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The Phenion® Full-Thickness Skin Model for Percutaneous Absorption Testing

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Key Words
Full-thickness skin model · Reconstructed human epidermis · Percutaneous absorption · Skin permeation, OECD standard compounds

Abstract
In recent years many efforts have been made to replace dermal toxicity testing of chemicals in the animal by in vitro assays. As a member of a German research consortium, we have previously contributed to the validation of an in vitro test protocol for percutaneous absorption studies on the basis of reconstructed human epidermis and both human and pig skin ex vivo. Aiming to assess the barrier properties of a newly developed reconstructed skin model, this protocol has now been transferred to the Phenion® Full-Thickness Skin Model (FT model). The permeation of testosterone and caffeine was quantified in parallel to that of pig skin using Franz-type diffusion cells. In addition, the permeation of benzoic acid and nicotine was studied. As expected, the FT model is more permeable than pig skin, yet its barrier properties are well in accordance with those of reconstructed human epidermis when compared to previous data. In fact, the FT model most efficiently retards testosterone as the compound of highest lipophilicity, which can be explained by an additional uptake by a reservoir formed by the dermis equivalent. Thus, the structure closely parallels human skin. In consequence, the Phenion FT model appears to be suitable for percutaneous absorption studies in hazard analysis and should be subjected to a catch-up validation study.

Introduction

Percutaneous absorption studies are essential for hazard analysis of compounds making contact with the skin surface as well as in the development of drugs for dermal or transdermal application. In order to standardise the predictive testing of chemical compounds and cosmetics for regulatory purposes, the Organisation for Economic Co-Operation and Development (OECD) adopted guideline 428 [1] and a corresponding technical guidance document [2] which describe methods for assessing absorption by using human and animal skin ex vivo. Excised human skin is preferable to animal skin (e.g. rat skin or pig skin), but it is clearly less available. To overcome this shortage, a protocol was developed and validated according to the OECD principles [3] by using three commercially available types of reconstructed human epidermis (RHE). Permeability of the Epiderm® (Mattek Corp., Ashland, Mass., USA), SkinEthic® and EPISKIN®

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(L'Oréal, Paris, France) models was compared to that of human epidermis, pig skin and bovine udder skin by using 9 compounds widely varying in physicochemical characteristics including the OECD standards testosterone, caffeine and benzoic acid. The results demonstrate that RHE can be used for skin penetration studies, taking product-specific overpredictability into account [4, 5]. Moreover, in vitro skin corrosion testing based on RHE has been approved by the OECD (guideline 341 [6]) and a retesting procedure for phototoxicity of compounds positive in the 3T3 fibroblast test yet of poor skin penetration has been adopted by the European Union (ECVAM Scientific Advisory Committee [7]). Recently, a method for skin irritation testing has also been validated [8], and is submitted to the ECVAM Scientific Advisory Committee for the scientific review process. This protocol has now been adapted for the development and validation of a stand-alone in vitro skin irritation test using the Epiderm model [9].

Besides RHE reconstructed human full-thickness (FT) skin models, e.g. Epiderm FT and Phenion® Full-Thickness Skin Model [10, 11], were introduced to the market. These consist of keratinocytes and fibroblasts and present an epidermis, a basement membrane and a dermis featuring morphology and tissue functionality very close to the characteristics of human skin. Both RHE and FT models have already been evaluated for the permeation and biotransformation of various compounds, e.g. of hair dye ingredients (N-acetylation [12]) and glucocorticoids (ester hydrolysis [13–15]). As genotoxicity and sensitisation are often linked to biotransformation of the compound of interest within the skin, which can vary considerably between the major cell types forming the skin [14, 16–18], FT skin models may be superior to RHE for the prediction of biotransformation-linked toxic endpoints [19, 20]. According to the outcome of first percutaneous absorption studies in the FT model [15], the validated protocol for skin absorption studies in RHE has now been transferred for a characterization of the Phenion Full-Thickness Skin Model (FT model) using a relevant selection of test compounds.

