Validation and testing of a new artificial biomimetic barrier for estimation of transdermal drug absorption

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ABSTRACT

Human skin remains the most reliable model for studying the transdermal permeation of active compounds. Due to the limited source, porcine skin has been used extensively for performing penetration tests. Performing penetration studies by using human and animal skin, however, would also involve a series of ethical issues and restrictions. For these reasons, new biomimetic artificial barriers are being developed as possible alternatives for transdermal testing. If appropriately optimized, such products can be cost-effective, easily standardized across laboratories, precisely controlled in specific experimental conditions, or even present additional properties compared to the human and animal skin models such as negligible variability between replicates. In this current work we use the skin mimicking barrier (SMB) for drug permeability tests. The aim was to evaluate the suitability of the new barrier for studying the percutaneous absorption of the lipophilic extract of the plant *Zingiber officinale Roscoe in vitro* and compare its permeability ability with the artificial membrane Permeapad® and porcine skin. Our results showed that the permeability values obtained through the SMB are comparable are comparable to those obtained by using the porcine skin, suggesting that the new barrier may be an acceptable *in vitro* model for conducting percutaneous penetration experiments.

1. Introduction

Transdermal drug administration is an interesting alternative route with several advantages compared to the classical oral and parental administrations. Indeed, transdermal drug delivery avoids gastrointestinal absorption and hepatic first-pass metabolism, minimizes adverse effects arising from peak plasma drug concentrations, avoids the risk of possible infections that often arise through more invasive administration routes thus providing a greater comfort for the patient, also through reducing the number of doses by developing sustained drug delivery systems (Tanner and Marks (2008)); (Schoellhammer et al. (2014)). In this context, the *stratum corneum* represents a major barrier to drug penetration through the skin into the blood circulation, making transdermal drug delivery challenging (Prausnitz and Langer (2008)). In recent years, various *in vitro* models have been introduced and investigated (Godin and Touitou, 2007); (Zsikó et al. 2019); (Moniz et al. (2020)) in order to evaluate the permeability of new active pharmaceutical ingredients (API) across the skin. Such *in vitro* absorption assays are generally performed by using diffusion cells where donor and receptor compartments are separated by a barrier (biological or artificial) (Cilurzo et al., 2007); (Bartosova and Bajgar 2012). Generally, skin absorption is a process that describes the passage of compounds across the skin and includes penetration (the mass of the test substance that enters the skin) and permeation (the mass that has transferred from the skin to the reservoir compartment fluid) (Hopf et al. 2020). Instead, permeability is the is the speed by which a specific molecule passes through a given biological membrane (Di and Kerns 2016). Human skin, either excised from surgical reduction or obtained from a cadaver, still represents the gold-standard barrier for evaluating the feasibility of transdermal and topical delivery (Franz 1975); however, the use of such

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biological products is difficult and arises a series of ethical issues. Porcine skin has been recognized as valid substitution for prediction of human skin permeability because of its physiological and histological similarities to human skin (Barbero and Frasch 2009); (Wester et al. 1998); (Simon and Maibach (2000)), although many ethical concerns still remain. Recently, an alternative organic solvent-free model named phospholipid vesicle-based permeation assay (PVPA) was introduced as a model mimicking the *stratum corneum* barrier of the skin. The PVPA consists of a immobilized layer of liposomes onto a cellulose ester filter support (Flaten et al. 2015). Two modifications of the PVPA skin model were developed to evaluate the permeability of many compounds: one prepared using the liposomes made of egg phosphatidylcholine (EPC) and cholesterol ($PVPA_c$),and another made of the main lipid components of the SC such as cholesterol, ceramide, free fatty acid, cholesterol sulfate and EPC ($PVPA_s$) (Flaten et al. 2015). Moreover, both $PVPA_c$ and $PVPA_S$ are able to distinguish between drugs with different degrees of lipophilicity, and permeation capacity (Engesland et al. 2013). The PVPA model may also be adopted to mimic the damaged skin barrier (Engesland et al., 2016). Even though the PVPA utilized 24-vells plates and might be potentially suited to medium throughput screening, the preparation process of these barriers is quite complex and time consuming. Moreover, the stability of these barrier over time is rather short (original PVPA lasted 2 weeks, biomimetic PVPA exhibits a 6-months stability) (Naderkhani et al. (2015)). As a consequence, the PVPA in all its formats is not commercially available nether appealing for industry. A different and commercially available artificial skin membrane is the Strat-M® membrane. This membrane is used as substitute for human or animal skin in Franz diffusion cells (Uchida et al. 2015). This membrane is composed of multiple layers of polyester sulfone impregnated with blends of synthetic lipids which, imparting hydrophobic skin-like properties to this synthetic membrane. Despite its wide implementation, the correlation between the skin and the Strat-M® results is often far from ideal (Arce et al. 2020). A new approach to investigate drug permeability is represented by the newly introduced biomimetic artificial barrier Permeapad® which consists of two regenerated cellulose membranes enclosing a layer of dry phospholipids between them, having a thickness 0.10 mm (Fig. 1). This product has been designed to study oral (Bibi et al. 2015); (di Cagno et al., 2015) and mucosal (Corazza et al. 2022) drug absorption. Once hydrated, this barrier forms a liposomal gels that in structure and composition reassemble quite closely a cellular monolayer and also accounting for paracellular drug transport (Eriksen et al. 2022). The Permeapad® technology has been used as a starting point for the manufacturing of a new skin-mimicking barrier (SMB) suitable for transdermal formulation testing (Fig. 1).

