000). Moreover, viability of the tissues was also determined after the stratum corneum was exposed to 11.3 μl/cm^2 of increasing concentrations of Igepal® under conditions of skin cultivation as suggested by the manufacturer. Formazan formation was quantified at 540 nm and 570 nm, respectively, using isopropanol as blank. The data are expressed as [%] of PBS treated control, which was set 100%. Moreover, other punch biopsies of skin and reconstructed epidermis pre-fixed in Karnovsky solution at 4–8 °C for transport were embedded in epoxy resin (Luft, 1961). Slices of 1 μm were prepared and stained with 1% pyronin G/1% toluidine. An observer blind inspection by light microscopy and classification according to a 8 point histological score describing the degree of damage followed.

2.2.3. Analysis

Specimens sampled for analysis of the substances were stored at −80 °C until analysed. Caffeine and testosterone concentrations in the receptor medium were quantified by scintillation counting (Microbeta Plus, Wallac, Turku, Finland). To overcome the quenching effect of BSA or media components calibration curves were obtained dissolving the spiked substance in identical solvents.

2.2.4. Statistics and data analysis

Human skin, pig skin and heat separated epidermis of three donators and reconstructed epidermis of three batches were used for experiments which were each performed in triplicate or duplicate. Permeated substance is given as μg/cm^2 skin surface. The permeability constant was calculated as described (Jaeckle et al., 2003; Moss et al., 2002) assuming equal conditions, i.e. constant skin thickness and diffusion area of the skin resulting in an equation only consisting of the slope of the linear portion of the permeation curve divided by the applied donor concentration (K_p (permeability constant) = $[V/A * C_i] * dC_A/dt$). It is expressed as cm/h where V is the volume of the receiver chamber (12 cm^3), A the area of the skin surface exposed to the receptor medium (1.768 cm^2), C_i the initial concentration of the applied substance in μg/cm^3 and dC_A/dt represents the increasing concentration of the substance in the receptor medium with increasing time. Sink conditions were also tested by total permeation of the test substances into the receptor medium which should not exceed 10% of the saturation solubility (SCCNFP, 2003). In fact, a linear increase of the testosterone concentration in the receptor medium was seen despite of the fact that the concentration in some cases (with ethanol/MIGLYOL® 812 (10/90 v/v) serving as donor medium) exceeded 10% of the saturation solubility. In all other experiments, sink conditions were guaranteed.

All data are presented as the arithmetic mean values ± standard deviation (mean ± SD). When indicated in the figure legends, significance of differences was analysed using Shapiro–Wilk, F-test and Student's t-test or Welch-test if necessary, $p \leqslant 0.05$ was considered to be significant.

3. Results

Since the OECD Guidance Document 28 (OECD, 2003) implicates that reconstructed epidermis will also be available for uptake studies given comparable results are to be obtained, we aimed to identify a test protocol promising for future interlaboratory validation experiments. Moreover the protocol should be as strictly defined as possible. Since the OECD Guidance Document 28 allows the use of the static and dynamic Franz cells permeation was compared using both approaches. Since permeation of the substances did not differ (Fig. 1) as Van de Sandt et al. (2004) also showed, handling and costs resulted in the final decision for the static set-up for the following experiments.

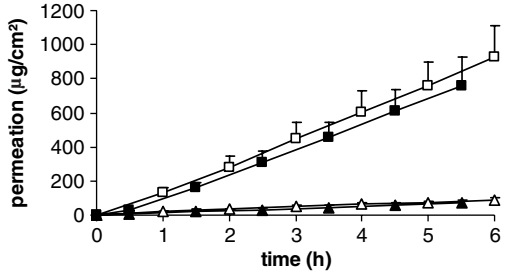

Fig. 1. Comparison of permeation from 1% solutions using the static (open symbols) and dynamic (filled symbols) approach. Permeation of radiolabelled caffeine (in PBS, squares) and testosterone (in MIGLYOL® 812/ethanol (90/10 v/v), triangles) with PBS serving as receptor compartment was measured using the SkinEthic® model inserted into Franz cells. The data represent the mean values ± SD from three independent experiments (three batches, each run in triplicate).

