Journal Pre-proofs

Review

How to define the experimental conditions of skin permeation assays for drugs presenting biopharmaceutical limitations? the experience with testosterone

Nicole Esposto Biondo, Débora Fretes Argenta, Gabriela Schneider Rauber, Thiago Caon

PII: S0378-5173(21)00793-6
DOI: https://doi.org/10.1016/j.ijpharm.2021.120987
Reference: IJP 120987

To appear in: *International Journal of Pharmaceutics*

Received Date: 7 June 2021
Revised Date: 5 August 2021
Accepted Date: 6 August 2021

Please cite this article as: N. Esposto Biondo, D. Fretes Argenta, G. Schneider Rauber, T. Caon, How to define the experimental conditions of skin permeation assays for drugs presenting biopharmaceutical limitations? the experience with testosterone, *International Journal of Pharmaceutics* (2021), doi: https://doi.org/10.1016/j.ijpharm.2021.120987

HOW TO DEFINE THE EXPERIMENTAL CONDITIONS OF SKIN PERMEATION ASSAYS FOR DRUGS PRESENTING BIOPHARMACEUTICAL LIMITATIONS? THE EXPERIENCE WITH TESTOSTERONE

Nicole Esposto Biondo; Débora Fretes Argenta; Gabriela Schneider Rauber; Thiago Caon*

Postgraduate Program in Pharmacy (PGFAR), Federal University of Santa Catarina, Trindade, 88040-900 Florianopolis, SC, Brazil.

*Corresponding author: T. Caon

Laboratory of Pharmaceutical Technology & Cosmetology
Department of Pharmaceutical Sciences
Federal University of Santa Catarina (www.ufsc.br)
Telephone number: +55 48 3721 5850
E-mail: caon.thiago@ufsc.br / thiagocaon@gmail.com
ORCID: 0000-0003-3030-6310

ABSTRACT

Cutaneous permeation assays are crucial to attest the performance or bioequivalence of topical or transdermal products. Although the official guidelines (e.g., FDA/EMA) play a key role in harmonizing the experimental design, alternative methods are often proposed by the scientific community, which makes it difficult to compare results from different studies. In this review, permeation assays with testosterone (TST) were selected to show this high variability in drug transport rate. The main sources of variation discussed were tissue thickness, animal model, donor and receptor fluid

constitution, type of solubilizing agent used in aqueous fluids, drug concentration, degree of supersaturation, skin lipid content, number of experimental times and the physical-chemical stability of the molecule in test fluids. This variation becomes even more critical for molecules that present biopharmaceutical limitations such as TST. In addition, the skin presents specific receptors for this hormone due to its physiological action in this region of the body, which makes the evaluation of the TST transport rate in this tissue even more challenging. The impact of each experimental parameter mentioned above on the flux or permeation coefficient of TST is discussed in detail in the review. Assays used to evaluate tissue integrity are also presented.

Keywords: permeation studies; Franz-type diffusion chamber; skin integrity evaluation; testosterone.

1. INTRODUCTION

Many topically administrated therapeutic agents present a limited or suboptimal efficacy due to low penetration into the skin. Solubility and partition coefficient are key aspects related to drug transport through the skin. Overall, high solubility results in high drug concentration in the donor phase (or region of application of the formulation), improving the permeation flux. The drug partitioning from the donor phase into skin layers, in turn, represents the rate-limiting step for drug flux when these molecules are characterized by a poor aqueous solubility taking into account the lipid constitution of biological membranes (Ceschel et al., 2005).

Steroidal hormones, for example, are characterized by a low aqueous solubility (Norman and Litwack, 1997), requiring the investigation of technological/formulation approaches aiming to increase the free drug concentration in the donor phase. Drug complexation (e.g. association with cyclodextrins), incorporation in vesicular or particulate systems (e.g. micro- and nanoparticles) and supersaturated systems have been often considered for this purpose (Ceschel et al., 2005).

On the other hand, the low aqueous solubility of hormones allows a drug partitioning from the donor phase into skin layers. Due to the high value of log P, these compounds bind so strongly to the tissues, particularly the more superficial layers (SC and viable epidermis), resulting in a reservoir effect and low transport rate to the dermis (Magnusson et al., 2006). This event is particularly noticed for TST after application of low concentrations of this agent on the skin (Schlupp et al., 2014).

In addition to the slow tissue diffusion because the high affinity by lipids and high molar mass, the skin presents receptors for these compounds due to the physiological action they play in that region of the body. The TST interacts directly with androgen receptors (ARs), which have been localized in most epidermis keratinocytes. In the

dermis, ARs were found in approximately 10% of fibroblasts. AR expression was also found in both basal cells and sebocytes of sebaceous glands whereas it was restricted to dermal papilla cell in hair follicles (Pelletier and Ren, 2004). A modest reduction in epidermal thickness is also observed in TST replacement therapies (Kao et al., 2001).

In view of this high affinity of sex hormones by skin tissues, the inclusion of chemical absorption enhancer combinations able of providing membrane fluidification or lipid extraction is often recommended during the development of novel formulations. These compounds may interact with both lipid and polar domains (Magnusson et al., 2006) and thus chemical absorption enhancers of both polarities should be considered.

The evaluation of percutaneous permeation of molecules is a key step when new dermal or transdermal delivery systems are purposed and different *in silico*, *ex vivo* and *in vivo* models may be considered. Although *in vivo* human assays represent the gold standard, ethical, practical, or economic reasons have limited its use. Alternatively, tissues from different animal models, particularly pig ear skin, have been selected in many permeation studies. These tissues are easier to obtain compared to human tissues, however, high experimental variability may be found. Barrier properties may also vary from human skin depending the selected animal model (Abd et al., 2016).