Materials and Methods

Experiments were performed close to the principles of good laboratory practice. The test procedures used are described in detail by study-oriented programmes devised by the skin absorption study group (http://www.gd-online.de/german/fgruppen.htm [4]) and were adopted taking the handling instructions of the manufacturer of the FT model into account (www.phenion.com).

Test Chemicals and Preparation of Solutions

For cutaneous absorption studies, the OECD proposes the use of caffeine (MW = 194; logP = 0.01) and testosterone (MW = 288; logP = 3.47) as reference compounds with a low or high lipophilicity, respectively. Moreover, the OECD standard compounds benzoic acid (MW = 122.1; logP = 1.90; $\log D_{pH\ 7.4} = -1.25$) and nicotine (MW = 162.2; logP = 0.72; $\log D_{pH\ 7.4} = 0.02$) are also included in the panel of test compounds.

Benzoic acid (65-85-0), caffeine (58-08-2), nicotine (54-11-5), testosterone (58-22-0), ethanol (64-17-54), Igepal® CA-630 [(octylphenoxy)polyethoxyethanol, 9043-52-1] and phosphate-buffered saline, pH 7.4 (PBS), were supplied by Sigma-Aldrich (Munich, Germany). The radiolabelled compounds [7-^{14}C]-benzoic acid (57 mCi/mmol), 1-methyl-^{14}C-caffeine (51.20 mCi/mmol), L-(–)-N-methyl-^{3}H-nicotine (66.9 Ci/mmol) and 2,4,6,7-^{3}H-testosterone (100 Ci/mmol; 6384-79-8) were purchased from Moravek Biochemicals (Brea, Calif., USA), ARC Inc. (St. Louis, Mo., USA), Perkin Elmer Life Sciences (Boston, Mass., USA) and Amersham (Freiburg, Germany), respectively.

Stock solutions of testosterone (40 μg/ml) were prepared by dissolving 10 mg of the compound in 1.0 ml of ethanol. Then, 200 μl of this ethanolic solution were added to 1.0 ml of Igepal CA-630 and expanded to 50.0 ml with PBS. Stock solutions of benzoic acid, caffeine and nicotine (1,000 μg/ml) were obtained by dissolving 50 mg of the compound in 50.0 ml of PBS. In order to prepare the solutions for permeation testing, stock solutions were spiked with an appropriate amount of the radiolabelled compound to achieve a total radioactivity of 2 μCi/ml. Solutions stored at 4°C are stable for at least 4 weeks [5].

Test Matrices

The Phenion FT model (Henkel, Düsseldorf, Germany) is a multilayered equivalent of the human skin. It has a diameter of 1.3 cm and consists of keratinocytes and fibroblasts derived from the same human donor. The tissue pieces were handled according to the instructions of the manufacturer. Immediately after arriving each specimen of the FT model was transferred from the delivery plate into a 3.5-cm Petri dish equipped with filter paper and filled with approximately 4–5 ml of preheated culture medium (37°C, Air Liquid Interface Medium). Tissue pieces were then incubated overnight at 37°C, with 5% CO_2 before being mounted into Franz-type diffusion cells or subjected to histological examination.

Pig skin was obtained from 3 donor animals (breed 'Deutsche Landrasse', 43–45 kg, from 12 to 14 weeks old) without soaking the cadaver in boiling water from the Department of Comparative Medicine and Facilities of Experimental Animal Sciences, Charité-Universitätsmedizin Berlin (Berlin, Germany). Immediately after stripping, the skin was placed in ice-cold cloth and transferred to the laboratory. There the skin was trimmed removing subcutaneous fat and connective tissue and then subjected to cryopreservation at –20°C for at least 1 day and up to a maximum of 6 months [5]. On the day of the experiment, the pig skin was thawed and skin sheets of 1,000 ± 100 μm thickness were prepared using a dermatome (Aesculap® GA 630, Aesculap, Tuttlingen, Germany). After checking the skin thickness using a calliper (Pocket Thickness Gage, Mitutoyo Corp., Kawasaki, Japan), the skin was punched to obtain discs of 2 cm in diameter, rehydrated in PBS for 30 min and inspected for integrity before being mounted into Franz-type diffusion cells.