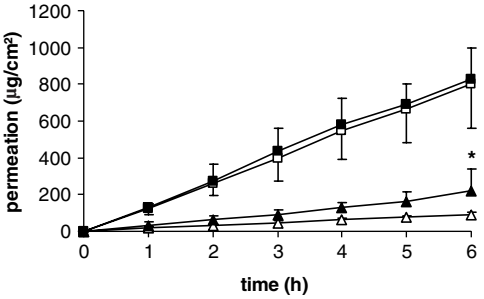

Fig. 2. Influence of the addition of 5% BSA (filled symbols) to the receptor fluid (PBS) on the permeation of caffeine (1%) solution in PBS (squares) and testosterone (1%) in MIGLYOL® 812/ethanol (90/10 v/v, triangles); open symbols = PBS alone. Permeation was measured using the SkinEthic® model. The data represent the mean values ± SD from three independent experiments (three batches, each run in triplicate). Increased permeation of testosterone to the BSA-containing receptor fluid compared to PBS alone was considered to be significant ($p \leqslant 0.05$).

Next the influence of the composition of the receptor medium on the permeation was tested. Because of restricted solubility testosterone permeation should be limited, if PBS serves as receptor medium. In order to overcome solubility restrictions, 5% BSA was added to the receptor medium according to the recommendations of the OECD (2003). As to be seen from Fig. 2, testosterone permeation more than doubled in the presence of 5% BSA (permeation after 6 h: 3.2% (90.7 µg/cm^2) with PBS and 7.9% (223.8 µg/cm^2) with addition of 5% BSA; $p \leqslant 0.05$) while it had no influence on permeation of the hydrophilic compound caffeine (permeation after 6 h: 28.5% (804.1 µg/cm^2) with PBS and 29.3% (826.6 µg/cm^2) with addition of 5% BSA). Since the addition of BSA to the receptor medium was denied by one of the future project partners because of analytical reasons when using HPLC, other solubilizers besides BSA were tested as well. Yet the addition of 0.5% of the non-ionic surfactant Igepal® to the receptor medium resulted in an inconsistent pattern of influence on permeation with respect to the different substances and tissues. Moreover, the viability of the EpiDerm™ model declined by about 85% (Table 1) when the receptor medium contained 0.5% Igepal®. Therefore, also the use of Igepal® in the receptor medium was omitted in further experiments. Instead we decided for a major decrease in the testosterone concentration which was now adjusted to the solubility in PBS. This also helped to overcome solubility problems in the donor medium.

Table 1
Influence of the solubilizer Igepal® in receptor and donor media on the viability of the reconstructed epidermis model EpiDerm™ after exposure for 6 h in the Franz cell

Igepal® [%] in the donor medium	0	2	0	2
Igepal® [%] in the receptor medium	0	0	0.5	0.5
Viability (%)	100 ± 0	77 ± 19.3	15 ± 13.9	5 ± 0.1

The skin samples not treated with Igepal® served as control and were set 100%. The data represent the mean values ± SD from skin samples from three independent batches.

While the OECD Guidance Document 28 favours the use of viable human skin for the permeation tests, skin from other species (preferentially pig skin) and non-viable skin are accepted, too (OECD, 2003). Therefore, we are obliged to relate our data generated with the reconstructed epidermis to those generated with human and porcine skin. First we evaluated the influence of freezing the skin and the influence of the anatomic region of withdrawal of porcine skin on permeation of the test substances. As to be expected (Harrison et al., 1984) cryopreservation was of no relevance to the flux of the test compounds (Fig. 3) and we can state that the use of cryopreserved skin is convenient for uptake studies. Moreover, also the withdrawal region of the skin is of minor importance (Fig. 4). For all further experiments, we decided for porcine skin from the back and for the use of cryopreserved human and porcine skin due to feasibility. Human skin permeability of heat separated epidermis sheets and full thickness skin were also very close for testosterone, while caffeine permeation was 4.7 times more efficient through heat separated epidermis as compared to full thickness skin. This difference, however, failed to be significant because of the very high variation