In addition to the inherent variability of the special animal considered, the tissue thickness and preparation method, composition of the receptor and donor fluid can also represent sources of experimental variation. All these aspects will be discussed detailly in this review article, which considered the TST as a drug model. This drug was selected based on its biopharmaceutical limitations (low solubility and reservoir effect), the various permeation studies already carried out with this compound and its wide use in hormone replacement therapies. Although TST therapy has effectively treated

hypogonadism for decades, therapies simpler and more convenient to use, safer and able to mimic physiological levels are still needed (Kaminetsky and Wynia, 2015).

The main objective of this study is to provide tools so that future permeation assays can be planned more rationally considering a series of problems that may be found in each step assay. Once free drug solutions are frequently used as the control during the evaluation of the performance of new topical formulations in skin permeation studies, this review focused on the analysis of studies with free TST solutions/suspensions. The solubility of this molecule in different solvents, the importance of this physicochemical parameter in the design of permeation studies as well as aspects related to the drug quantification step are described in detail.

2. SOLUBILITY STUDIES

Prior to the cutaneous permeation assay, a study of the drug's solubility in different media should be performed. Drug should be soluble in both media used for donor and receptor phases of diffusion chambers and in solvents used to extract the drug from tissues or analytical/quantification step. If a drug is not soluble in the receptor phase, for example, low drug partitioning into this phase with consequent low transdermal flux is obtained (permeation rate can be underestimated) and solubilizing agents or cosolvents need to be included in these situations. For this reason, many permeation guidelines provide information on the need to carry out these assays under “sink conditions” (OECD, 2004). In other words, the drug concentration should not reach values >10% of its saturation to assure that concentration in the receptor fluid does not limit the drug permeation across the tissue (Azarmi, Roa, Löbenberg, 2007).

For ionizable drugs, solubility study at different pH values should be carefully performed. The ionized-non-ionized drug fraction changes with pH, which impacts not only on solubility but also on the affinity by the biological tissues.

In general, the thermodynamic solubility is evaluated via a long duration incubation (24-72 h) starting with solid materials, which is known as shake-flask method (Zhou et al., 2007). After adding an excess of solid material in given amount of solvent (above saturation), the flasks are placed on an orbital shaker with controlled temperature for 18-72 h (until the system reaches a thermodynamic equilibrium condition). Temperature close to that used in permeation assays should be preferentially selected. Finally, an aliquot of this medium is removed and then centrifuged or filtered through a 0.45 mm membrane for drug quantification.

Ethanol has been one of the most used solvents for the solubilization of drugs. It may also play a role as a skin penetration enhancer (Williams and Barry, 2012), but disruption in the SC may be observed depending on used ethanol concentration. Overall, the permeation assays reported for the TST use high concentrations of ethanol, or even the solvent pure in the donor receptor (Hewitt et al., 2020). Although permeation of lipophilic molecules is less affected by ethanol concentration than hydrophilic molecules (Horita et al., 2012), the establishment of a maximum amount of solvent is an important criterion for not having overestimated permeation values. The use of ethanol pure has been suggested to markedly alter skin permeability (Thomas and Panchagnula, 2003). The solvent evaporation is another concern regarding the use of ethanol in the donor phase. This fact leads to an increase in the TST concentration with formation of precipitates, which impairs the entry of the molecules into the SC. Thus, the evaporation rates depend on ethanol concentration, duration of the assay and temperature used.

Although the solubility of TST is higher in pure ethanol, its combination with water or buffer solutions can be considered for skin permeation studies using Franz-type diffusion cells. Determination of experimental conditions as concentration of TST in the donor chamber and the temperature used in the assay are relevant for the selection of hydroethanolic solution. An increase in ethanol concentration from 20 to 50%, for example, increases the TST solubility in approximately 31-fold. When this parameter is changed from 50 to 70 %, in the same conditions of temperature (37 ^{o}C) and agitation time (48 h), the hormone solubility increases about 6 times (Table 1).

Propylene glycol has also been used in various percutaneous permeation studies with TST. The hormone solubility in PBS/propylene glycol (1:1) is approximately 24-fold higher than in pure PBS (Table 1). PG can also act as a chemical permeation enhancer. This agent permeates through human SC and can change the thermodynamic activity of drugs (Williams and Barry, 2012). Carrer et al., (2020) investigated its effect on the transport of molecules with different physicochemical properties. Interestingly, the permeation enhancing effect of PG was more pronounced for hydrophilic compounds. Protein denaturation (solvation the α-keratin structures of the cells) is the most probable mechanism of action, which can contribute to the barrier disruption and fluidization of intercellular lipids or intracellular expansion (Haq and Michniak-Kohn, 2018).

[Table 1]

3. RECEPTOR PHASE SELECTION

The diffusion cell apparatus has been widely used for percutaneous permeation studies of drugs and it may be categorized into static or flow-through cells. In both classes, formulations are applied to the surface of a membrane, which is sandwiched between a donor and a receiver compartment of the diffusion cell (Moser et al., 2001). In static

diffusion cells, samples are collected and the same volume of fresh perfusate is added at each time point (Bronaugh and Stewart, 1985). In these systems, the membrane, donor and receiver chambers may be placed either "vertically" as in the popular Franz diffusion cell or "horizontally" (Zsikó et al., 2019). In flow-through diffusion cells, a pump continuously supplies the receiver compartment with perfusate, simulating the blood flow from the dermis (Bronaugh and Stewart, 1985). When more physiologically relevant assessments of percutaneous permeation for lipophilic compounds are needed, flow-through diffusion cells should be prioritized (Clowes et al., 1994).

