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Comparison of human skin or epidermis models with human and animal skin in in-vitro percutaneous absorption

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Abstract

For the study of in-vitro skin penetration of candidate drugs, excised animal skin is frequently used as a replacement for human skin. Reconstructed human skin or epidermis equivalents have been proposed as alternatives. We compared the penetration properties of human, pig and rat skin with the Graftskin™ LSE™ (living skin equivalent) and the Skinethic™ HRE (human reconstructed epidermis) models using four topical dermatological drugs (salicylic acid, hydrocortisone, clotrimazole and terbinafine) with widely varying polarity. In agreement with published data, pig skin appeared as the most suitable model for human skin: the fluxes through the skin and concentrations in the skin were of the same order of magnitude for both tissues, with differences of at most two- or fourfold, respectively. Graftskin™ LSE™ provided an adequate barrier to salicylic acid, but was very permeable for the more hydrophobic compounds (e.g. about 900-fold higher flux and 50-fold higher skin concentrations of clotrimazole as compared to human skin), even more than rat skin. In the case of the Skinethic™ HRE, we found similar concentrations of salicylic acid as in human skin and an approximately sevenfold higher flux. In contrast, the permeation of hydrophobic compounds through the epidermal layer was vastly higher than through split-thickness human skin (up to a factor of about 800). To conclude, currently available reconstituted skin models cannot be regarded as generally useful for in-vitro penetration studies. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Clotrimazole; Human-skin culture; Hydrocortisone; Percutaneous absorption; Salicylic acid; Terbinafine

1. Introduction

Skin-penetration studies play an essential role in the selection of drugs for dermal or transdermal application. Therefore, the choice of predictive in-vitro penetration models is highly important. Ideally, one would like to use human skin to evaluate penetration properties of candidate drugs. However, specimens of human skin of sufficient size and quality for penetration experiments are not readily accessible to most investigators and in any case are only available in limited amounts. Human reconstructed skin models could, in principle, offer an alternative, if the

Abbreviations: HRE, human reconstructed epidermis; LSE, living-skin equivalent; PBS, phosphate-buffered saline.

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penetration barrier of these skin equivalents were similar to that of human skin. In fact, such skin equivalents have been suggested for penetration studies (reviewed in Kriwet and Parenteau, 1996).

We measured the penetration of four topical dermatological drugs of widely varying polarity (terbinafine, hydrocortisone, clotrimazole and salicylic acid) into (1) human skin; (2) a human living skin equivalent (Graftskin™ LSE™); (3) human reconstructed epidermis (Skinethic™ HRE), (4) rat skin; and (5) pig skin. The data allow us to assess the usefulness of artificial skin equivalents and of animal skin as a model for human skin in studies of in-vitro percutaneous absorption.

2. Materials and methods

2.1. Skin donors and skin preparation

Rat skin was obtained from 8–12 week old hairless female animals (strain ICO:OFA hr–hr) supplied by Iffa-Credo (Lyon, France). After suffocation with CO_2, the skin was dissected, and subcutaneous tissue was removed as described previously (Schmook et al., 1993).

Skin of domestic pigs (Landrasse × Edelschwein, obtained from a local breeder) and of Göttingen minipigs (Ellegaard, Denmark) (all females, aged 5 months) was dissected after euthanasia with Ketavet/T-61. The skin was dermatomed to 0.6 mm with an Aesculap dermatome.

Human abdominal cadaver skin was obtained from the National Disease Research Interchange (Philadelphia, PA), kept frozen at −80°C and used within 2 months after autopsy. After thawing, the skin was dermatomed, as for porcine skin.

Specimens of Graftskin™ LSE™ (round pieces, diameter 7 cm) were obtained from Organogenesis (Canton, MA). Punches (2.6 cm^2) were cut out, the support membrane was removed, and the samples were mounted in Franz chambers.

HRE (specimens of 4 cm^2) was obtained from Skinethic (Nice, France); samples were used as delivered, i.e. with the filter support attached. In control experiments, it was shown that the filters did not influence the flux of the test compounds.

2.2. Penetration assay

Test compounds and internal standards were bought from Sigma, except for terbinafine and *N*-methyl-*N*-[[4-(1-methyl-1-phenylethyl)phenyl] methyl]-1-naphthalenemethanamine (SDZ880-586) (Nussbaumer et al., 1993), which were synthesized by P. Nussbaumer, Novartis, Vienna.