The new SMB is composed by a thin lipid layer mixture that is comprised between a layer of regenerated cellulose (cut-off size 10–14 KDa) and a porous filter (Fig. 1). The porous filter stabilizes the liposomal gel formed upon hydration (Fig. 1) and it also allows the direct contact of a transdermal formulation applied with the lipid (fundamental for testing efficiency of transdermal formulation). The thickness of the SMB was $< 0.30 \pm 0.02$ mm. The first aim of this work therefore was to evaluate the suitability of the new SMB for drug penetration testing. To examine and validate the SMB as useful membrane for transdermal studies, diclofenac sodium was used as a model drug for preliminary permeation assays comparing these data with previously published skin permeation experiments performed using Franz's diffusion cells and human SC and epidermis as a model membrane (Minghetti et al., 2007a). The second aim of this work was to test the lipophilic extract of the plant *Zingiber officinale Roscoe* which contains 18 % of [6]-gingerol utilizing the new SMB and compare its permeability ability using other two membranes: (i) the artificial membrane Permeapad® and (ii) porcine skin. *Zingiber officinale Roscoe (Zingiberaceae)*, commonly known as ginger, has been traditionally used as a treatment for common diseases, including headaches, colds, nausea, emesis and inflammatory diseases, in particular arthritis (Grzanna et al., 2005). Various bioactive compounds in ginger have been identified, such as gingerols and their main dehydration products shogaols (Butt and Sultan, 2011); (Stoner 2013). Both gingerols and shogaols exhibit a wide array of biological activities, including anti-inflammatory, anti-oxidant antimicrobial anti-allergic, and anticancer (Vijendra Kumar et al. 2014); (Nile and Park (2015)); (Surh 2002); (Citronberg et al. 2013); (Surh et al. (1998)). The [6]-gingerol, as the primary pungent ingredient of ginger, possesses substantial antioxidant and anti-inflammatory properties and inhibits the tumor necrosis factor-α (TNF-α) production in mouse skin (Park et al. 1998), as well as the epidermal growth factor (EGF)-induced neoplastic transformation in mouse epidermal JB6 cells and the epidermal ornithine decarboxylase activity. It also shows inhibitory effect on nitric oxide synthase expression in LPS-treated cell lines (Ippoushi et al. 2003). Cutaneous application of ginger extract appears interesting and noninvasive alternative for the delivery of ginger ingredients. It could circumvent "first-pass" inactivation by the liver, reduce gastrointestinal irritation, and provide a steady absorption of the medication over long periods of time (Durand et al., 2012). Moreover, [6]-gingerol and its derivatives have appropriate physicochemical properties for dermal absorption such as a low molecular-weight (less than 300 Da), a log P between 2.5 and 3.8 and a moderate solubility in water and in oil (Minghetti et al., 2007b).

2. Material and methods

2.1. Chemicals

All chemicals were of analytical grade: acetonitrile (ACN), Ethanol

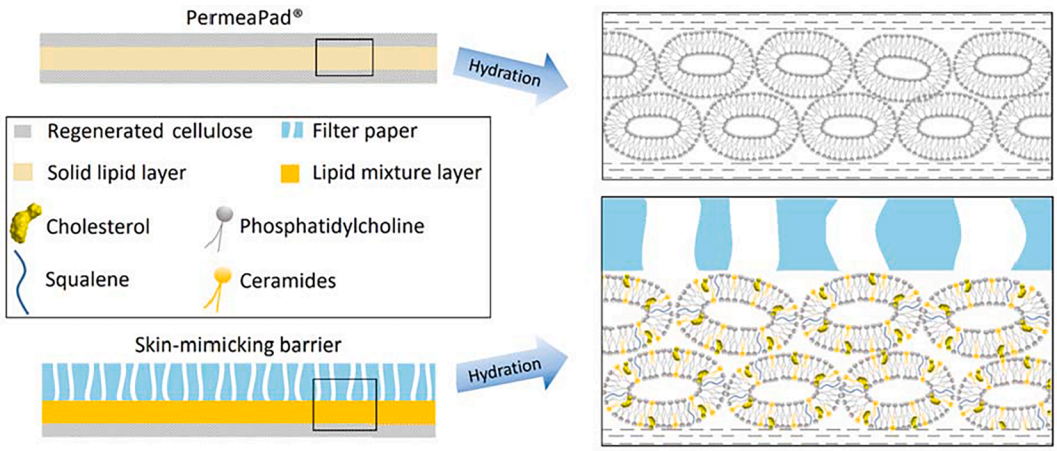

Fig. 1. Representation of Permeapad® and skin-mimicking barrier before and after hydration.