In static diffusion cells (most used in skin permeation studies), the volume of diffusion cell receiver chamber should be selected to guarantee detectable concentrations of the permeant in the receiver medium. In this context, the receptor phase should present a reduced volume for the evaluation of drugs with low permeation rate as TST. Likewise, the smaller the amount of TST added in the donor phase, the smaller volumes of receiver medium are recommended. The system agitation should be enough to obtain a homogenous distribution of the permeant and temperature equilibrium in this medium.

The composition of receiver phase should ensure that "sink conditions" may be achieved and barrier properties of biological tissues are preserved (Cilurzo et al., 2018; Ng et al., 2010). In an acceptable sink condition, the maximum concentration of drug in the receptor fluid in the permeation assay should not exceed 10% of its maximum solubility (EMA, 2012). It is calculated by the relationship between C_S and C_D ("C_S" is the saturated solubility of the compound in the medium whereas "C_D" represents the concentration of compound in the bulk medium).

Isotonic saline or buffered isotonic saline (pH=7.4) are often used for highly soluble drugs to mimic the physiological environment; however, solubilizer agents need to be included when poorly water-soluble drugs are considered (Finnin et al., 2012). Azone®

and *N*-methyl-2-pyrrolidone, for example, increased the hormone solubility from 0.02 mg/mL (in water) to 92.29 mg/mL and 518.89 mg/mL, respectively (Table 1). Although the solubilization of TST by these agents allows sink conditions to be achieved, the impact on the skin structure discourages their application (Fang et al., 2003). Thus, aqueous solutions containing less aggressive agents such as ethanolic solutions, bovine serum albumin or propylene glycol should be alternatively considered for the composition of receptor fluid (Table 2). Sodium azide was also added to the receptor solution in some studies with an experimental time of 24 h (Table 2). This compound acts as a preservative and is therefore recommended for long-term permeation assays (Bartosova and Bajgar, 2012).

4. EFFECT OF DRUG CONCENTRATION FROM DONOR PHASE

According to Fick's 1st law (Equation 1), the permeation flux of a drug is proportional to its concentration in the vehicle (or thermodynamic activity, A_v), occurring in favor of a concentration gradient. High drug solubilization leads to a high thermodynamic activity in the donor phase, improving its permeation flux through the SC (Ceschel et al., 2005).

$$J = \frac{K.C_0.D}{L} = \frac{\gamma_v.C_0.D}{\gamma_s.L} = \frac{A_v.D}{\gamma_s.L} \quad \text{(Equation 1)}$$

Where: K, C_0, D, γ_v and γ_s represent the partition coefficient, initial concentration in the formulation, diffusion coefficient, activity coefficient in the formulation and that from the skin barrier, respectively. L is the tissue thickness (Ishii et al., 2010).

The maximum permeation flux (J_{max}), in turn, is achieved at the maximum solubility (S_s) of a solute in the SC (Couto et al., 2014), which also corresponds when solute solubility in vehicle achieves its saturation condition.

$$J_{max} = \frac{D.S_s}{L}$$ (Equation 2)

In general, the permeation profile of TST through the skin follows a Fickian diffusion given the absorbed drug fraction or rate in specific period is constant. When the diffusion of the TST in the skin is analyzed, its transfer rate to the SC is faster whereas a slower rate from the dermis to the systemic route is showed. As already discussed, this fact is attributed to reservoir effect of TST in tissue. The greater the interaction or partitioning of the drug with the tissue, the slower the diffusion rate.

The fluid composition of the donor phase appears to impact on the permeation rate more significantly than the TST concentration (Table 2). In a study that considered 0.22, 6.31 and 5.14 mM of TST in only PBS, ethanol/PBS and propylene glycol/PBS in the donor phase of diffusion cells, permeation coefficient (K_p) values from equine skin were 6.82, 1.59 and 2.04 x 10^{-3} cm/h, respectively (Mills, 2007). Although the donor phase containing only PBS had a lower drug concentration, it provided a higher K_p value. These findings may be associated with solubility differences of TST in the donor phase, which affect the supersaturation degree (greater in aqueous solutions). In another study considering a similar donor phase composition (solubilizer type and ratio as well as TST concentration) and canine skin as membrane model, donor solutions without solubilizer agent and with lower TST concentration also provided higher K_p values (Mills et al., 2006). In the same way, in a permeation study carried out with different concentrations of ethanol in water (20, 40, 50, 60, 70, 80 and 100 %) and TST (0.45, 8.53, 18.52, 38.95, 68.32, 140.01 and 33.04 mg mL^{-1}), lower K_p values through rat skin were found for increasing amounts of ethanol and TST (Kim et al., 2001). In these analyzed studies, the inclusion of propylene glycol seems to be more advantageous than ethanol in increasing the cutaneous transport of TST.

In permeation studies, authors commonly mention that an increased thermodynamic activity provided by a state of supersaturation results in higher permeation rate (Schwarb et al., 1999), however, it is necessary to be careful with this assumption. In a study comparing the permeation rate of TST from saturated solution and suspensions presenting different supersaturation degrees (1.4, 2.1 and 2.6), only the suspension with a supersaturation degree of 1.4 provided a higher flux permeation than control. For the other conditions, a permeation flux similar to the saturated solution was found because drug precipitation phenomena would be observed, reducing the free drug amount available to be absorbed (Leichtnam et al., 2006) Considering these findings, it is important to consider the inclusion of anti-precipitating agents in tested medium so that the advantages resulting from the increase in the degree of supersaturation can be observed. This may also explain why membrane-based systems (e.g., polymeric films or transdermal patches) can provide higher rates of cutaneous permeation compared to the solution or other liquid systems in specific situations.