Permeation Assay

The first experiments (in 2006) were performed with pig skin and the FT model applying testosterone and caffeine. In 2007 the FT model was retested for the permeation of caffeine and in addition for that of benzoic acid and nicotine.

Using the static set-up and the infinite dose approach, permeation tests (Franz-type diffusion cells, diameter 15 mm, volume 12 ml; Permegear Inc., Bethlehem, Pa., USA) were performed in triplicate, with pig skin from 3 donors and with 3 batches of the skin model, respectively. Thus, with each compound a total of at least 9 experiments were performed for each matrix. Since the size of the FT model was too small (between 11 and 12 mm) to be directly mounted into the Franz-type diffusion cells, special inserts constructed for the Episkin model [21] were used. The surface area was thereby reduced from 1.767 to 0.358 cm^2. Both pig skin and skin model were mounted into Franz-type diffusion cells with the stratum corneum facing the air.

Experiments were performed using PBS as receptor fluid (12 ± 0.2 ml) which was maintained at 33.5 ± 1°C and magnetically stirred (500 rpm). The system was allowed to equilibrate for 30 min before a sample of receptor fluid was collected. Subsequently, 500 and 110 μl of spiked test compound solutions were applied to the pig skin and the FT model, respectively, resulting in an identical dose of 284.1 μg/cm^2 of caffeine, benzoic acid or nicotine and 11.3 μg/cm^2 of testosterone. Then the donor compartment of Franz-type diffusion cells was covered by Nescofilm® (Carl Roth, Karlsruhe, Germany) for 26 h to avoid donor fluid evaporation. Receptor fluid was sampled repeatedly for up to 26 h and each time replaced by fresh PBS. Samples were immediately stored at –20°C until analysis by scintillation counting.

Tissues were discarded if there was a wet skin surface caused by the appearance of receptor fluid during the equilibration phase. Moreover, tissue integrity as well as the avoidance of air bubbles was monitored throughout the experiment.

Liquid Scintillation Counting

Amounts of the test compounds in the receptor fluid were quantified using radiochemical detection (Microbeta Plus, Wallac, Turku, Finland) as described [5, 22].

Morphology Investigation of the Skin Model

In order to prove skin model conformity to the standards defined by OECD guideline 431 [6] and to investigate stress effects due to the experimental conditions, 7 specimens of each batch were also subjected to histology. One specimen was inspected as received. The others were mounted into Franz-type diffusion cells and exposed to 110 μl of the PBS vehicle or caffeine and testosterone solution for 8 and 26 h, respectively (1 specimen each). Then punch biopsies of 8 mm in diameter were taken from the centre of the tissue (treated area), fixed in 2 ml Karnovsky solution [23] and stored at +4°C. After several washing steps and a gradual dehydration procedure, the samples were embedded into epoxy resin [24], cut into 1- to 2-μm semi-thin sections, stained with 1% toluidine (108-44-1)/1% pyronine G (92-32-0) and then evaluated under blind conditions. A scoring system with up to 8 points (table 1) defined the degree of damage, which is an extension of the scoring system set-up for morphological analysis of reconstructed human epidermis exposed to the donor vehicles [22]. Moreover, the number of keratinocyte layers building up the epidermis of the FT model was derived from the histology of untreated specimens.

Table 1. Score system used to define the degree of damage of the FT model

8 = Normal	– Normal morphology
7 = Normal to mild damage	– From normal morphology to mild damage
6 = Mild damage	– Mild irritation – Hyperkeratosis of the stratum corneum – Oedema of the viable cells
5 = Mild to moderate damage	– From mild to moderate damage
4 = Moderate damage	– Irritation – Appearance of intra- and intercellular vacuoles in viable keratinocytes – Partial loss of corneocytes
3 = Moderate to severe damage	– From moderate to severe damage
2 = Severe damage	– Severe irritation – Presence of many intra- and intercellular vacuoles in viable keratinocytes – Massive loss of corneocytes – Detachment of the viable keratinocytes from the underlying dermis-like structure
1 = Keratinisation	– Keratinisation of the upper skin layers

Data Evaluation and Statistics

For each individual experiment, cumulative amounts of the permeated compounds in the receptor medium were plotted versus time (means ± standard deviation, SD). The apparent permeability coefficient P_{app} (equation 1) and lag time were calculated from a regression line based on mean values of the experiment [25, 26] assuming a constant tissue thickness and diffusion area resulting in an equation only consisting of the slope of the linear portion of the permeation curve divided by the applied donor concentration.

