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Research Paper

A custom tailored model to investigate skin penetration in porcine skin and its comparison with human skin

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ABSTRACT

Reliable models for the determination of skin penetration and permeation are important for the development of new drugs and formulations. The intention of our study was to develop a skin penetration model which (1) is viable and well supplied with nutrients during the period of the experiment (2) is mimicking human skin as far as possible, but still is independent from the problems of supply and heterogeneity, (3) can give information about the penetration into different compartments of the skin and (4) considers specific inter-individual differences in skin thickness. In addition, it should be quick and inexpensive (5) and without ethical implications (6). Using a chemically divers set of four topically approved active pharmaceutical ingredients (APIs), namely diclofenac, metronidazole, tazarotene, and terbinafine, we demonstrated that the model allows reliable determination of drug concentrations in different layers of the viable epidermis and dermis. For APIs susceptible for skin metabolism, the extent of metabolic transformation in epidermis and dermis can be monitored. Furthermore, a high degree of accordance in the ability for discrimination of skin concentrations of the substances in different layers was found in models derived from porcine and human skin. Viability, proliferation, differentiation and markers for skin barrier function were surveyed in the model. This model, which we call 'Hamburg model of skin penetration' is particularly suited to support a rational ranking and selection of dermatological formulations within drug development projects.

1. Introduction

Reliable models for the determination of skin penetration and permeation are important for the development of new drugs and formulations. For the development of new chemical entities (NCE), an early generation of meaningful skin penetration data allows to incorporate physico-chemical properties that are favorable for skin penetration as a selection criteria for new drug candidates. In the context of innovative formulation development, it allows to select the best performing formulation principle and to justify the choice of excipients. Further, such models are helpful in providing supportive data with regard to equivalence or non-equivalence of safety and efficacy of formulations. The mostly used method to investigate skin penetration and permeation is human or porcine skin mounted in Franz cells. Often frozen skin or heat separated epidermis is used for these measurements. This method has the advantage of being very well established, thus many reference data exist. It has even been proven to be suited to be used as surrogate for in vivo bioequivalence studies [1]. In addition, due to the usage of frozen skin, shortage of supply can be reduced. However, frozen skin and heat separated epidermis are not viable, therefore active processes in the tissue which may influence penetration and permeation and especially metabolism cannot be monitored. Further, freeze-thaw or heating procedures can induce damage or variability. In addition, in a typical setup, a jacket of 3 cm diameter is used which requires around 7 cm^2 of skin. This means that for parallel experiments large amounts of skin are

Abbreviations: API, active pharmaceutical ingredient; Ck, cytokeratin; Cldn, claudin; Ocln, occludin; SC, stratum corneum; SD, standard deviation.

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needed, and that central areas of the skin are badly supplied, even if medium supply was to be provided. This can be circumvented by using fresh (porcine) skin in specific inserts with tissue medium as an acceptor medium, but for this, specific equipment is needed which is not routinely present in every laboratory. Further, it is difficult to control humidity – which is of importance for skin barrier function [2] – in Franz cells and application of highly viscous formulations or patches is problematic. In recent years, alternative/complementary models have been developed. The Saarbrücken model has the important advantage that it does not have a backflow of substances into the model because it rests on a filter paper. Due to the permanent presence of a punch in the skin sample, evaporation of the test item is equally avoided as unphysiological hydration of the tissue [3,4]. This renders the model suitable for mimicking conditions of full occlusion, in case this is desired. However, it also often uses frozen skin, and the skin is not supported by medium, again reducing its viability. Bovine Udder Skin (BUS) was also introduced as a valuable model to investigate skin penetration but it has the limitation of a lower skin barrier function compared to human and porcine skin [5,6]. Reconstructed human skin and reconstructed human epidermis also have shown to be promising for permeation and penetration studies, including metabolism, however, barrier function of these artificial reconstructs is still impaired compared to human and porcine skin and reconstructed human epidermis does not contain a dermal compartment [7,8]. Artificial membranes, used e.g. in "skin"-PAMPA (parallel artificial membrane assays) are useful for permeation studies [9], but lack the equivalent of compartments like epidermis and dermis which renders them unsuited for penetration studies. Furthermore, there are restrictions in the usage of formulations containing oils or higher concentrations of certain organic solvents for the risk of the extraction of lipids from the artificial membranes. In addition, of course, no aspects that require viability can be addressed. Finally, often in vivo tests are used for permeation and penetration, mostly in rats. Even though this has the immense advantage of a viable system with epidermis, dermis and subcutis and blood supply, the disadvantage is that rat skin is very thin and therefore data gained with this model are only of limited use and still have to be corrected by a "human factor" (bridging). Pigs, which have a skin morphology and barrier much more similar to human, are due to their size very expensive and need a lot of space. Thus their usage for in vivo investigations is limited.