5. IMPACT OF TISSUE PREPARATION ON DRUG PERMEATION

Permeation assays may be performed by using epidermal membranes, dermatomed skin, or full-thickness skin. The use of epidermis provides a greater correlation with *in vivo* situation (in humans). The presence of dermis in dermatomed skin and full thickness skin acts as an unreal barrier since continuous blood flow occurs within watery dermis only in *in vivo* environment. Although the use of the epidermis provides more realistic information on drug transport (Barbero and Frasch, 2016), the tissue preparation is more difficult and susceptible to structural damages. Currently available methods to separate the epidermis from the dermis use heating or chemical treatment (e.g. treatment with salts, detergents, enzymes) (Abd et al., 2016), which can affect both tissue integrity and

skin metabolic activity. The tissue treatment with trypsin, for example, may lead to a SC more heterogeneous, contributing to the partitioning of more polar solutes (Magnusson et al., 2006). Another disadvantage is that hair follicles may be damaged during the tissue separation process, leading to drug leakage (Barbero and Frasch, 2016).

The transappendageal route is preferentially used to transport hydrophilic drug, high molecular weight compounds and drug delivery systems as nanoparticles (Knorr et al., 2009); however, Hueber et al. (1992) has also demonstrated its contribution in transport of TST (via sebaceous glands). In another study performed by the same research team (Hueber et al., 1994), the absorption of TST from the human normal skin was approximately 2.4-fold higher than in human scar skin (without skin appendages) at the end of 8 h. These findings showed the importance of preserving the appendages of the skin in permeation studies.

Transfollicular drug delivery may be evaluated by comparing different body regions, use of specific animal models, artificial introduction of hair follicles in skin equivalents and/or blockage of hair follicles compared to untreated skin (Knor et al., 2009). Porcine skin is regarded as the most representative animal model to determine the contribution of the follicular route in drug transport due to the similarity to human skin. In fact, porcine and human skin present about 20-30 follicles per cm^2 of skin area, and a hair density of 11-25 hairs/cm^2 with a diameter of 58-97 μm. Animal models such as rat and mouse skin exhibit much more follicles, with smaller diameters when compared to human skin (Lauterbach and Müller-Goymann, 2015). Follicle-free skin models as EpidermFT™ (for comparison with human skin presenting follicles) and *in vitro* models of human fibroblasts or keratinocytes with hair follicles may represent alternatives to animal use in these studies (Krugluger et al., 2005; Michel et al., 1999).

"Full-thickness skin" is prepared by removal of connective tissue and subcutaneous fat and its thickness may be reduced with a dermatome. This procedure reduces experimental variability (Abd et al., 2016). Although dermatomed and full-thickness skin present an additional barrier that is not found *in vivo*, particularly for lipophilic drugs, the tissue integrity is preserved (Barbero and Frasch, 2016). As a result, the absorption rate of drugs could be underestimated. The presence of this additional layer represents a situation analogous to vasoconstriction (Abd et al., 2016).

Interestingly, a study testing only the dermis layer as membrane model and TST was also performed (Kretsos et al., 2008). The permeation coefficient value found from this study showed to be higher than other studies performed with full-thickness or dermatomed skin as well as only with human epidermis (Baert et al., 2012; Guth et al., 2015; Hewitt et al., 2020; J R Heylings et al., 2018; Kretsos et al., 2008; Netzlaff et al., 2006; Qvist et al., 2000; Schreiber et al., 2005; Veryser et al., 2015), confirming that both SC and viable epidermis play key barrier properties.

A significant difference in permeation flux of TST through human dermatomed skin (most of the dermis removed) and full-thickness skin was observed, with a TST flux of approximately 10-fold higher in the thinner skin (Wilkinson et al., 2006). Thus, membrane thickness should be a critical variable for TST permeation across human skin. The intra- and inter-laboratory study between 10 laboratories found higher flux rates of TST using skin samples presenting thickness between 300 and 500 µm (2.82 to 5.39 $\mu g.cm^{-2}.h^{-1}$) compared to skin samples with thickness between 700 and 900 µm (0.40 to 0.80 $\mu g.cm^{-2}.h^{-1}$) (Van de Sandt et al., 2004). This finding is consistent with the lipophilic nature of TST, which difficult its transport through the dermis. Therefore, permeation results obtained for lipophilic agents after the transport in full-thickness or dermatomed tissue preparations should be carefully analyzed (Magnusson et al., 2006).

The use of epidermal membranes, in turn, may overestimate the drug absorption in humans because of insufficient barrier function. The use of cultured and reconstructed human skin models (e.g. constructed from keratinocytes) is not recommended for the determination of dermal penetration as these models have not been validated for dermal absorption studies and there are reports that their barrier properties are not comparable with those of 'natural origin' skin (SCCS, 2010; WHO, 2006).