Equation 1: $P_{app} = (V/A \cdot C_i) \cdot (dC_a/dt)$

where P_{app} = apparent permeability coefficient (cm/s), V = volume of the receiver chamber (12 cm^3), A = area of the skin surface exposed to the donor medium (1.767 or 0.358 cm^2), C_i = initial concentration of the applied substance (μg/cm^3) and dC_a/dt = increasing concentration of the substance in the receptor fluid with increasing time.

Using the published algorithm and spread sheets [27], P_{app} and lag time were calculated conforming to the standards of the validation study [4] which requires at least 6 data points in the linear range. If 6 valid data points were not available, P_{app} was estimated by approximating a linear increase in the permeation curve within the first 4 h (5 data points).

Results are presented as the arithmetic mean value, SD and coefficient of variation (%). Differences in skin permeation were evaluated by the Wilcoxon test; $p \leq 0.05$ was regarded to indicate a statistically significant difference.

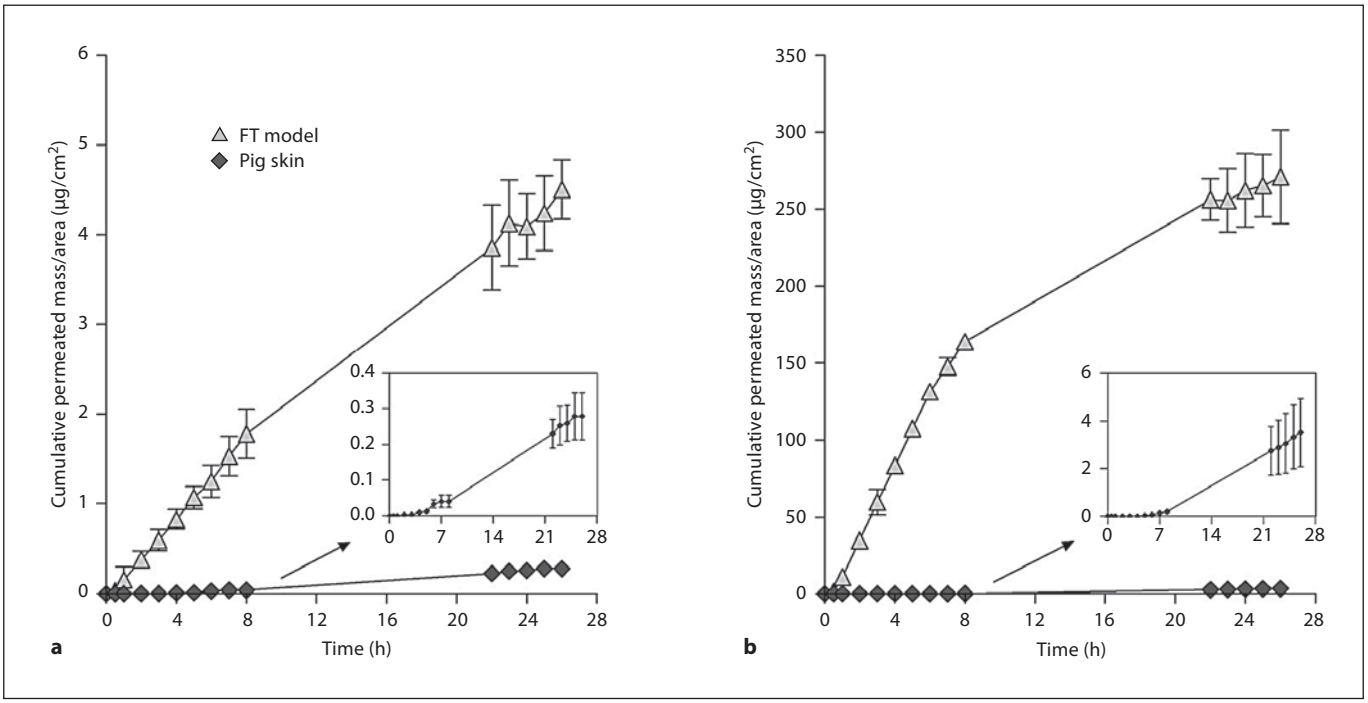

Fig. 1. Cumulative permeation (mean values ± SD) of the FT model and pig skin by the test compounds. Experiments were performed in reconstructed skin of 3 batches and pig skin from 3 donor animals in triplicate. **a** Testosterone. **b** Caffeine.

Results

Permeation Tests

The first experiments with the Phenion FT model (3 batches) were based on testosterone and caffeine which are OECD standard compounds for high and low lipophilicity. The permeability of the FT model was compared to that of pig skin (fig. 1). To study the reproducibility of the barrier over time, another 3 batches of the model produced 1 year later were subjected to another testing series using caffeine but also benzoic acid and nicotine (fig. 2). Thus, results of caffeine retesting should indicate batch-related overpredictability over time.