(EtOH), ethyl acetate and diclofenac sodium salt powder were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Ginger lipophilic extract containing 18 % [6]-gingerol was provided from Indena (Milan, Italy). The physicochemical properties of [6]-gingerol and diclofenac sodium, are reported in Fig. 2. Molecular weight (MW), Log P and pKa were extracted from (Haq et al. 2018) and (Minghetti et al., 2007b). Sodium chloride, sodium hydrogenphosphate, potassium dihydrogenphosphate were purchased from Carlo Erba (Milan, Italy). Propylene glycol (PG) was obtained from BASF (NY, USA). Water reagent grade was produced with a Millipore purification pack system (MilliQ water). Permeapad® and Skin-mimickingbarriers (SMB) were generous gifts from Phabioc InnoME GmbH (Espelkamp, Germany).

2.2. Diclofenac sodium solution preparation

Diclofenac sodium salt powder was dissolved in 1.0 mL of propylene glycol (PG) and sonicated at 37 °C for 2 h to obtain a clean solution with a concentration of 0.6 mg/mL, using a previously reported procedure (Minghetti et al., 2007a). The obtained solution was used for the permeability assay to validate the skin-mimicking barrier.

2.3. Membranes preparation

2.3.1. Porcine skin membranes

Piglet ears were collected immediately after animals suppression. They were stored at –25 °C on aluminum foil for a period of up to 4 months. Porcine skin was used as a model of human skin in the penetration test due to its similarity in terms of morphology and permeability to human skin (Schmook et al. (2001)); (Barbero et Frasch 2009); (Wester et al. 1998); (Simon and Maibach 2000). On the day of the experiment, the piglet ears were thawed in a physiological solution at room temperature and the skin samples were cut into 4 cm^2 square pieces. The thickness was measured with a micrometric caliper (Mitutoyo, Roissy en France, France). The thickness of pig ear skin membranes was $< 0.97 \pm 0.03$ mm. To evaluate skin integrity, Trans Epidermal Water Loss (TEWL) was measured on each skin piece after one hour of equilibration using a Vapometer (Delfin Vapometer, Delfin Technologies, Sweden) already used in our previous work (Magnano et al. 2022): the average TEWL values of skin samples was found to be below 10 $g{\cdot}m^{-2}{\cdot}h^{-1}$ (Guth et al. 2015).

2.4. In-vitro absorption studies

2.4.1. Permeation assay of diclofenac sodium solution through skin-mimicking barrier

The permeation test for diclofenac sodium, as a model drug was performed for the validation of the SMB and were performed at Logan Instrument Laboratories (Logan Instruments, Somerset, NJ). The diffusion study was conducted using static vertical glass Franz diffusion cells with a donor area of 0.64 cm^2 and a receptor volume of 7.0 mL (Logan Instruments, Somerset, NJ). SMB was used immediately with no pretreatment. Each membrane was mounted on the Franz diffusion cells with the filter side facing the donor compartment and the regenerated cellulose side facing the receptor. The receptor compartment of each cell was filled with phosphate buffer saline (PBS) pH 7.4, previously filtered by 0.45 μm nylon filter and the water bath was maintained at 37 °C to ensure 32 ± 1 °C at the surface of the skin whereas the surface of the donor side of the barrier was kept unoccluded at room temperature. At time 0 (beginning of the experiment), 100 μL of diclofenac sodium solution formulation were added to the donor compartment of each Franz diffusion cell and was left uncovered. At pre-specified time intervals (0, 1, 2, 3, 4, 5, 6, 7, and 8 h), 500 μL of receptor solution was withdrawn and immediately replaced with an equal volume of fresh buffer. Samples were analyzed by high performance liquid chromatography (HPLC). Experiments were conducted in six replicates.

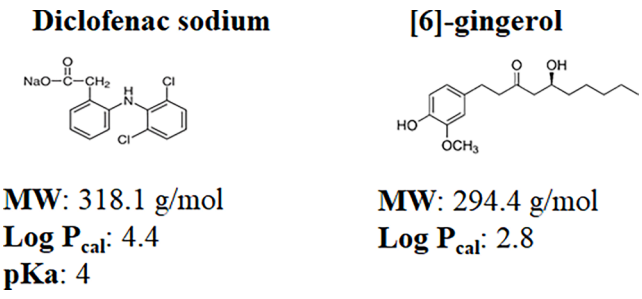

Fig. 2. Physico-chemical properties of the compounds investigated: diclofenac sodium and [6]-gingerol extracted from (Haq et al. 2018) and (Minghetti, Sosa, et al. 2007).