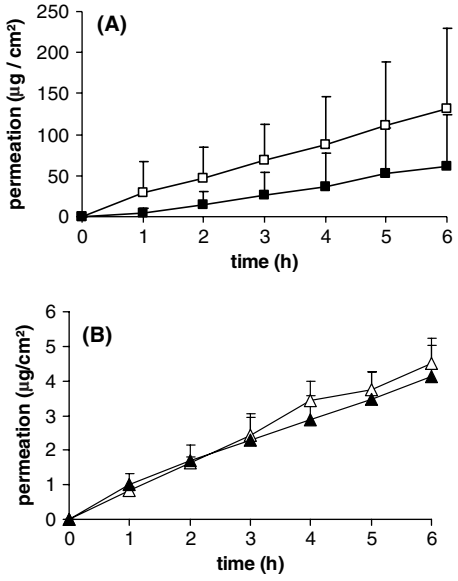

Fig. 3. Influence of freezing on the permeation of (A): 1% caffeine solution in PBS with PBS serving as receptor compartment and (B): 1% testosterone in MIGLYOL® 812/ethanol (90/10 v/v) with PBS + 5% BSA serving as receptor compartment. Permeation was measured using back skin (pig, 1000 µm) both fresh (open symbols) and cryopreserved for 24 h to 6 months at −20 °C (filled symbols). The data represent the mean values ± SD from three independent experiments (three donators) each run in triplicate.

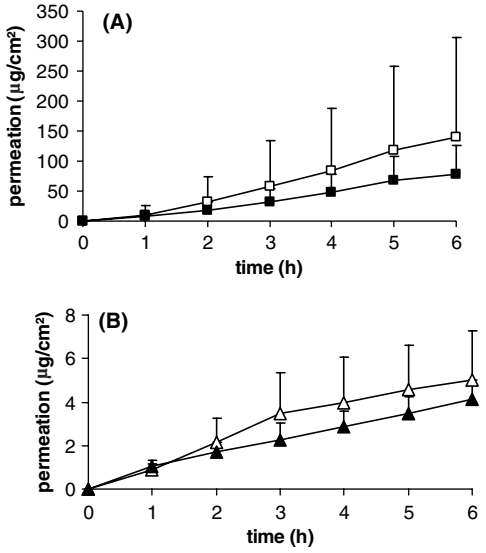

Fig. 4. Comparison of permeation using different anatomic regions of withdrawal of cryopreserved porcine skin: abdominal (open symbols) and back (filled symbols) skin. Solutions of 1% caffeine in PBS with PBS serving as receptor compartment (A) and 1% testosterone in MIGLYOL® 812/ethanol (90/10 v/v) with PBS + 5% BSA serving as receptor compartment (B) were used for permeation. The data represent the mean values ± SD from three independent experiments (three batches) each run in triplicate.

(Fig. 5) owed to one abdominal skin sample in this single experiment of otherwise only breast skin samples which increased the total permeation and of course the variability. Without these data not even the tendency of an increase is to be seen.

Next, we evaluated the influence of the donor medium on the permeation. Hydrophilic substances freely soluble in water can be applied in isotonic buffer solutions. Highly lipophilic substances, however, are often dissolved in non-physiological solvents which may influence the integrity and viability of the skin. Here we compared the permeation of testosterone dissolved in ethanol 96% and in ethanol/MIGLYOL® 812 (10/90 v/v) through the SkinEthic® model. Using pure ethanol as a solvent testosterone permeation amounted to about 20% of the permeation applying a solution in ethanol/MIGLYOL® 812 (Fig. 6A; $p \leqslant 0.05$). The permeation after 6 h was 1.6% (46.1 µg/cm^2) with ethanol 96% and 7.9% (223.8 µg/cm^2) with ethanol/MIGLYOL® 812 (10/90 v/v). Adding the solubilizer Igepal® (2%) to PBS (at a reduced testosterone donor concentration of 0.004%) increased permeation through the EpiDerm™ model compared to the PBS solution (Fig. 6B; $p \leqslant 0.05$). After 6 h 4.4% (0.5 µg/cm^2) of testosterone was recovered from the receptor medium when dissolved in PBS but 24.2% (2.9 µg/cm^2), if 2% Igepal® was added.

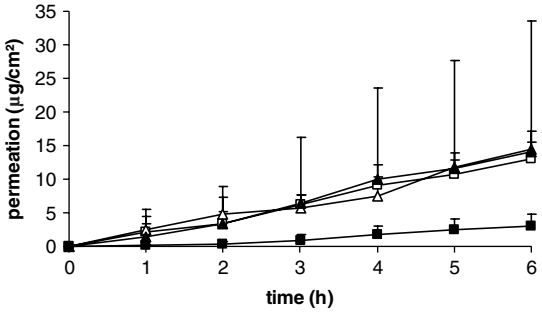

Fig. 5. Comparison of the permeation using differently treated human skin: heat separated epidermis (open symbols) versus full thickness skin (filled symbols). Solutions of caffeine (1%) in PBS with PBS serving as receptor compartment (squares) and testosterone (1%) in MIGLYOL® 812/ethanol (90/10 v/v, triangles) with PBS + 5% BSA serving as receptor compartment were used for permeation. The data represent the mean values ± SD from three independent experiments (three donators, each run in triplicate). Permeation of caffeine and testosterone through heat separated compared to full thickness skin was not considered to be significantly different.