With pig skin the maximum concentration of caffeine and testosterone attained in the receptor fluid was always lower than the permitted range of 10% saturation solubility described previously [28]. Results conform to the infinite dose approach, and permeation profiles are approximated to be linear up to 26 h (fig. 1). At this time 1.4 ± 0.3% of the applied caffeine and 3.1 ± 1.3% of testosterone had permeated into the receiving compartment. With the FT model, however, testosterone results did not conform to the infinite dose approach over time, since the limit of the 10% saturation solubility was already attained at 8 h (22.6% after 26 h) indicating restricted diffusion in the long time range (fig. 1). In contrast, the maximum concentration of benzoic acid, caffeine and nicotine attained in the receptor fluid was always lower than the permitted range of 10% saturation solubility [28]. Efficient permeation of the FT model resulted in a depletion of the test compounds in the donor compartment (fig. 1, 2). In fact, 95.7 ± 8.7% (first series) or 95.2 ± 4.5% (second series) of the applied caffeine, respectively, 91.0 ± 6.0% of the applied benzoic acid, 70.4 ± 4.3% of the applied nicotine and 39.4 ± 2.9% of the applied testosterone were recovered in the receptor fluid at the end of the experiment (26 h). To avoid depletion-related error, P_{app} values and lag time had to be derived from amounts permeated within 6 h (caffeine, benzoic acid, nicotine) and 8 h (testosterone). The respective data indicate a linear increase in the amount of the agent in the receptor fluid with time despite the still rather high fraction of permeated compound. Once more, interbatch variability was low.

As expected with the FT model, the lag times (table 2) were clearly lower than with pig skin. With the latter, lag