2.4.2. Permeation and retention study of [6]- gingerol

Permeation studies of [6]-gingerol from ginger lipophilic extract with diffusion cells were conducted with two artificial barriers (SMB and Permeapad®) and full thickness porcine ear skin, according to OECD guidelines (OECD 2004). Differently from Permeapad®, the new SMB is polarized, therefore it was mounted with the paper filter side facing the donor compartment and the regenerated cellulose side facing the receptor. In the same way, while the pig skin was mounted between the donor and receptor chamber of Franz-type static diffusion cells with the *stratum corneum* facing the donor chamber. The effective area for diffusion was 0.95 cm^2. The receptor fluid (RF) was composed of a freshly prepared 50/50 % (v/v) water/ethanol solution continuously stirred using a Teflon coated magnetic stirrer. The receptor compartment had a mean volume of 4.5 mL filled with RF. Mounted Franz cells were maintained at 32 ± 1 °C. For the porcine-skin experiments: at time 0, infinite dose of 7 droplets of ginger lipophilic extract solution (51.4 mg/mL) were deposited in direct contact with the porcine skin surface in the Franz cell. This resulted in a theoretical applied dose of $Q_0 = 19.3$ mg/cm^2. The donor compartment was sealed with parafilm during the whole time of the experiment. The permeation study was then carried out for 8 h, in order to determine the permeation profile of [6]-gingerol remaining and permeating through the skin. At selected time points (0, 1, 2, 3, 4, 5, 6, 7, and 8 h) 1.0 mL of each receptor sample was collected and analyzed. An equal volume of fresh receptor fluid was immediately replaced in each sample in order to maintain sink conditions. All the experiments were conducted on 6 independent biological replicates.

Experiments were performed following the same procedure described above also for the biomimetic barrier but replacing the porcine skin by the biomimetic barriers (SMB and Permeapad®).

The amounts of [6]-gingerol in RF as well as in each skin layer or in the entire biomimetic barriers after 8 h were quantified by HPLC (see later section 2.6.2.).

2.5. Collection and treatment of samples

The skin or, alternatively, the biomimetic membrane surface was washed three times with 1.0 mL of EtOH. Skin layers were separated as follows: the *stratum corneum* (SC) was isolated from viable layers by tape stripping (7 strips) using D-Squame tape (Monaderm, Monaco) and placed in vials each containing 5.0 mL of EtOH and stirred overnight. Then, the explant epidermis and dermis (E + D) were cut into small pieces with a scalpel, immersed in 2.0 mL of EtOH and stirred overnight. Concerning the biomimetic barriers, the membranes were cut into small pieces with scissors, immersed in 2.0 mL of EtOH, incubated overnight and diluted 1:10 in EtOH before HPLC analysis. The [6]-gingerol was extracted from each fraction (*stratum corneum*, epidermis + dermis, and whole biomimetic barriers) at room temperature overnight. After each extraction, aliquots of 1.0 mL were filtered through a 0.45 μm polytetrafluoroethylene (PTFE) membrane filter, Whatman® Maidstone, United Kingdom) before analysis by UV-HPLC. Six replicates were performed for each experiment.

2.6. High performance liquid chromatography (HPLC)

For the analysis of diclofenac sodium, a Shimadzu LC-20ADXR HPLC with UV DAD detector was employed equipped with ZORBAX Extend-C18 column with the particle size of 5 μm and length of 4.6 × 150 mm (Agilent Technologies, CA, USA). The temperature of the column was set to 40 °C. The mobile phase was composed of 60 % to 40 % of Acetonitrile [containing 0.1 % Trifluoroacetic anhydride (TFA)] and water, in gradient elution mode, at a flow rate of 1 mL/min. The injection volume was 20 μL, and the detection wavelength was 281 nm. The calibration curve of diclofenac was linear ($R^2 = 0.999$) in the concentration range of 0.5–50 μg/mL. Limit of quantification (LOQ) and limit of detection (LOD) were 0.3 μg/mL and 0.1 μg/mL respectively.

The [6]-gingerol concentrations were quantified by an Agilent 1260 chromatograph (Santa Clara, CA, USA) equipped with diode array (DAD). Agilent Eclipse XDB C-18 (3.0 × 150 mm) 3.5 μm was used as stationary phase, with temperature set to 23 °C. The mobile phase was composed of acetonitrile (A) and water 1 % formic acid (B), in gradient elution mode, at a flow rate of 0.03 mL/min. The gradient of elution was used as: 0 min 30 %A,70 %B; 10 min 100 %A,0%B; 15 min 100 %A,0%B; 15.50 min 30 %A,70 %B, till 18 min. The injection volume was 10 μL, and the detection wavelength was 280 nm. The retention time of [6]-gingerol was 8.8 ± 0.02 min and the total run time was 18 min. The calibration curve of [6]-gingerol was linear ($R^2 = 0.999$) in the concentration range of 0.5–100 μg/mL. Limit of quantification (LOQ) and limit of detection (LOD) were 0.3 μg/mL and 0.1 μg/mL respectively.

2.7. Permeability calculations and data analysis

The cumulative amount of permeated drug (dQ, expressed in μg) was plotted as a function of time (dt expressed in s). The linear portion of the slope, corresponding to the steady-state (Hopf et al. 2020) was utilized to calculate the flux according to Eq. (1).

$$J = \frac{dQ}{A*dt} \tag{1}$$

Where A represents the surface area of the barrier (expressed in cm^2). The calculated flux was used to calculate the apparent permeability coefficient (P_{app}) as Eq. (2):

$$P_{app} = \frac{J}{C_d} \tag{2}$$

where P_{app} (cm/s) is the apparent permeability coefficient, J (μg/cm^2 per s) is the flux at the steady state and C_d is the drug donor concentration (μg/cm^3). The resistivity to permeation (R, units of s/cm) therefore the resistance that the barrier opposes to drug permeation was calculated utilizing Eq. (3).

$$R = \frac{1}{P_{app}} \tag{3}$$

2.8. Statistical analysis

Data from skin absorption experiments were expressed as mean ± standard deviation (SD) and calculated by normalizing the thickness of the SMB. Statistical analysis of differences between two groups were analyzed by Student *t*-test and those between multiple groups were performed using the analysis of variance (ANOVA, one-way) The significance level was set at $p < 0.05$.