The OECD recommends the use of dermatomed skin with a thickness between 200 and 400 μm for harmonization of *ex vivo* studies with human skin. In these range of thickness, the membranes tend to have significantly lower levels of residual material than full-thickness preparations. For permeation assays with TST (Table 1), dermatomed and full-thickness skin models appears in most of these studies. The use of membranes with 400 μm of thickness was prevalent among the studies with human skin, even though, different values of permeated drug and rate flux were showed. Higher permeability coefficient values (K_p) have been reported with the use of only epidermis compared to dermatomed skin as already mentioned. Correlations can be established by regarding studies performed in similar experimental conditions (donor and receptor fluid). Netzlaff et al. (2006) and Schreiber et al. (2005), for instance, found K_p values of 9.4×10^{-4} and 8.3×10^{-4} $cm.h^{-1}$, respectively, with human epidermis separated by heat. In contrast, lower K_p values, 4.5×10^{-4} and 3.92×10^{-4} $cm.h^{-1}$, were showed by Baert et al. (2012) and Veryser et al. (2015), respectively, using skin samples with a thickness of 400 μm. In fact, the tissue preparation has a significant effect on the TST permeation. The studies showed an increase of about 2-fold in permeation rate of the hormone without dermis layer.

The tissue storage conditions also impact on drug permeation. As the skin preparation step is laborious, the tissue is commonly frozen for a specific period before the use in

the permeation assays. The freezing does not affect the skin barrier integrity and it is a suitable procedure to measure the passive permeation of drugs (Barbero and Frasch, 2016). One the other hand, it is worth mentioning that frozen tissue is not appropriated to investigate the metabolic activity of drugs (Fahmy et al., 1993).

In general, the permeation assays with TST were performed using similar storage conditions. The tissues were frozen at -20 ºC in all studies with human skin, which is according with regulatory agencies guidelines (OECD, EU Scientific Committee on Consumer Products, US Environmental Protection Agency, International Programme on Chemical Safety). Tissue preparation for permeation studies was performed prior to freezing. The human skin storage time, in turn, often ranged from 3 (Baert et al., 2012; Hewitt et al., 2020) or 6 months (Schreiber et al., 2005; Veryser et al., 2015). A consensus among regulatory agencies regarding the freezing time of tissues prior the use has not yet been reached. For example, the International Programme on Chemical Safety (IPCS) states that human skin can be stored to one year, whereas the US Environmental Protection Agency (EPA) allows the storage for up to 3 months. The skin thawing should also be standardized in assays since the use of frozen skin without hydration provides different permeation rate values when compared to fresh tissue. Thus, the tissues should be appropriately rehydrated before use (Swarbrick et al., 1982).

6. SELECTION OF ANIMAL MODELS OR MEMBRANE TYPE

Human skin is the preferred membrane model to predict the *ex vivo* permeation of compounds. Excised skin is commonly obtained from autopsies (cadaver skin) or plastic surgery. Overall, the permeation assays of TST were performed with tissues from abdomen and breast. Several animal models have been alternatively considered to human skin, including pig, mouse, rat, guinea pig and rabbit skin. Porcine skin has been

widely used given its histological similarity to human skin. It presents a comparable SC thickness, which varies from 21 to 26 μm, and similar hair-follicle density (20 vs. 14-32/cm^2 for porcine ear skin and human forehead skin, respectively) (Jacobi et al., 2007). Porcine SC lipids are organized as a hexagonal lattice whereas human SC lipids are arranged in the denser orthorhombic lattice (Caussin et al., 2008).

The easy availability and relatively low cost make the group of rodents (mice, rat, and guinea pigs) widely used in *ex vivo* percutaneous permeation studies. Although rat skin is most structurally similar to human skin among the rodents, rat dermis is thicker than human and does not present a defined limit between papillary and reticular dermis. In addition, rats have no subcutaneous fatty tissue or subcutaneous muscle tissue (McFarlane et al., 1965). In terms of permeability, significant differences between rat and human skin have been found for various compounds presenting different physicochemical properties (Abd et al., 2016). *Ex vivo* permeation studies with saturated and supersaturated solutions of TST applied in rat skin have been performed (Kim et al., 1999; Leichtnam et al., 2006). Overall, higher values of flux and permeation coefficient were found with rat skin compared with human skin (Table 1). Nevertheless, the rat is also often used for *in vivo* studies because the extensive pharmacokinetics/ pharmacodynamics data that may be obtained with this species.

Netzlaff et al. (2006) performed *ex vivo* permeation studies with TST and skin models from different species. The permeation coefficient values of TST through bovine udder, human and pig skin were 5.42, 2.31 and 1.29 (x 10^{-7}) cm.s^{-1}, respectively. Differences in skin composition explain these findings. Human and pig skin are characterized by a much higher lipid fraction (triglycerides and free fatty acids) than udder skin, which increase the affinity of TST by the tissue. Although these tissues significantly differ in relation to the number of follicles per tissue area (6, 30-36 and 207-338 mm.cm^{-2} for

human, pig and bovine udder skin, respectively), this permeation route is not used for lipophilic molecules such as TST. The epidermal thickness also varied with the animal species (57-82, 40 and 54-92 μm for human, pig and bovine udder skin, respectively), but this parameter would present a marginal contribution on permeation rate of TST.

TST has been used as lipophilic molecule model in several percutaneous permeation studies. When the different studies are compared (Table 2), mice, amphibian and equine skin seem to be more permeable to TST than human or porcine skin (K_p values increased from 10 to 100 times). In fact, amphibian skin, for example, presents only 1-2 cell layers of stratum corneum with no intercellular lipid layers, offering less resistance to TST transport (Lillywhite, 2006). In the same way, mice also have a stratum corneum that is thinner than humans (5 vs. 10-20 μm) (Wei et al., 2017), which may explain the greater permeation rate.

As amphibian skin is much more permeable than other vertebrates and more sensitive to environmental contaminants, this skin model has not only been considered to assess the absorption of molecules but also as an indicator of the relative health of an ecosystem (Llewelyn et al., 2019). Kaufmann and Dohmen (2016) performed permeation assays with TST in the African clawed frog skin (X. laevis, wild type). The permeation coefficient values ranged from 1.3 to 3.0 x 10^{-3} cm.h^{-1}. Regarding the same composition of donor and receptor fluid, Baert et al. (2012) evaluated the TST permeation across human skin and found a permeation coefficient of 4.5 x 10^{-4} cm.h^{-1}. Therefore, the permeation of TST through amphibian skin was 2.9 to 6.7-fold higher than human skin.