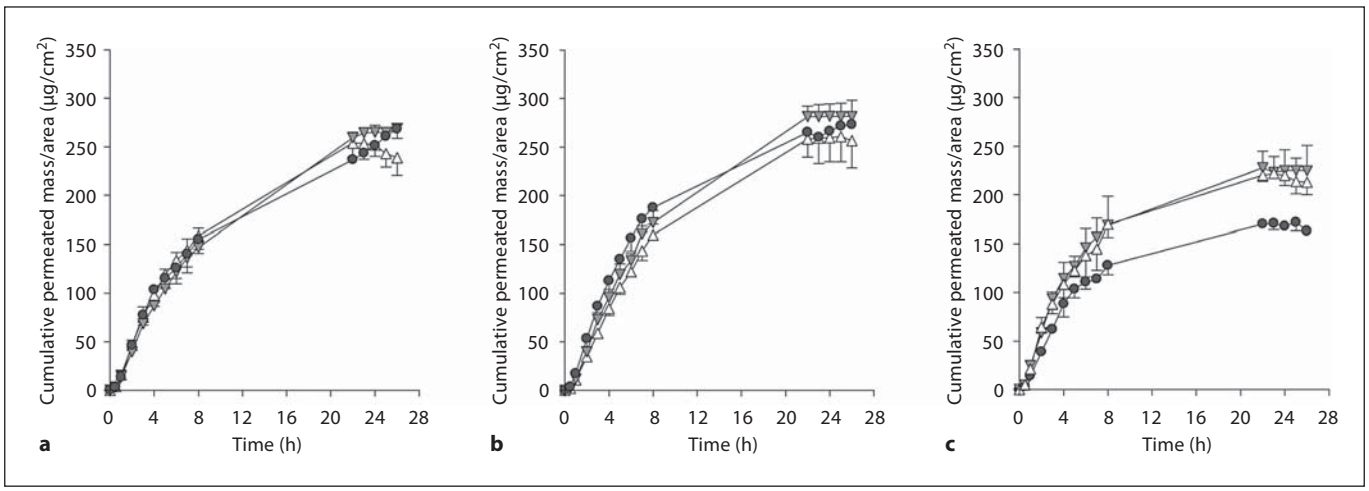

Fig. 2. Cumulative permeation (mean values ± SD) of the FT model by the test compounds. Each compound was tested in 3 batches (3 different symbols) in triplicate. **a** Benzoic acid. **b** Caffeine. **c** Nicotine.

Table 2. P_{app} values, lag time and drug permeated into the receptor fluid following 0.1% solution of benzoic acid, caffeine and nicotine and 0.004% testosterone/2% Igepal applied to pig skin and the FT model (mean values ± SD)

Skin matrix	Permeation 6 h		P_{app} $\times 10^{-6}$ cm/s	CV %	Lag time h
	µg/cm²	%			
Testosterone					
Pig skin	0.08 ± 0.06	0.03 ± 0.02	0.08 ± 0.02	25.0	5.60 ± 3.28
FT model	1.25 ± 0.18	10.9 ± 1.60	1.58 ± 0.26	16.5	0.56 ± 0.11
Caffeine					
Pig skin	0.24 ± 0.26	0.08 ± 0.09	0.03 ± 0.01	33.3	6.73 ± 2.44
FT model (1)	131.4 ± 4.03	46.0 ± 1.40	6.70 ± 0.14	2.08	0.55 ± 0.18
FT model (2)	140.9 ± 23.5	49.6 ± 8.3	6.73 ± 0.33	9.68	0.43 ± 0.09
Benzoic acid					
FT model	127.1 ± 6.14	44.8 ± 2.16	6.93 ± 0.39	5.63	0.28 ± 0.04
Nicotine					
FT model	131.5 ± 18.5	46.3 ± 6.52	6.58 ± 0.50	7.60	–0.03 ± 0.02

CV = Coefficient of variation.

times of all test compounds were well in accordance with those reported previously [4]. Except for testosterone, lag times of the FT model were shorter than lag times observed previously with the EPISKIN model (caffeine 0.81 h, benzoic acid and nicotine 0.62 h [4]) which can be explained by the structure of the reconstructed tissues: the dermis of the FT model is built up from a bioartificial scaffold which contains fibroblasts embedded into loosely packed newly synthesized ECM fibres. In contrast the EPISKIN keratinocytes are grown on a densely packed collagen layer. Thus, the lag times of the FT model undershoot those of the RHE model. Even shorter were lag times observed with the Epiderm and SkinEthic reconstructed human epidermis (<0.35 h) which are built up from keratinocytes grown on a synthetic supporting membrane [4].

In accordance with the results of the RHE validation study, too, the P_{app} value of testosterone calculated for the FT model (table 2) is one order of magnitude higher than the P_{app} value found with pig skin but is in the same range as P_{app} values found with RHE (2.10 ± 0.46 to 5.70 ± 1.51 × 10^{-6} cm/s [4]). This conforms to an additional reservoir formed by the dermis or dermis equivalent in the FT model, respectively [29]. Retesting caffeine, the P_{app} value