3. Results

3.1. Resistance of the skin-mimicking barrier to diclofenac sodium' s permeability

The resistance (R) of the SMB to diclofenac sodium solution permeation was measured and compared with human skin values obtained from the literature (Minghetti et al., 2007a). These results are summarized in Fig. 3. As it can be seen, the resistances measured with the intact human skin and the SMB are in the same order of magnitude, ranging from ranging from $0.9 \times 10^5 \pm 0.60 \times 10^5$ s/cm to $1.04 \times 10^5 \pm 0.10 \times 10^5$ s/cm.

3.2. Skin permeation of [6]-gingerol through different membranes

The ginger lipophilic extract is obtained by supercritical CO_2 technology, containing 28.84 % of total gingerols and 2.24 % of total shogaols. [6]-gingerol is the main gingerol with a content of 18 %. The concentrations of [6]-gingerol in the receptor fluid expressed in μg/cm^2, normalized by the thickness of the SMB are reported in Fig. 4. Normalization was given by the following Eq. (4).

$$\text{X value}^* \left(\frac{h\,porcine\,or\,h\,Permeapad®}{h\,SMB} \right) \tag{4}$$

where X value represents the concentration of [6]-gingerol in RF (expressed in μg/cm^2), h is the thickness of each membrane (expressed in cm), having a value of: 0.03 cm; 0.09 cm; 0.01 cm for SMB, porcine and Permeapad® respectively.

In the experimental condition, the mean amounts of [6]-gingerol observed in RF using SMB were closed to porcine skin samples, reaching 1918 ± 78.22 μg/cm^2 and 1450 ± 484.4 μg/cm^2, respectively, at the end of the contact time (8 h). Notably, steady state transdermal flux of [6]-gingerol in both membranes (SMB and porcine skin) was found to be similar with a value of 0.07 μg/cm^2*s (Table 1). On the other hand, the flux and the concentration of [6]-gingerol measured through Permeapad® barriers were 0.01 μg/cm^2*s and 120 ± 9.30 μg/cm^2, respectively (Table 1). These values are relatively lower compared to the other artificial skin-mimicking barrier and may be quite difficult to compare these results to those observed in porcine skin. Such discrepancy can be explained by the similar composition of SMB with the structure of the skin, due to presence of a lipid layer enriched with ceramides, squalene and cholesterol, simulating the *stratum corneum* barrier, compared to the composition of Permeapad® barrier. Importantly, the different RF data of [6]-gingerol at 8 h, obtained by SMB the other two membranes (porcine and Permeapad®) are statistically different.

The apparent permeability coefficient P_{app} (cm/s) of [6]-gingerol though each membrane was determined through dividing the flux by the equilibrium concentration of [6]-gingerol in the donor solution (C_d). Both SMB and porcine skin tests produced the highest values of P_{app} ranging around $1.4*10^{-6}$cm/*sec*, while Permeapad® showed a P_{app} approximately 14 times lower (Table 1).

3.3. In vitro skin distribution of [6]-gingerol

The amount of [6]-gingerol retained by the different skin layers and by the whole biomimetic barriers was also quantified (Fig. 5). The obtained values were normalized over the thickness of the SMB using the Eq. (5).

$$\text{X value}^* \left(\frac{h\,porcine\,or\,h\,Permeapad®}{h\,SMB} \right) \tag{5}$$

where X value represents the concentration of [6]-gingerol in the different skin layers (expressed in μg/cm^2), h is the thickness of each membrane (expressed in cm): SMB 0.03 cm; porcine 0.09 cm; Permeapad 0.01 cm.

Concerning the two artificial barriers, it is important to point out that it is meaningless to separate drug accumulation through the different layers, so only the total amount of [6]-gingerol retained by the skin was measured. The results reported in Fig. 5 clearly show that the total amount of [6]-gingerol after 8 h of contact retained by SMB and porcine