In vitro permeation studies in equines and canines have also been performed (Mills, 2007; Mills et al., 2006) since the skin is an administration route used for the TST in these animals. The main clinical use of TST in horses aims to increase libido and treat

hypogonadism (Snow, 1993). Its application to improve the performance of animals in competitions is illegal (Houghton and Maynard, 2010). In dogs, the hormone is particularly used for treating TST-responsive urinary incontinence and for suppression of oestrus in racing greyhounds (Blythe et al., 1994; Plumb, 2002).

Synthetic membranes are structurally simpler and demonstrate superior permeation data reproducibility since *in vivo* variables as skin age, race, sex and anatomical site are eliminated. In contrast, they do not exhibit the lipid perturbation effects undergone by biological samples (Ng et al., 2010). For the TST, all synthetic silicone-based membranes demonstrated lower barrier properties and thus greater permeation compared to biological tissues. The fact that synthetic membranes do not have receptors for TST, and present greater permeability may raise the hypothesis of a probable relationship between physiological receptors and the reservoir effect observed for this hormone; however, further investigations should be performed.

Reconstructed skin models are interesting substitutes for human and animal skin because they overcome the problems of availability of human skin and the ethical issues of using animals. On the other hand, the OECD has not yet approved the use of these models for skin absorption assays (only for *in vitro* skin irritation assays). Therefore, more studies are needed to validate the models for this purpose. In addition, reconstructed skin is usually more permeable than human skin. The low barrier property of reconstructed skin was demonstrated by Schreiber et al. (2005). The authors founded higher permeation coefficient values for TST using reconstructed epidermis (K_p= 176.4 x 10^{-5} and 777.6 x 10^{-5} cm.h^{-1}) compared to human epidermis (K_p= 7.6 x 10^{-5} cm.h^{-1}) and porcine skin (K_p= 31.7 x 10^{-5} cm.h^{-1}). The assays with porcine skin and human epidermis were performed in 24 h whereas the use of reconstructed skin reduced this time to 6 h. The short lag time and high flux values are associated with the reduction of

experimental time. For this reason, the reconstructed skin model has been considered advantageous for routine and screening assays, particularly when several compounds or topical formulations should be analyzed at the same time.

The efforts to improve the barrier function of these models is constant. Simard et al. (2019), for instance, evaluated the effect of different lipids on skin barrier properties by considering a 3D reconstructed skin model. A slower permeation rate of TST was found after supplementation with alpha-linolenic acid. On the other hand, the supplementation with linoleic acid did not affect the drug absorption. Thus, a proper composition and proportion of fatty acids in culture media should be considered to achieve similar barrier properties to human skin.

7. ASSAY TOTAL DURATION & SAMPLING INTERVALS

Considering that the TST presents a low diffusion rate through different skin layers, it is rational to consider a long experimental time in permeation studies. Moreover, the more effective barrier effect of the membrane, the longer the time of experiment required to obtain the permeation data for a certain compound. In fact, most of the permeation assays with TST were performed over a time equal or greater than 24 h (Table 2). One the other hand, a time exposure of 12 h or less was enough for permeation assays performed with tissues characterized by greater permeation as mice, rat and amphibian skin. Therefore, the assay total duration depends mainly on the selected membrane model. The distribution of the sampling intervals should be as homogeneous as possible and should not consider too many points. Permeation studies with a sampling time at 12 h and the next at 24 h can be found, however, if any operator error occurs during sampling, all permeation parameters (flux, permeation coefficient and lag time) may be erroneously calculated. Therefore, a homoscedastic distribution of sampling intervals

should be designed. Overall, the concentration of TST in receptor fluids was evaluated at time intervals of 0, 1, 2, 4, 8, 12, 16 18, 20, 21 and 24 h in studies with human skin (Baert et al., 2012; Hewitt et al., 2020; Veryser et al., 2015) Another problem for drugs as TST is the inclusion of many sampling intervals. For each replacement of the medium of the receptor phase, the drug is diluted. In these situations, if the quantification method is not sensitive enough, TST may not be detected. This problem occurs particularly when various initial sampling intervals are defined (drug is found in the least amount in the medium in these periods).

8. SKIN INTEGRITY ASSAYS

The evaluation of tissue integrity is strongly recommended for permeation tests involving long incubation periods, such as those considered for the TST. Permeation assay itself can provide indications of the impairment of barrier properties. A change in tissue structure can result in permeability changes (Bennion et al., 2017). If the permeation rate is modified over the course of the assay or an extremely large amount of drug permeates through membrane in specific intervals, this fact may indicate a compromise in the barrier properties and further investigations should be carried out.

OECD, WHO and FDA guidelines recommend the evaluation of skin integrity prior to the permeation study to identify samples that affect the tissue barrier function. In addition to a visual inspection of the skin, the OECD proposes the measure of transepidermal electrical resistance (TEER), transepidermal water loss (TEWL) and tritiated water. No guidance on how to perform and interpret these integrity tests or then reference values for each assay is available in official guidelines.

While physical methods evaluating TEER and TEWL are useful for the selection of tissues for assays (Bartosova and Bajgar, 2012), histological studies allows to identify the region of tissue most affected (e.g. epidermal desquamation, dermal vacuolization).