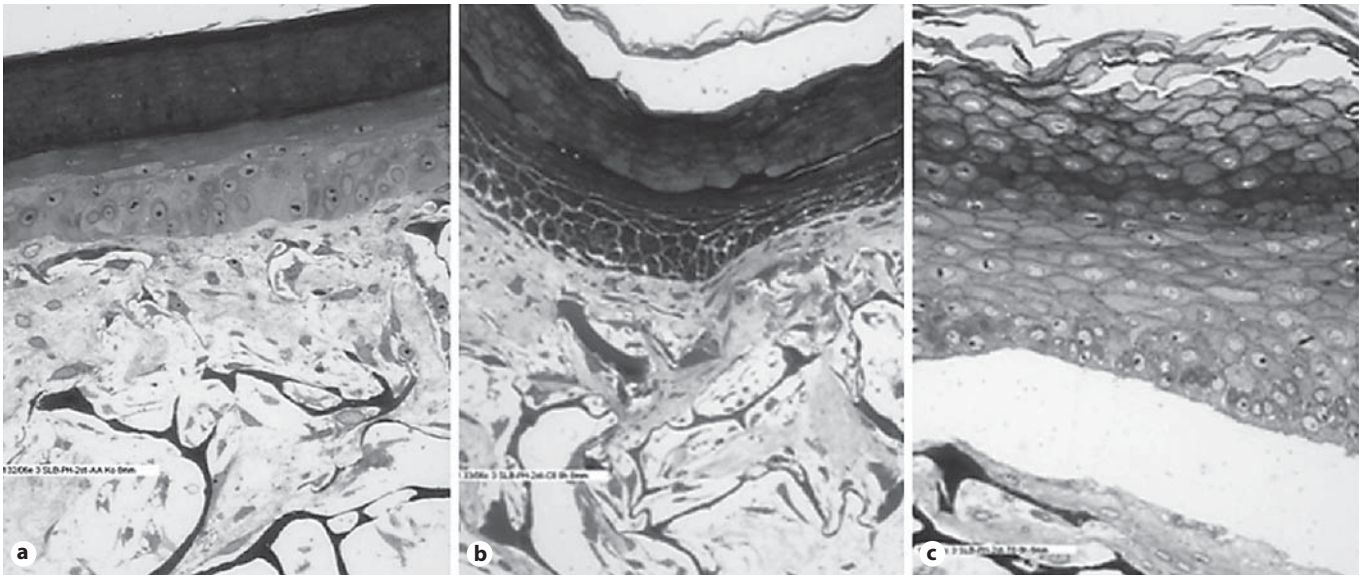

Fig. 3. Histological appearance of the FT model (toluidine/pyronine staining). Specimens of an identical batch were examined as received (**a**) and treated for 8 h with 0.1% caffeine/PBS (**b**) and 0.004% testosterone/PBS + 2% Igepal CA-630 (**c**), respectively.

Table 3. Histological score describing the damage undergone by the FT model subjected to permeation testing (mean values ± SD; n = 3)

Donor medium	8 h	26 h
PBS	4 ± 1 (moderate)	3 ± 1 (from moderate to severe)
Caffeine/PBS	6 ± 2 (mild)	3 ± 1 (from moderate to severe)
Testosterone/PBS + 2% Igepal CA-630	2 ± 1 (severe)	3 ± 2 (from moderate to severe)

did not deviate significantly from the one of the first series, which appears to indicate a consistent development of barrier properties of the FT model over a production period of 1 year. The P_{app} values of benzoic acid, caffeine and nicotine were rather close (table 2), which holds true for RHE only with benzoic acid and caffeine, with nicotine being more permeable. The P_{app} values of benzoic acid and caffeine calculated for the FT model slightly exceeded the P_{app} values found with reconstructed human epidermis but were close for nicotine. As with RHE [4], the FT model surmounted by one order of magnitude the permeability of pig skin. Interestingly, the coefficients of variation of P_{app} values generated for the FT model (table 2) are less than with pig skin [4] and RHE. However, it should be noted that the data reported here were generated by a single laboratory, while previous data were obtained by a joint group.

Morphology

Morphology of the FT model was investigated studying vertical sections of models subjected to light microscopy. As received, the FT model presented an overall normal tissue architecture and morphology (score 8.0, table 1, fig. 3) which confirms the model claims. The average number of viable epidermal cell layers was 8.3 ± 0.6, with the thickness of viable epidermis and stratum corneum being 9.0 ± 1.7 and 4.5 ± 3.3 μm, respectively.