Percutaneous penetration was studied in vitro using static Franz-type diffusion cells (Franz, 1978; Schmook et al., 1993). With the cells used here, the exposed skin area was 2.54 cm^2, and the volume of the receptor chamber was 5.8 ml. Phosphate-buffered saline (PBS)/ethanol 3:1 was used as receptor phase. All experiments were performed at 32°C in triplicates for 48 h. Test compounds were applied to the epicutaneous side of the skin in propylene glycol or in propylene glycol/water 9:1 (for salicylic acid) at a concentration of 1% in a volume of 300 μl.

2.3. Sampling and sample processing

Samples of 100 μl were withdrawn from the receptor phase at eight time points during the 48 h experiment and replaced by fresh receptor fluid as described previously (Schmook et al., 1993). After addition of an internal standard (see Table 1), these samples were analyzed directly by HPLC (see Section 2.4).

At the end of the experiment, the skin was taken from the diffusion cells, and the stratum corneum was removed by five strippings (rat skin, LSE™ and HRE samples) or 20 strippings (porcine and human skin) with transparent adhesive tape (bought from Kores, Barcelona, Spain). Specimens from the stripped skin (round segments of diameter 7 mm) were taken with a biopsy punch, weighed and then homogenized in 0.2 M ammonium phosphate buffer (pH 7; for salicylic acid: pH 2.5) or 0.2 M borate buffer (pH 9; only for terbinafine). Following addition of an internal standard (see Table 1), the homogenates were extracted with ethyl acetate, the extracts were evaporated, and the residues were subjected to analysis by reversed-phase HPLC.

2.4. High-performance liquid chromatography (HPLC)

For isocratic chromatography, prepacked columns (Merck Lichrosorb RP18; 7 μm; 250 × 4 mm) were used at 70°C with a Waters 510 HPLC-pump (flow rate: 2.0 ml/min), a Waters WISP-710B autoinjector and a Kratos Spectroflow 757 absorbance detector. The mobile phase and the wavelength for UV detection are specified in Table 1.

Skin concentrations were calculated by comparing the area of drug vs. the area of 10 μg of internal standard. Calibration curves were prepared for the test compound in the respective skin homogenates and analyzed by linear regression. Calculation of flux was done as described by Schmook et al. (1993). Depending on the compounds, the limits of quantitation were 20–100 ng/ml for the receptor fluid and 2–10 ng per sample (equivalent to 50–250 ng/g) for skin concentrations.

Capacity factors ($\log k_0$) were determined according to Yamana et al. (1977), by HPLC using an RP C-18 column and acetonitrile/water mixtures as the mobile phase.

3. Results

In the model system used here, skin is mounted in Franz-type diffusion cells, and solutions of the test compounds are applied to the epicutaneous side of the skin. Two parameters are obtained: (1) the rate of permeation (flux) of the test compound through the skin into the receptor fluid; (2) the concentration of the compound in the skin or skin equivalent. Permeation rates are calculated by taking samples from the receptor phase at various time points, while the skin concentration reported here refers to the 48 h time point at the end of the experiment. We chose to test four compounds (salicylic acid, hydrocortisone, clotrimazole, and terbinafine), which cover a wide range of polarity with capacity factors ($\log k_0$) of 1.19, 2.04, 3.08 and 3.83, respectively.

For terbinafine (see Table 2A), the rate of permeation through human and pig skin was at or below the detection limit of our analytical method, but was at least 24-fold higher through Graftskin™ LSE™ and Skinethic™ HRE and ~ 50-fold higher through rat skin. Drug concentrations in LSE™ and in the Skinethic™ epidermis were 14- and 55-fold higher than in human skin, while levels in pig skin were about twofold lower than in human skin. Drug levels in the skin equivalents were four- to 15-fold higher than in rat skin.

A similar situation was encountered for clotrimazole (Table 2B), where a very low flux through human and pig skin was measurable and also a low degree of permeation was detected with rat skin, while rapid permeation through the skin or epidermis equivalents was observed. Concentrations of clotrimazole in the LSE™ and HRE were substantially higher than in the ex-vivo skin: levels were seven- to 17-fold higher than in rat skin and about 20- to 200-fold higher than in human and pig skin.

For hydrocortisone (Table 2C), high permeation rates through the skin/epidermis equivalents and through rat skin and low fluxes through pig and human skin were observed. Skin concentrations were of the same order of magnitude for human, pig and rat skin, and 14- to 25-fold higher for LSE™ and HRE.