TEER represents a simple, rapid, and cost-effective method of cellular barrier integrity. TEER measurements based on impedance spectroscopy have proven to be more reliable and provide more information about the cells when compared to the Ohm's law method (Srinivasan et al., 2015). The measured resistance is dependent on the device, applied frequency, resulting current, ionic strength of the solution, temperature and surface area of the tissue (Guth et al., 2015). Skin samples from different animal models may also affect TEER values. In a study with human, mouse, guinea pig, pig, rat and rabbit skin, TEER values were ≥10, 5, 5, 4, 3 and 0.8 kΩ, respectively (Davies et al., 2004). Kaufmann and Dohmen (2016), for instance, used TEER to evaluate the integrity of fresh excised and frozen amphibian skin. The higher impedance measurements for fresh skin suggested that the freezing step affected the epidermal integrity. In fact, the ice crystal formation in the frozen skin can lead to the disruption of epidermal cells, impairment the tight junctions, and hence impedance decreases. The K_p value of TST through the fresh dorsal skin was lower (1.3×10^{-3} $cm.h^{-1}$) than frozen skin (1.9×10^{-3} $cm.h^{-1}$). On the other hand, a minor transition between intact and damaged skin can be found due to the low impedance of amphibian skin and thus methods such as TEWL would be recommended for analysis of amphibian skin integrity.

TEWL methods measure water vapor flux in the air above the SC, which is an indicator of water diffusion through SC and its barrier properties. A temperature and moisture stabilization time around the probe are required before measurements (Neupane et al., 2020). Another caution refers to the complete removal of moisture on the skin surface generated from rehydration of the frozen skin samples or TEER measurements given

that the assay objective is to measure exclusively the water loss from the skin sample (Guth et al., 2015). For human skin, TEWL values ranges from 4 to 10 $g/m^2/h$ depending on the tissue area. When the epidermis is damaged, these values may increase up to 30-times (Boer et al., 2016).

The transepidermal water flux (TWF), which involves the measurement of tritiated water (a radioactive form of water), is another assay able to evaluate the skin barrier function. This test can be carried out before, during or after the permeation assay. When it is performed only before permeation study, eventual tissue damages caused by treatments will not be identified. In this same way, limitations may be found when TWF is performed only at the end of permeation assays. Once the tissue is exposed to infinite amounts of water or hydration for many hours during the permeation and it is often washed after its removal of diffusion cells, which may lead to tissue deterioration, TWF measurements at this stage may reject previously intact tissues (Fabian et al., 2017).

Although these assays detect only changes in the polar transport pathway (Flynn et al., 1974), Guth et al. (2015) considered them during the evaluation of tissue integrity prior to permeation studies with TST. The limit to distinguish damaged and intact tissues was 10 $g\ m^{-2}\ h^{-1}$, 4.5×10^{-3} $cm\ h^{-1}$ and 2 kΩ for TEWL, TWF and TEER, respectively.

The inclusion of chemical markers (e.g., mannitol) whose permeation is already well known have also been considered. In general, two reference compounds presenting different physicochemical characteristics are selected. The results of these tests should be carefully analyzed given that the presence of additional compounds in the donor phase may affect the absorption characteristic of the test compound due to changes in solubility or saturation levels (Guth et al., 2015).

Absorption chemical enhancers are the main agents responsible for compromising the skin barrier properties; however, it is important to highlight that the drug itself may also

affect them. For TST, studies have demonstrated that fluctuations in its level modulate barrier function and that hormone replacement with TST can exert a negative consequence for permeability barrier homeostasis (Kao et al., 2001).

9. SAMPLE STORAGE BEFORE QUANTIFICATION

Samples should be quantified immediately after the permeation test has been completed; however, some studies have stored the samples under refrigeration until quantification step (Table 2). It is well known that the storage of drugs presenting low aqueous solubility under refrigeration can lead to the precipitation and that analytical errors can increase because of a non-soluble drug fraction. On the other hand, storage at room temperature increases the chances of drug degradation by hydrolysis, oxidation and/or enzymatic reactions. Thus, a prior analysis of the drug's stability in different aqueous media and conditions (presence and absence of enzymes, temperature, pH, etc.) is recommended.

10. DRUG QUANTIFICATION STEP

The quantification of steroids from biological matrices (e.g. blood, plasma, urine and skin homogenate) usually involves extraction as the first step and such procedures are carried out by selecting specific solvents. The liquid-liquid extraction may lead to emulsion formation, which would be avoided by a centrifugation step; however, this latter is time-consuming. During the selection of extraction solvent, both polarity of the steroid and its interaction with binding proteins should be considered. Solvents such as acetonitrile and methanol act by providing the disruption of steroid-protein binding through a mechanism of protein denaturation. After solvent addition, the mixture is vortexed, and the protein plug removed by centrifugation (Makin et al., 2010). The

protein precipitation efficiency is associated with the solvent type and solvent/biological matrix ratio used. Overall, a ratio 2:1 of solvent (acetonitrile or trichloroacetic acid) to biological matrix is able to precipitate more than 90% of proteins (Polson et al., 2003). In human skin matrices containing TST, Baert et al. (2012) removed proteins by adding a ratio 1:1 of acetonitrile/matrix. No analytical problems were reported in this study.

The absence of proteins in the supernatant may be confirmed by adding a dye capable of binding proteins. When proteins are found, Coomassie blue dye under acidic conditions changes the color from brown to blue (Rodger and Sanders, 2017). The dye binds to basic amino acids by a combination of hydrophobic interactions and heteropolar bonding (Drabik et al., 2016).