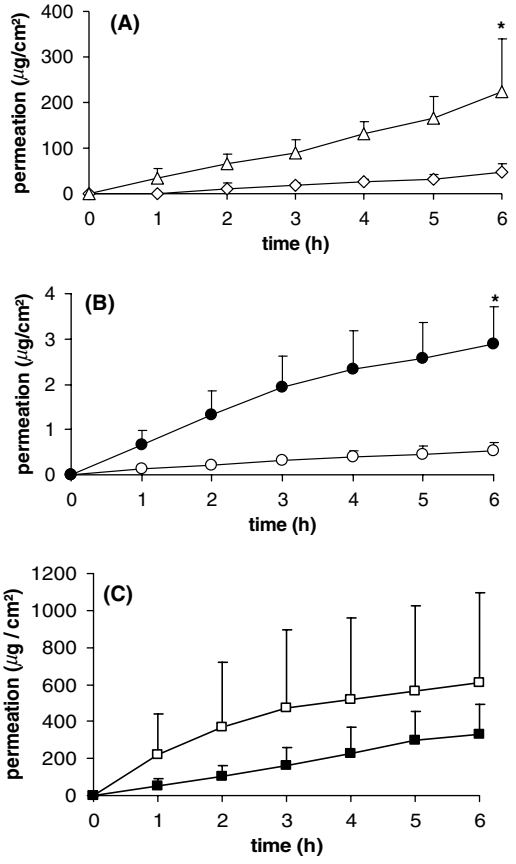

Fig. 6. Influence of the donor composition on the skin permeation. Testosterone permeation through the SkinEthic® model (A) was significantly less ($p \leqslant 0.05$) if a 1% solution in ethanol (96%, diamonds) was applied as compared to a 1% solution in MIGLYOL® 812/ethanol (90/10 v/v, triangles). While testosterone (0.004% in PBS, open circles) permeation through the EpiDerm™ model was significantly increased ($p \leqslant 0.05$) by 2% Igepal® (filled circles) (B), this was not true with caffeine. Solutions of caffeine (1%) in PBS without (open squares) or with 2% Igepal® (filled squares) were tested (C). The data represent the mean values ± SD from three independent experiments (three batches/donators, each run in triplicate).

Since permeation of the hydrophilic compound caffeine should not be influenced by the solubilizer, this is an appropriate test to evaluate the influence of the solubilizer on the integrity of the skin barrier. This way toxic effects and other interferences of the solvent/solubilizer on the skin and skin models can be revealed. Fig. 6C hints at an interference of 2% Igepal® because of the reduced caffeine permeation, although the differences failed to become significant. This might be connected with the Igepal® toxicity to reconstructed epidermis observed in the MTT-test (Table 1) and by histological evaluation. The skin damage, however, is less severe than if Igepal® is added to the receptor medium or to both receptor and donor medium. Viability declined by about 23% with Igepal® in the donor medium compared to a decline of about 85% with Igepal® in the receptor medium and 95% with Igepal® in both receptor and donor medium. To verify Igepal® toxicity we also measured the viability of the EpiDerm™ model following 24 h exposure to different concentrations of Igepal® (0.5%, 1%, 2%, 4%, 5%, 20%) in the donor compartment in transwells. The lowest concentration of the solubilizer already affected the viability of the epidermis model, decreasing it to about 13%. Higher concentrations showed stronger effects. In contrast to reconstructed epidermis, adding Igepal® to the donor solution applied to porcine skin did not influence caffeine permeation (data not shown). At low concentrations Igepal® added to the donor medium appears tolerable and can be used for the application of highly insoluble test substances at the lowest concentration possible.