Table 1
Conditions for HPLC analysis of the test compounds

Test compound	Mobile phase	Internal standard	UV detection
Terbinafine	10 mM ammonium sulfate, pH 6.0/acetonitrile 65:35	SDZ880-586	283 nm
Clotrimazole	10 mM ammonium sulfate, pH 6.0/acetonitrile 50:50	Ketoconazole	220 nm
Hydrocortisone	10 mM ammonium sulfate, pH 2.8/acetonitrile 35:65	Hydrocortisone-21-acetate	236 nm
Salicylic acid	10 mM ammonium sulfate, pH 2.8/acetonitrile 25:75	Naphthoic acid	236 nm

Table 2
Permeation rate (flux) and skin concentration after 48 h of test compounds measured with various skin types mounted in static Franz-type diffusion cells at 32°C

Skin	Flux (μg/cm²/h)	Skin concentration (μg/g)
(A) Terbinafine, applied as a 1% solution in propylene glycol		
Human	<0.01	175 ± 36
Graftskin™ LSE™	0.28 ± 0.21	2400 ± 200
Skinethic™ HRE	0.37 ± 0.02	9600 ± 570
Domestic pig	0.010 ± 0.004	90 ± 33
Minipig	0.011 ± 0.005	75 ± 10
Rat	0.55 ± 0.21	650 ± 158
(B) Clotrimazole, applied as a 1% solution in propylene glycol		
Human	0.02 ± 0.01	93 ± 6
Graftskin™ LSE™	20.4 ± 3.1	2250 ± 260
Skinethic™ HRE	18.8 ± 2.7	4980 ± 312
Domestic pig	0.02 ± 0.01	23 ± 10
Minipig	0.02 ± 0.01	30 ± 14
Rat	0.055 ± 0.027	300 ± 88
(C) Hydrocortisone, applied as a 1% solution in propylene glycol		
Human	0.023 ± 0.007	47 ± 6
Graftskin™ LSE™	4.86 ± 0.07	662 ± 121
Skinethic™ HRE	5.29 ± 0.09	826 ± 165
Domestic pig	0.011 ± 0.009	33 ± 11
Minipig	0.010 ± 0.03	22 ± 5
Rat	1.16 ± 0.73	40 ± 20
(D) Salicylic acid, applied as a 1% solution in propylene glycol/water 9:1		
Human	21.9 ± 1.8	592 ± 110
Graftskin™ LSE™	45.5 ± 15.7	585 ± 200
Skinethic™ HRE	152.8 ± 15.7	745 ± 216
Domestic pig	12.7 ± 0.5	612 ± 101
Minipig	9.6 ± 2.1	591 ± 11
Rat	24.2 ± 9.0	1183 ± 78

Finally, we tested salicylic acid (Table 2D). For this compound, differences of the fluxes between human, pig, and rat skin on the one hand and LSE™ and Skinethic™ HRE on the other hand were not so pronounced: permeation through LSE™ was higher by factors of 2–4, and through HRE by factors of 6–12. Skin concentrations were similar within error limits for human and pig skin and the HRE and LSE™ models.

We also asked whether the barrier function of the Skinethic™ HRE model would be impaired by the receptor fluid or the application solution, which contain ethanol or propylene glycol. In a first experiment with salicylic acid, we replaced the PBS/ethanol 3:1 receptor fluid by PBS alone: no significant change in permeation rate (153 vs. 148 μg/cm²/h) or skin concentrations (745 vs. 779 μg/g) was seen as compared to the data obtained when using the alcohol-containing receptor. In a second experiment, we applied salicylic acid as a 1% solution in PBS (instead of propylene glycol/water 9:1). Here, the permeation rate (277 vs. 153 μg/cm²/h) and skin concentrations (2308 vs. 745 μg/g) were increased, obviously due to a better penetration of the compound from the aqueous solution. Thus, no detrimental effect of ethanol or propylene glycol on the barrier function of the model was detectable.

Finally, we compared the percutaneous absorption of the test compounds using skin of domestic pigs vs. skin of Göttingen minipigs, which are frequently used for toxicological investigations. Both transdermal fluxes and drug concentrations in the skin were identical within the error limits for both types of skin.