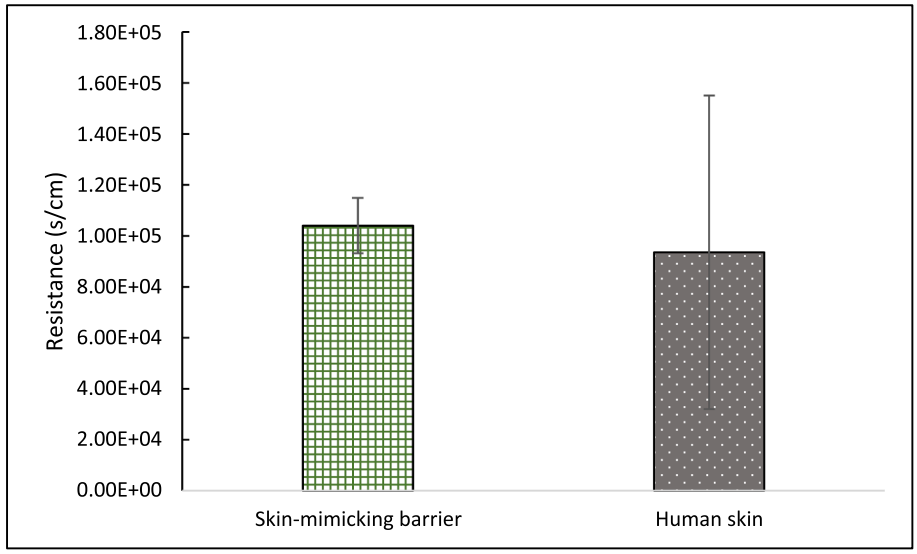

Fig. 3. Resistance (R) to penetration offered by the skin-mimicking barrier (SMB) and human skin to diclofenac permeation. Human skin R values were obtained from the literature (Minghetti, Cilurzo, et al. 2007).

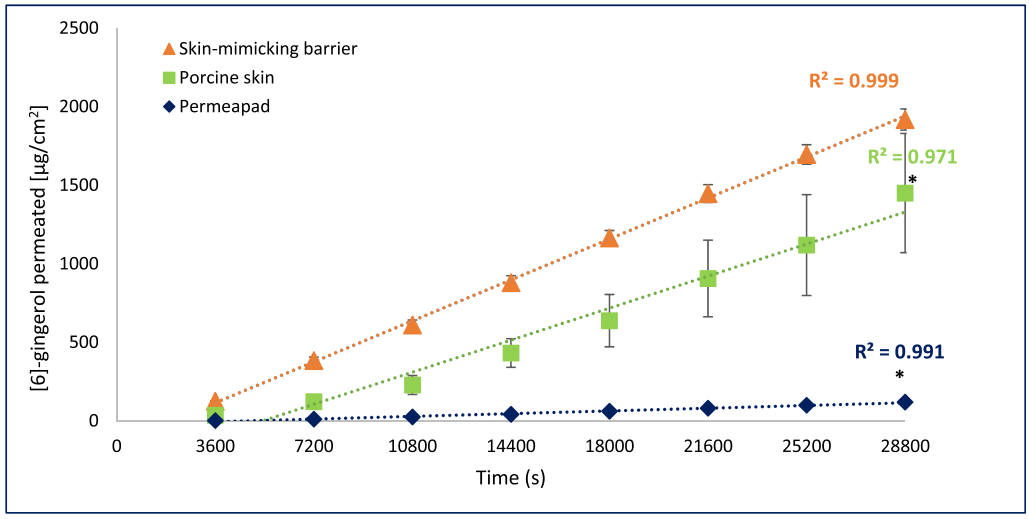

Fig. 4. [6]-gingerol amount (µg/cm^2) that permeated in the receptor fluid at specific extraction times through SMB, Permeapad®, and porcine skin. Values are normalized by the thickness of the SMB and expressed as mean ± SE (n = 6). Asterisk (*) indicates statistically significant differences between SMB and the other two tested membranes ($p < 0.05$).

Table 1
Flux (J), apparent permeability coefficients (P_{app}) and [6]-gingerol concentration measured for each membrane. Values are expressed as mean ± SE (n = 6). Asterisk (*) indicates statistically significant between SMB and the other two tested membranes ($p < 0.05$).

Membrane	J (µg/cm^2*s)	P_{app} (cm/s)	Normalized [6]-gingerol concentration in RF at 8 h (µg/cm^2)
Skin-mimicking barrier	0.07 ± 0.00	1.41x10^{-6} ± 0.05x10^{-6}	1918 ± 78.22
Porcine skin	0.07 ± 0.02	1.36x10^{-6} ± 0.35x10^{-6}	1450 ± 484.4*
Permeapad®	0.01 ± 0.00	0.10x10^{-6} ± 0.07x10^{-6}	120 ± 9.30*

skin samples was significantly higher (6242 ± 1417 µg/cm^2 and 2463 ± 1591 µg/cm^2, respectively) compared to the Permeapad® samples (44.3 ± 2.8 µg/cm^2). Additionally, in the case of porcine skin samples, [6]-gingerol indicated similar accumulation both in the viable epidermis and dermis (up to 1352 ± 1196 µg/cm^2) and in the *stratum corneum* (up to 1111 ± 1200 µg/cm^2) (Fig. 5). A statistically significant difference between the SMB and Permeapad® barrier was found.

Finally, the full absorbed recovered amount (Q_{abs}) was calculated utilizing Eq. (6).

$$Q_{abs} = SC + (E + D) + RF \quad (6)$$

Where SC is the sum of the recovered amounts of [6]-gingerol in the *stratum corneum*, E + D is the sum of the recovered amounts of [6]-gingerol in the epidermis and dermis, RF is the sum of the recovered amounts of [6]-gingerol in the receptor fluid.