Extended lag-times in permeation experiments with human and porcine skin have been reported in the literature (Lotte et al., 2002; An et al., 2003). Considering this fact and to exclude our data obtained with human and pig skin lying in the lag-phase we also performed experiments with prolonged exposure periods (24 h) in all skin types and skin models. Because of an increased number of measuring points due to a longer duration of the experiments we were enabled to make an accurate determination of lag-times and permeation parameters. After defining the linear portion of the permeation curve (Cooper and Berner, 1985) the lag-time describes the intersection of the accordant regression line with the x-axis. For infinite dose applications as in our case, K_p represents the rate at which a substance penetrates the skin under steady-state conditions (Nokhodchi et al., 2003). Due to saturation effects with the extended test period, negligible lag-times of less than one hour in the skin models (Table 2) and including viability data, we decided for a shorter exposure (6 h) in further studies with these models. Heat separated epidermis showed very short lag-times with both caffeine and testosterone. In contrast to that, we observed lag-times of up to 10 h in porcine skin (Table 2).

4. Discussion

To prepare for a large scale validation study for the use of reconstructed epidermis in routine skin absorption tests with maximal success, several pretrial studies are necessary. Test conditions have to be optimized stepwise and harmonized in the partner laboratories. Only with strictly controlled experimental conditions variability due to the different test materials will become serious. For this purpose we selected two substances widely differing in their lipophilicity, caffeine ($\log P = -0.01$) and testosterone ($\log P = 3.32$). Both substances are extensively studied in penetration and permeation studies (Lotte et al., 2002; Dreher et al., 2002; Potard et al., 1999; Dias et al., 1999; Hueber et al., 1994) and are therefore recommended as reference substances by the current OECD Guidance Document 28 for skin absorption studies (OECD, 2003). Since permeability of a test matrix is most precisely quantified by the permeability constant we decided for infinite dose experiments although these do not reflect actual experience. Finite dose experiments will be performed at the end of the validation study.

One of the first experiments was the evaluation of the differences between the static and dynamic Franz cell approach. Calculating the amount of the permeated substances resulted in almost identical curve progressions (Fig. 1) irrespective of the used Franz cell type, which fully agrees with existing literature (Bosman et al.,

Table 2
Permeation parameters of different skin types obtained from 24 h permeation experiments with 0.1% caffeine in PBS or 0.004% testosterone in PBS + 2% Igepal® serving as donor and PBS serving as acceptor medium

Skin type	Caffeine		Testosterone	
	$K_p \times 10^{-5}$ [cm/h]	Lag-time [h]	$K_p \times 10^{-4}$ [cm/h]	Lag-time [h]
Human epidermal sheet	7.6	0	9.4	0.03
Porcine skin	31.7	10.7	11.5	7.63
EpiDerm™	176.4	0.16	122.4	0
SkinEthic®	777.6	0	212.4	0.01

0.4 ml medium was withdrawn and replaced each removal time. The data represent the mean values from three independent experiments (three batches, each run in duplicate).

1998; Van de Sandt et al., 2004). Because of higher concentrations of permeated substance into the receptor fluid using the static model, the analytical variability declines. Moreover, taking also expenses for the experiment into consideration, the preference was given to the less error-prone and more economic static approach.

In the next series of experiments the appropriate receptor media were identified. Since we aimed at the establishment of a routine test for percutaneous absorption only, tissue viability is not a matter of concern. We decided in favour of simple and inexpensive media which also avoid analytical problems occurring quite frequently in the case of complex media. While hydrophilic substances such as caffeine do not show solubility restrictions, there is a risk to violate the sink conditions if flux is high (Bosman et al., 1998). Decreasing permeation with proceeding incubation time is a sign for this violation and should be looked over carefully especially when testing lipophilic substances. The recommended addition of 5% BSA (OECD, 2003) to the receptor medium increased the flux of testosterone several times (Fig. 2). Since, however, BSA may induce analytical problems with respect to online monitoring of the amount of permeated substances, the alternative solubilizer Igepal® was also tested for suitability. Morphology of skin and skin models which were exposed to Igepal® especially in the receptor medium showed clear signs of damage (data not shown). This is well explained by the close contact of the solubilizer to viable cells on the dermal side. Therefore, addition of Igepal® to the receptor medium cannot be advocated. Instead the use of 5% BSA is to be preferred, if the analytics will permit its addition. Alternatively, the concentration of the applied substance might be lowered by several orders of magnitude in order to avoid the risk of solubility restrictions in PBS as receptor medium. We decided for the second way to avoid albumin-caused analytical problems occurring in partner laboratories using online HPLC-technique. Therefore, the concentration of applied testosterone was reduced to 0.004%.