The histological score describing the degree of damage undergone by the FT model when subjected to permeation testing is reported in table 3, histological pictures of the models are shown in figure 3. After 8 h the damage induced was clearly dependent on the donor fluid. Specimens treated with PBS (not shown) or caffeine/PBS showed only mild to moderate damage ranging from normal morphology to oedema of the viable cells. Some tissues presented also intra- and intercellular vacuoles and the beginning of the detachment of keratinocytes from the underlying dermis. Well in accordance with the previous experience obtained with RHE [22], severe dam-

age was seen in the specimens treated with 2% Igepal CA-630 donor fluid, which can be derived from the presence of intra- and intercellular vacuoles, massive loss of corneocytes and detachment of multiple keratinocytes from the underlying dermis. After 26 h, all specimens presented moderate to severe damage.

Discussion

Aiming to investigate the barrier properties of a new FT human skin model, the permeation of 4 standard compounds was studied. Taken together, the Phenion FT model appears to have a slightly weaker barrier against benzoic acid and caffeine than the previously tested RHE models [4], whereas the FT model more efficiently retards the permeation of lipophilic compounds such as nicotine and in particular testosterone (table 2). A detailed comparison of the reconstructed tissues is given by table 4 which relates the P_{app} values obtained using the FT model and those gained with RHE in the validation study [4] to P_{app} values obtained with pig skin [4]. The comparison demonstrates that the barrier properties of the FT model appear to increase with lipophilicity of the test compounds while they appear to decrease with the EPISKIN and SkinEthic models taking pH-related lipophilicity (logD) into account. As already stated, this conforms to an additional reservoir or physical barrier formed by the dermis equivalent, respectively, in particular if built up from dense material. Interestingly, the same was observed with bovine udder skin when compared to human and pig skin [5]. With bovine udder skin the increase in permeability of hydrophilic compounds was explained by a less lipophilic lipid pattern. These findings suggest that the FT model might also contain a lipid pattern which differs from the pattern of human epidermis and RHE models used for the validation study.

The experimental set-up in Franz-type diffusion cells obviously damaged the structure of the FT model; the degree of damage (fig. 3, table 3) was dependent on the donor medium and the time of exposure. The severest changes were seen with the admixture of Igepal CA-630, which is also well in accordance with alterations seen with reconstructed human epidermis. An even more dramatic effect with the testing of RHE was seen when Igepal CA-630 was added to the receptor fluid [22], thus making contact with viable keratinocytes. While these effects do not seem to impair the barrier properties in the relevant time frame, this should be different with respect to skin biotransformation, as can be derived from impaired glucocorticoid ester cleavage observed previously [30]. Therefore, the composition of donor and receptor fluid has to be selected with great care when studying biotransformation or biotransformation-related endpoints such as skin genotoxicity and sensitisation. Relevant metabolite formation in human skin ex vivo was observed for example with steroids [16] and retinoids [31].

Table 4. Ratio of P_{app} values obtained with the FT model and RHE models over P_{app} values obtained with pig skin (data from Schäfer-Korting et al. [4])

	P_{app} ratio in relation to pig skin			
	benzoic acid	caffeine	nicotine	testosterone
$logD_{pH\ 7.4}$	–1.25	0.01	0.02	3.47
FT model	24.8	21.6/21.7	8.23	6.32
Epiderm	13.0	1.90	6.06	11.1
SkinEthic	17.8	13.9	22.9	22.8
EPISKIN	3.57	9.48	8.25	8.40

Conclusions

The barrier of the Phenion FT model was found to be weaker than the barrier of pig skin, yet close to the barrier of RHE. Since data reproducibility was good, the FT model appears to be suitable for percutaneous absorption studies of compounds in hazard analysis. Given the same result is also obtained by another independent laboratory, the model should be subjected to a catch-up validation study within a joint project to quantify reproducibility and (over-)predictability based on a relevant number of compounds.

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Conflict of Interest

M. Schäfer-Korting is a member of the scientific advisory board of Biological and Clinical Research, Henkel GmbH.

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