In the case of porcine skin samples, Q_{abs} is the sum of [6]-gingerol observed in receptor fluid + skin layers (epidermis, dermis and *stratum corneum*), while Q_{abs} for the two artificial barriers corresponds to the sum of the recovered amounts of [6]-gingerol in receptor fluid + the whole barriers. Q_{abs} of [6]-gingerol values obtained through SMB was similar to that obtained through porcine skin, reaching values of 8160 ±

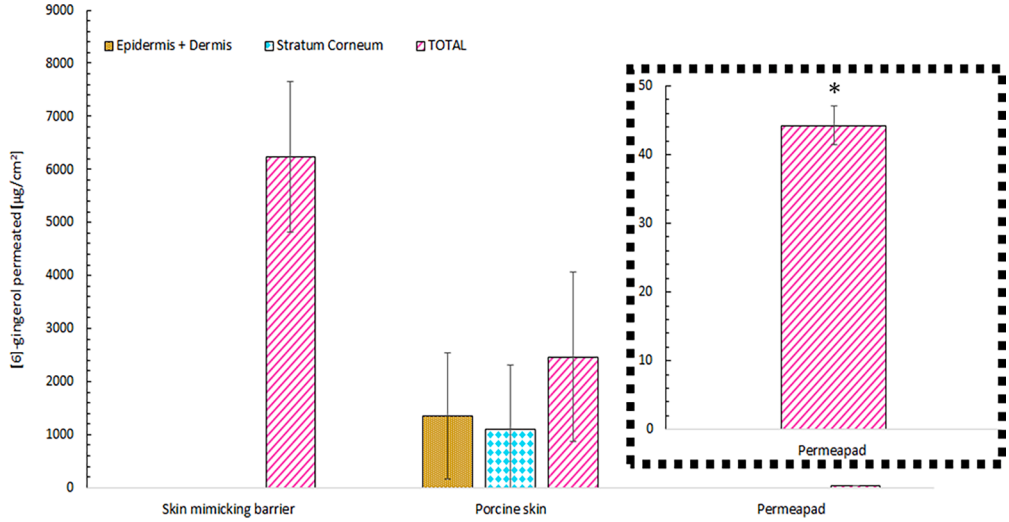

Fig. 5. [6]-gingerol concentration found in skin layers or in the entire barrier after 8 h exposure. Applied dose was 19.3 mg/cm^2. Data are normalized by the thickness of the SMB and given as mean ± SD (n = 6). Asterisk (*) indicates statistically significant ($p < 0.05$).

1400 µg/cm^2 and 8154 ± 5071 µg/cm^2, respectively. On the contrary, Q_{abs} of [6]-gingerol through Permeapad® barrier was 158 ± 28.1 µg/cm^2.