Of additional interest is the comparison of permeation via heat separated human epidermis and human full thickness skin which contains dermis as well. With the skin of each donator experiments were performed in parallel. Neither caffeine nor testosterone showed significant differences in the flux data (Fig. 5), stressing the known fact that the main barrier is the stratum corneum (Regnier et al., 1993). In contrast to the literature (Cross et al., 2003) the additional barrier of the dermis for lipophilic compounds was not detected here.

A very delicate question is the selection of an appropriate donor solution in the case of lipophilic substances. To enable dissolution of the lipophilic substances in water-based solvents, solvents or solubilizers have to be introduced. With increasing concentrations there is an interference with the highly ordered intercellular lipid structure of the stratum corneum (Krishnaiah et al., 2002), and consequently the barrier function decreases (Goto et al., 1993; Levang et al., 1999). This, however, applies to topical treatment of skin diseases as well and is often even intended in order to increase the uptake rate. Therefore, when comparing different matrices for their suitability for permeation experiments the amount of solvent or solubilizer has to be carefully balanced versus the toxic effects on the skin against the background of intended use of the substance. Compromises have to be made according to the wanted effects. Keeping in mind the 24 h viability data with Igepal® even after the optimal storage (exposition to culture medium in the transwell system) we decided for the addition of 2% Igepal® to the donor medium in case of testosterone application to reach adequate solvation. Taking together negligible lag-times and high flux data allowing to obtain sufficient data with 6 h permeation experiments make skin models elegant tools for routine testing of percutaneous absorption. The extended lag-time we observed in pig skin is probably due to modified experimental conditions (decreased concentration of donor solution; different vehicle in the donor solutions and different volume of withdrawal) in comparison to the 6 h experiment which are again ascribed to the optimization process of the methods resulting in a stepwise change of the test conditions. Experiments with porcine skin and heat separated human epidermis will therefore be run with prolonged exposure times of 24 h.

Now we will proceed with the formal validation study for testing the applicability of reconstructed epidermis models in skin absorption studies. The test conditions which are fixed in standard operating procedures will be transferred to the partner laboratories for further testing.

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References

An, N.M., Kim, D.D., Shin, Y.H., Lee, C.H., 2003. Development of a novel soft hydrogel for the transdermal delivery of testosterone. Drug Development and Industrial Pharmacy 29, 99–105.

Bauerova, K., Matusova, D., Kassai, Z., 2001. Chemical enhancers for transdermal drug transport. European Journal of Drug Metabolism and Pharmacokinetics 26, 85–94.

Bosman, I.J., Avegaart, S.R., Lawant, A.L., Ensing, K., de Zeeuw, R.A., 1998. Evaluation of a novel diffusion cell for in vitro transdermal permeation: effects of injection height, volume and temperature. Journal of Pharmaceutical and Biomedical Analysis 17, 493–499.

Cooper, E.R., Berner, B., 1985. Methods in Skin Research. Skin Permeability. Skerrow & Skerrow, Chichester Westsussex, p. 407 (Chapter 15).

Cross, S.E., Magnusson, B.M., Winckle, G., Anissimov, Y., Roberts, M.S., 2003. Determination of the effect of lipophilicity on the in vitro permeability and tissue reservoir characteristics of topically applied solutes in human skin layers. The Journal of Investigative Dermatology 120, 759–764.

Dias, M., Farinha, A., Faustino, E., Hadgraft, J., Pais, J., Toscano, C., 1999. Topical delivery of caffeine from some commercial formulations. International Journal of Pharmaceutics 182, 41–47.

Diembeck, W., Beck, H., Benech-Kieffer, F., Courtellemont, P., Dupuis, J., Lovell, W., Paye, M., Spengler, J., Steiling, W., 1999. Test guidelines for in vitro assessment of dermal absorption and percutaneous penetration of cosmetic ingredients. European Cosmetic, Toiletry and Perfumery Association. Food and Chemical Toxicology 37, 191–205.

Dreher, F., Fouchard, F., Patouillet, C., Andrian, M., Simonnet, J.-T., Benech-Kieffer, F., 2002. Comparison of cutaneous bioavailability of cosmetic preparations containing caffeine or α-tocopherol applied on human skin models or human skin ex vivo at finite doses. Skin Pharmacology and Applied Skin Physiology 15 (Suppl. 1), 40–58.

EC, 2003. 7th Amendment to the EC Cosmetic Directive (L66/26–35). 27th February 2003, Brussels, Belgium.