4. Discussion

The scope of this study was to provide a side-by-side comparison of various skin types that could serve as a replacement for human skin in in-vitro penetration studies. We conclude from the data with four different test compounds, which cover a wide range of polarity, that pig skin was the most suitable model of those available in the absence of human tissue. The difference in penetration between human and pig skin, depending on the compound, was up to twofold in flux and up to fourfold in skin concentration, with human skin yielding either similar or higher values than pig skin. Therefore, overestimation of drug penetration into human skin by extrapolation from experiments with porcine skin appears unlikely. This is in accordance with published reports of an adequate correlation of skin penetration into human and porcine skin (Hawkins

and Reifenrath, 1986; Dick and Scott, 1992). A comparison of the skin of Göttingen minipigs, which are frequently used in toxicological investigations, with the skin of domestic pigs did not reveal any significant differences for the four test compounds of this study. This is in line with the report that the skin of Yucatan pigs, another strain of minipigs, yields permeation coefficients similar to human skin (Fujii et al., 1997).

Continuous efforts to reconstruct human skin in vitro are reflected by numerous reports on the development of different culture systems and their assessment as penetration models (Ernesti et al., 1992; Gay et al., 1992; Régnier et al., 1992; Yang and Krueger, 1992; Roy et al., 1993; Hager et al., 1994; Kriwet and Parenteau, 1996; Parenteau et al., 1996; Lotte et al., 1997a,b,c; Monteiro-Riviere et al., 1997; Rosdy et al., 1997). These include investigations with commercialized products consisting of either reconstructed dermis/epidermis (Testskin™ LSE™; Ernesti et al., 1992; Kriwet and Parenteau, 1996; Lotte et al., 1997a), Skin2ZK2000 (Yang and Krueger, 1992), Epiderm™ (Monteiro-Riviere et al., 1997), or epidermis (Episkin™ Lotte et al., 1997b,c; and Skinethic™ HRE; Lotte et al., 1997c; Marty et al., 1997; Rosdy et al., 1997). As an overall conclusion from these studies, the barrier properties of the model systems are weak when compared to fresh or frozen human abdominal skin; the quality of the barrier was, in most cases, equivalent, or even inferior, to that of mouse, rat or guinea-pig skin.

We decided to study Graftskin™ LSE™, since it is a successor product to Testskin™ LSE™. In studies of Parenteau et al. (1996) on its use as a penetration model, diffusion of tritiated water was seen to be four- to 10-fold higher than for human skin. In our study, LSE™ provided an adequate barrier to salicylic acid, but was very permeable for the more hydrophobic compounds. This is a serious shortcoming since in our experience, the majority of drug candidates for both topical or transdermal applications have $\log k_0 > 2.0$. Thus, Graftskin™ LSE™ cannot be recommended as a general model for human skin in routine penetration studies.

Rosdy et al. (1997) reported that the reconstituted epidermis model Skinethic™ HRE provided a similar penetration barrier to tritiated water as human foreskin epidermis. In contrast, fluxes of trinitroglycerol and estradiol through Skinethic™ HRE were measured to be about 20-fold higher than through split-thickness human skin (Marty et al., 1997). In our study, we found similar concentrations of salicylic acid in human skin and in HRE and an approximately sevenfold higher flux through HRE. In contrast, the permeation of hydrophobic compounds through the epidermal layer was vastly higher than through split-thickness human skin (up to a factor of about 800). One might argue that, especially for the more hydrophobic compounds, at least part of the barrier in human skin could be due to the dermal layers, which are absent from the HRE. However, all test compounds showed almost unimpeded diffusion through split-thickness human skin from which the stratum corneum was removed by tape-stripping (data not shown). Therefore, the stratum corneum/epidermis of human skin provides an effective barrier, while the reconstructed epidermis does not. In control experiments with salicylic acid, we also demonstrated that the presence of ethanol or propylene glycol in the receptor fluid or application solution, respectively, did not lead to increased penetration. Thus, the weak barrier function of the model is an intrinsic property and not induced by interaction with the solvents.

It has been argued that rates of water penetration are “the most meaningful assessment of barrier competence” (Gay et al., 1992). This study highlights that even when similar fluxes for water or for polar compounds (such as salicylic acid) are encountered, permeation rates for hydrophobic drugs may be vastly different.

To conclude, currently available reconstituted skin models cannot be regarded as generally useful for in-vitro penetration studies, and pig skin can be recommended as a suitable substitute for human skin.

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