4. Discussion

Permeation studies are usually performed using excised human and animal skin models such as porcine skin. It is generally described in literature that skin from adult pigs and piglets showed positive correlations to human skin, providing the most suitable experimental model for dermatological research (Schenk et al. 2018); (Barbero et Frasch 2009); (Schmook et al. (2001)); (Hawkins and Reifenrath, 1986). However, human skin is expensive and has intra- and inter-lot variability in data as high as 37 % and 50 %, respectively (Barry et al., 1984). A number of synthesized artificial membranes have been proposed as alternatives to human and animal skins (Hatanaka et al. 1990); (Sugibayashi et al. 2010). The *stratum corneum*, the outmost layer of the skin, is a heterogeneous membrane composed of dead protein rich cells (corneocytes) and intercellular lipids (Bouwstra and Ponec, 2006); (Bouwstra 2003) providing both hydrophilic and lipophilic domains for molecules transport. It is well reported, that this layer is regarded as the rate-limiting barrier for passive drug diffusion across the skin (Scheuplein 1965). Therefore, any artificial membrane used to assess skin absorption should mimic the *stratum corneum* structural properties as closely as possible. As it is now, the only synthetic, non-animal based barriers commercially available for studying drug permeation through skin is the Strat-M® and the Skin-PAMPA. Strat-M® barrier is composed by a polyethersulfone membrane in which the pores are impermeabilized with a lipid mixture. The synthetic Strat-M® membrane is an ultrafiltration membrane composed of polymeric layers impregnated with blends of synthetic lipids (Neupane et al. 2020). The Skin-PAMPA is quite similar in design to the Strat-M® (filter soaked with lipids) however, in this membrane mixture, the presence of other components such as free fatty acids and cholesterol simulate the *stratum corneum* barrier more closely (Sinkó et al. 2012); (SinkÃ3 et al. (2009)). The SMB used in this study has been designed to closely mimic not only the lipid composition but also the physical structure of the *stratum corneum* (Fig. 1). To validate this barrier, a diclofenac sodium solution (lipophilic compound) was used for assessing the resistivity of this membrane in comparison to human skin. Our results demonstrated that the resistance measured with the intact human skin and the SMB are in the same order of magnitude, ranging from $0.9 \times 10^5 \pm 0.60 \times 10^5$ s/cm to $1.04 \times 10^5 \pm 0.10 \times 10^5$ s/cm (Fig. 3). It is however important to point out some technical limitations of the SMB. Variability within batch to batch in the production remains relatively high (even though comparable or lower than human/pig skin, reported in Fig. 2). The manufacture process of the barrier is currently been improved to reduce the batch to batch variability. From the practical point of view, the barrier needs to be handled with cautions to prevent layer separations. However, steady state transdermal flux of diclofenac solution in the SMB was found to be close to human skin ($5.82*10^{-3}$ µg/cm^2*s for skin-mimicking barrier *vs* $6.06*10^{-3}$ µg/cm^2*s for human skin). Similarly, the resistivity values (P_{app}^{-1}) observed were $9.70*10^{-6}$ cm/s for SMB and $10.69*10^{-6}$ cm/s for human skin. Thus, the results demonstrate that the SMB and the human skin express comparable resistivity when low viscosity solutions are applied. This good correlation is by design and should be attributed to the characteristics of this new biomimetic barrier. To further scrutinize and compare the usefulness of the recently proposed SMB, percutaneous permeation study of [6]-gingerol after exposure to ginger lipophilic extract solution was conducted using the porcine ear skin and the commercially available artificial Permeapad® barrier. As it can be noticed from our data (Fig. 4), permeation of [6]-gingerol through SMB was comparable to that observed in porcine skin samples but not with Permeapad®. Notably, the flux measured was 0.07 µg/cm^2*s for both SMB and porcine skin. Conversely, the flux calculated through Permeapad® membrane was much lower compared to the other two tested membranes (0.01 µg/cm^2*s). This may be explained by the similarity of the new artificial membrane to human skin compared to the composition of the Permeapad® barrier. In fact, in Permeapad® the lipid layer is composed only by phosphatidylcholine S-100 that is comprised within two layers of regenerated cellulose. This structure allows only the free fraction of the drug to penetrate through, preventing any direct contact of the formulation excipients with the lipid layer. On the other hand, the top layer of the SMB is formed by a porous filter that allows the deep penetration of the ginger extract up to the hydrated lipid mixture layer (Fig. 1). This direct contact proves to be essential for a proper estimation of the drug penetration through the skin. The results demonstrated that the permeability coefficients measured through the SMB and porcine skin are almost identical ($1.41*10^{-6}$ cm/s for the SMB *vs* $1.36*10^{-6}$ cm/s for porcine skin) whereas, by using Permeapad® barrier the value was 13-times lower ($0.10*10^{-6}$ cm/s). The total amounts of [6]-gingerol found in whole porcine skin samples were lower compared to those quantified in SMB (2463 ± 1591 µg/cm^2 *vs* 6242 ± 1417 µg/cm^2) (Fig. 5). In accordance with the flux studies, also the accumulated [6]-gingerol into Permeapad® barrier was significantly lower (44.3 ± 2.8 µg/cm^2) than the SMB and the porcine skin (6242 ± 1417 µg/cm^2 for

SMB and 2463 ± 1591 μg/cm^2 for porcine skin). A probable explanation may be due to the structure of the Permeapad® barrier, which is most similar to the mucosal tissue in terms of permeation profile and permeability coefficient values (Bibi et al., 2016); (di Cagno et al., 2015) and cannot provide an insight into the interaction of the compound with the skin. Notably, the total absorbed amounts (Q_{abs}) of [6]-gingerol was comparable for SMB and porcine skin (8160 ± 1400 μg/cm^2 *vs* 8154 ± 5071 μg/cm^2), and a much lower Q_{abs} was observed in the case of the Permeapad® barrier (up to 152 ± 36.8 μg/cm^2). The differences between the two artificial barriers suggest that Permeapad® membrane cannot mimic the biological structure or performance of the *stratum corneum*, while the newly introduced SMB could be a possible alternative to diffusion cells in permeation studies.

5. Conclusion

The current study aimed to investigate the potential of SMB in predicting absorption of [6]-gingerol following exposure to ginger pure extract. This newly introduced system consists of a lipid layer containing ceramides, cholesterol, and squalene, and can effectively simulate the real human *stratum corneum*, making this methodology promising to assess other drugs. Our findings demonstrated that this synthetic barrier has a high and positive correlation with porcine skin compared to the artificial barrier design to study mucosal absorption (Permeapad® barrier). The SMB tested is cost effective, no pretreatment is required and, due to the uniform structure, it reduces the variability typically present in skin permeation studies or in other polymeric membranes such as cellulose acetate. Although further investigations need to be performed, the SMB tested in this work is able to provide a rapid initial estimation of the amount of compound absorbed through the skin. This suggests its potential use as a good alternative to *ex-vivo* animal skin for skin penetration measurements.

CRediT authorship contribution statement

Greta Camilla Magnano: Investigation, Data curation, Writing – original draft. **Stefania Sut:** Investigation. **Stefano Dall'Acqua:** Investigation. **Massimiliano Pio Di Cagno:** Conceptualization, Supervision, Writing – review & editing. **Luke Lee:** Investigation. **Ming Lee:** Investigation. **Francesca Larese Filon:** Funding acquisition, Supervision. **Beatrice Perissutti:** Supervision. **Dritan Hasa:** Supervision. **Dario Voinovich:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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