Goto, S., Uchida, T., Lee, C.K., Yasutake, T., Zhang, J.B., 1993. Effect of various vehicles on ketoprofen permeation across excised hairless mouse skin. Journal of Pharmaceutical Sciences 82, 959–963.

Hadgraft, J., 2001. Modulation of the barrier function of the skin. Skin Pharmacology and Applied Skin Physiology 14 (Suppl. 1), 72–81.

Harrison, S.M., Barry, B.W., Dugard, P.H., 1984. Effects of freezing on human skin permeability. Journal of Pharmacy and Pharmacology 36, 261–262.

Hartung, T., Bremer, S., Casati, S., Coecke, S., Corvi, R., Fortaner, S., Gribaldo, L., Halder, M., Hoffmann, S., Roi, A.J., Prieto, P., Sabbioni, E., Scott, L., Worth, A., Zuang, V., 2004. A modular approach to the ECVAM principles on test validity. Alternatives to laboratory animals: ATLA 32, 467–472.

Heylings, J.R., van de Sandt, J.J.M., Gilde, A.J., Ward, R.J., 1998. Evaluation of SkinEthic® human reconstituted epidermis for in vitro percutaneous absorption testing. Poster presentation, ICT Australia 1998.

Höfer, T., Gerner, I., Gundert-Remy, U., Liebsch, M., Schulte, A., Spielmann, H., Vogel, R., Wettig, K., 2004. Animal testing and alternative approaches for the human health risk assessment under the proposed new European chemicals regulation. Archives of Toxicology 78, 549–564.

Hueber, F., Schäfer, H., Wepierre, J., 1994. Role of transepidermal and transfollicular routes in percutaneous absorption of steroids: in vitro studies on human skin. Skin Pharmacology 7, 237–244.

Huggins, J., 2003. Alternatives to animal testing: research, trends, validation, regulatory acceptance: Validation of alternative methodology. Altex 20 (Suppl. 1), 55–61.

Indans, I., 2002. The use and interpretation of in vitro data in regulatory toxicology: cosmetics, toiletries and household products. Toxicology Letters 127, 177–182.

Jaeckle, E., Schaefer, U.F., Loth, H., 2003. Comparison of effects of different ointment bases on the penetration of ketoprofen through heat-separated human epidermis and artificial lipid barriers. Journal of Pharmaceutical Sciences 92, 1396–1406.

Krishnaiah, Y.S., Satyanarayana, V., Karthikeyan, R.S., 2002. Effect of the solvent system on the in vitro permeability of nicardipine hydrochloride through excised rat epidermis. Journal of Pharmaceutical Sciences 5, 123–130.

Levang, A.K., Zhao, K., Singh, J., 1999. Effect of ethanol/propylene glycol on the in vitro percutaneous absorption of aspirin, biophysical changes and macroscopic barrier properties of the skin. International Journal of Pharmaceutics 181, 255–263.

Liebsch, M., Traue, D., Barrabas, C., Spielmann, H., Uphill, P., Wilkins, S., McPherson, J.P., Wiemann, C., Kaufmann, T., Remmele, M., Holzhütter, H.G., 2000. The ECVAM prevalidation study on the use of EpiDerm™ for skin corrosivity testing. Alternatives to Laboratory Animals 28, 371–401.

Lotte, C., Patouillet, C., Zanini, M., Messager, A., Roguet, R., 2002. Permeation and skin absorption: Reproducibility of various industrial reconstructed human skin models. Skin Pharmacology and Applied Skin Physiology 15 (Suppl. 1), 18–30.

Luft, J.H., 1961. Improvements in epoxy resin embedding methods. Journal of Biophysical and Biochemical Cytology 9, 409–414.

Lundehn, J.R., 1992. Uniform principles for safeguarding the health of applicators of plant protection products (uniform principles for operator protection). Mitteilungen aus der Biologischen Bundesanstalt Heft 277, Berlin.

Mahmoud, A., Haberland, A., Dürrfeld, M., Heydeck, D., Wagner, S., Schäfer-Korting, M., 2005. Cutaneous estradiol permeation, penetration and metabolism in pig and man. Skin Pharmacology and Applied Skin Physiology 18, 27–35.

Mattie, D.R., Grabau, J.H., McDougal, J.N., 1994. Significance of the dermal route of exposure to risk assessment. Risk Analysis 14, 277–284.