The intention of our study was to develop a skin penetration model which (1) is viable and well supplied with nutrients during the period of the experiment (2) is mimicking human skin as far as possible, but still is independent from the problems of supply and heterogeneity, (3) can give information about the penetration into different compartments of the skin and (4) considers specific inter-individual differences in skin thickness. In addition, it should be quick and inexpensive (5), use routine cell culture laboratory equipment (6) and be without ethical implications (7). For this aim we started to develop an ex-vivo penetration model with human skin which fulfills the conditions (1), (3), (4) and (6) and then transferred the model to porcine skin which fulfills in addition points (2), (5) and (7) to a better extent. We call this model "Hamburg model of penetration". To determine penetration into the model we used four test substances, *diclofenac, tazarotene, metronidazole,* and *terbinafine* as a representative set of active pharmaceutical ingredients (APIs) approved for topical use. These APIs cover a wide space of physico-chemical properties, they are contained in approved topical drugs and references for their skin distribution are available [10–13]. For molecular characterization of the model we investigated viability, proliferation, differentiation and marker molecules for skin barrier function. In addition, we checked for inside-out barrier function.

2. Materials and methods

2.1. Tissues

The human samples for this study were obtained from our clinical departments of the UKE or the Agaplesion Diakonie-Klinikum Hamburg in the course of weight/breast reduction surgeries. They were used anonymously after surgical procedures had been completed. The local medical ethics committee (Aerztekammer Hamburg) approved this study (028/12, 029/12). Pig ears were derived from the local slaughterhouse. Ears were removed immediately after slaughtering of the pigs for human consumption (crossbred Yorkshire/Deutsches Edelschwein, age: 6 month) and transported to the laboratory.

2.2. Chemicals and test items

Methanol (HPLC gradient grade) and trifluoroacetic acid (TFA) (analytical reagent grade) were purchased by Merck KG (Darmstadt, Germany). Acetonitrile (HPLC gradient grade), tetrahydrofuran (THF) (HPLC gradient grade), and formic acid (analytical reagent grade) were purchased by Th. Geyer (Renningen, Germany). Water was Pharm. Eur. HPLC quality. Diclofenac sodium (Pharm. Eur. Quality) was purchased from Dipharma, Milan, Italy. Tazarotene (Pharm. Eur. Quality) and Terbinafine hydrochloride (Pharm. Eur. Quality) were purchased by Otto Brandes GmbH (Hamburg, Germany). Metronidazole (analytical reagent grade) was purchased by Sigma-Aldrich (Hanover, Germany). Lamisil Crème, Metrocreme, and Voltaren Emulgel were ordered from Phoenix Pharmahandel GmbH, Hamburg, Germany. Tazorac cream was ordered from Runge Pharma, Lörrach, Germany. The qualitative composition of the formulations is as follows: Tazorac cream: carbomer, edetate, medium chain triglycerides, mineral oil, sodium thiosulfate, sorbitan monooleate, sodium hydroxide, water; Voltaren emulgel: water, 2-propanol, propylene glycol, cocoyl caprylocaprate, liquid paraffin, macrogol cetostearyl ether, carbomer 974 P, diethyl amine, perfume; Lamisil cream: benzyl alcohol, cetyl alcohol, cetyl palmitate, isopropyl myristate, polysorbate 60, sorbitan monostearate, stearyl alcohol, sodium hydroxide, water; and Metrocreme: Emulsifying wax, cetylstearyl alcohol, polysorbate 60, sorbitol, glycerol, isopropyl palmitate, benzyl alcohol, lactic acid, water.

2.3. Antibodies

Antibodies specific for Cldn1 (Jay.8, 51-9000) were purchased from Life Technologies (Darmstadt, Germany), for Ocln (N-19; sc8145) from Santa Cruz (Heidelberg, Germany), for CK5 (GP5.2) and CK10 (DE-K10) from Progen (Heidelberg, Germany), for Filaggrin (NBP1-21310) from Novus Biologicals (Littleton, CO, USA), for Loricrin (PRB-145P) from Covance (Munich, Germany) and for Ki67 (MIB-1) from DAKO (Glostrup, Denmark).

2.4. Hamburg model of penetration

12 mm punch biopsies were taken from human abdominal skin (after removal of subcutis) or pig ears (thickness 1 mm) and placed dermis side down on gauze. 50 µl of the formulations was topically applied by using a pipette or a syringe to a central area of 8 mm which was limited by a polypropylene ring with an inner diameter of 8 mm and a thickness of 1 mm. The ring was fixed with Histoacryl (Braun Aesculap, Tuttlingen, Germany) (see also Fig. 1A). The models were incubated air–liquid interface in 1.5 ml medium (Dulbeccós MEM with stable L-Glutamin, 2% FCS + 1% Penicillin/Streptomycin) for 24 h at 32 °C and 60% humidity and subsequently frozen plainly. The outer part (Fig. 1B) was used to