Moser, K., Kriwet, K., Naik, A., Kalia, Y.N., Guy, R.H., 2001. Passive skin penetration enhancement and its quantification in vitro. European Journal of Pharmaceutics and Biopharmaceutics 52, 103–112.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63.

Moss, G.P., Dearden, J.C., Patel, H., Cronin, M.T., 2002. Quantitative structure–permeability relationships (QSPRs) for percutaneous absorption. Toxicology in Vitro 16, 299–317.

Münster, U., Nakamura, C., Haberland, A., Jores, K., Mehnert, W., Rummel, S., Schaller, M., Korting, H.C., Zouboulis, C.C., Blume-Peytavi, U., Schäfer-Korting, M., 2005. RU58841-myristat—a prodrug for acne and androgenetic alopecia. Pharmazie 60, 8–12.

Nokhodchi, A., Shokri, J., Dashbolaghi, A., Hassan-Zadeh, D., Ghafourian, T., Barzegar-Jalali, M., 2003. The enhancement effect of surfactants on the penetration of lorazepam through rat skin. International Journal of Pharmaceutics 250, 359–369.

OECD, 2003. Guidance Document No 28 for the conduct of skin absorption studies. Adopted at 35th Joint Meeting August 2003.

OECD, 2004a. Test Guideline 428: Skin absorption: in vitro Method. Adopted on 13th April 2004, Paris, France.

OECD, 2004b. Test Guideline 427: Skin absorption: in vivo Method. Adopted on 13th April 2004, Paris, France.

OECD, 2004c. Test Guideline 431: In vitro skin corrosion: Human Skin Model Test. Adopted on 13th April 2004, Paris, France.

Poet, T.S., McDougal, J.N., 2002. Skin absorption and human risk assessment. Chemico-Biological Interactions 140, 19–34.

Ponec, M., Boelsma, E., Gibbs, S., Mommaas, M., 2002. Characterization of reconstructed skin models. Skin Pharmacology and Applied Skin Physiology 15 (Suppl. 1), 4–17.

Potard, G., Laugel, C., Baillet, A., Schaefer, H., Marty, J.P., 1999. Quantitative HPLC analysis of sunscreens and caffeine during in vitro percutaneous penetration studies. International Journal of Pharmaceutics 189, 249–260.

Regnier, M., Caron, D., Reichert, U., Schaefer, H., 1993. Barrier function of human skin and human reconstructed epidermis. Journal of Pharmaceutical Sciences 82, 404–407.

Santos Maia, C., Mehnert, W., Schaller, M., Korting, H.C., Gysler, A., Haberland, A., Schäfer-Korting, M., 2002. Drug targeting by solid lipid nanoparticles for dermal use. Journal of Drug Targeting 10, 489–495.

SCCNFP, 2003. The SCCNFP's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation. Adopted at 25th plenary meeting October 2003.

Simon, G.A., Maibach, H.I., 2000. The pig as an experimental animal model of percutaneous permeation in man: qualitative and quantitative observations an overview. Skin Pharmacology and Applied Skin Physiology 13, 229–234.

Sivaramakrishnan, R., Nakamura, C., Mehnert, W., Korting, H.C., Kramer, K.D., Schäfer-Korting, M., 2004. Glucocorticoid entrapment into lipid carriers—characterisation by parelectric spectroscopy and influence on dermal uptake. Journal of Controlled Release 97, 493–502.

Suhonen, T., Pasonen-Seppanen, S., Kirjavainen, M., Tammi, M., Tammi, R., Urtti, A., 2003. Epidermal cell culture model derived from rat keratinocytes with permeability characteristics comparable to human cadaver skin. European Journal of Pharmaceutical Sciences 20, 107–113.

Van de Sandt, J.J., van Burgsteden, J.A., Cage, S., Carmichael, P.L., Dick, I., Kenyon, S., Korinth, G., Larese, F., Limasset, J.C., Maas, W.J., Montomoli, L., Nielsen, J.B., Payan, J.P., Robinson, E., Sartorelli, P., Schaller, K.H., Wilkinson, S.C., Williams, F.M., 2004. In vitro predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study. Regulatory Toxicology and Pharmacology 39, 271–281.

Van Ravenzwaay, B., Leibold, E., 2004. The significance of in vitro rat skin absorption studies to human risk assessment. Toxicology in Vitro 18, 219–225.