In addition to liquid-liquid extraction, solid-phase extraction (SPE) has been often applied to separate the steroids from biological interferences. It is composed by a sorbent such as microparticulate silica coated with octadecasilane, which is packed into syringes or cartridges (Makin et al., 2010). In the case of TST, which has an extremely low permeation rate and the volumes collected cannot be large, the application of this method is limited. The large number of samples also makes this method expensive.

HPLC has been the most important technique to quantify steroids in different matrices; however, not enough sensitivity could be achieved when a UV detector is used (Makin et al., 2010). Baert et al. (2012), for example, found a quantification limit of 0.17 µg/mL for TST by using this technique. Once the TST has an extremely low initial permeation rate (in the nanogram range) and several authors have considered HPLC as a quantification method, initial time intervals are more susceptible to analytical errors.

If reversed-phase columns are selected, guard column (30-70 mm in length) should be included to avoid the accumulation of non-polar material on the columns or time-

consuming clean-up procedures of sample or column. LC-MS-MS (i.e. tandem mass spectrometry) may represent an alternative to overcome this extensive need of clean-up of samples and also is able to provide a lower quantification limit than HPLC-UV (Makin et al., 2010).

Another alternative to improve the sensitivity of the quantification method is to increase the amount of sample injected, however, this must be compatible with the size of column used. In our database for TST (Table 2), most of the authors used an injection volume of 20 μL and a non-processing of the samples before injection (without solvent extraction or SPE). When the injection volume is increased, a greater need for sample clean-up/purification steps should be considered.

11. DRUG RETENTION

Androgen receptors are expressed in sebocytes, hair follicle, dermal papilla and keratinocytes. Hence, TST plays an important role in the sebum production, control hair growth as well as hair loss, epidermal growth and differentiation (Choudhry et al., 1992). The mechanism of action of TST occurs by its local conversion to dihydrotestosterone (DHT) by 5α-reductase (type 1). DHT then binds to the androgen receptor. This means that the increase of TST lead to an increase of DHT concentrations, which is associated with hair loss (alopecia) (Riggs et al., 2002; Shapiro and Price, 1998). The action of this hormone is regulated by cell-type-specific activation or deactivation in human skin. In physiological conditions, fibroblasts present in the dermis activate mainly TST to DHT. In contrast, keratinocytes present in the epidermis deactivate TST, forming androstenedione (Münster et al., 2003).

The higher concentration of TST in men is associated with a thicker epidermis, and greater amount of collagen and moisture compared to women skin (Markova et al., 2004). On the other hand, the higher levels of TST in male stimulate a large production of sebum, leading to fatty glow and coarser pores of the skin (Baumann, 2002). TST can also perturbs the epidermal barrier homeostasis considering that studies showed a retard in barrier development of skin fetus and slowed barrier recovery in adult skin (Kao et al., 2001). Other negative effects include the inhibition of skin wound healing in males and the TST association with an enhanced inflammatory response (Ashcroft and Mills, 2002). In contrast, a reduction in physiological levels of the hormone has also a negative impact on epidermal skin moisture, elasticity, and thickness (Bernard et al., 2012). In these cases, the local hormone replacement with TST can be advantageous to improve the aspect of skin aging in men.

Transdermal products containing 1% TST are commonly used to reproduce diurnal physiological variations of the hormone in the treatment of male hypogonadism (Mazer, 2000). Once application of TST in skin is focused on the systemic treatment, many *in vitro* studies do not evaluate the presence of the hormone in skin homogenates. Magnusson et al. (2006), for instance, evaluated the distribution of TST in human skin using excised epidermis, dermis, and full-thickness skin. TST retention was higher in the uppermost skin layers (SC > epidermis> dermis), which is attributed to its lipophilicity. In hormone retention assays performed with viable epidermis *versus* full-thickness skin, the dermis layer seemed to restrict TST permeation. The TST retention in viable epidermis from full-thickness skin as a membrane model was 3.36-fold higher than only in epidermis (isolated tissue). Heylings et al. (2018) also reported a lower affinity of the TST for dermis. The authors found 0.41 and 0.32% of dose applied of TST (1.0 mg/mL) in epidermis and dermis, respectively.

TST has a great affinity for the lipophilic layers of the skin, contributing for its accumulation in the tissue. This could generate important adverse effects from a topical administration. However, studies have not thoroughly investigated the possible toxic effects of this hormone in skin.

12. FINAL CONSIDERATIONS

The interplay/tradeoff solubility-permeation should not ignored and researchers need to achieve an optimal solubility-permeation balance to maximize the permeation of poorly soluble drugs such as TST. The inclusion of ethanol in aqueous medium increased the solubility or free fraction of TST in the solution, which solves problems of solubility in receptor fluids and maintains sink conditions; however, a reduction in its permeation rate was found when compared to aqueous solutions without solubilizing agents. In this same way, an optimal supersaturation degree should be found to maximize permeation and avoid drug precipitation phenomena. Infinite doses have been frequently used in commercial preparations of hormone due to its low cutaneous absorption; however, an increase in permeation is not always achieved with this approach as reported here.

The type of tissue selected as the membrane model as well as its preparation has also been shown to strongly affect the TST permeation rate. When full-thickness skin is considered, the dermis acts as a barrier that restricts the diffusion of TST, significantly reducing the partitioning into the receptor chamber. Therefore, the amount of absorbed TST is underestimated. The drug retention in epidermis is also increased in an unrealistic way. This fact represents the main reason for prioritizing dermatomized skin. Substantial differences in the rate of transport of the hormone were found depending on the animal model and between biological and artificial membranes/tissues. Despite its widespread use, mouse/mice skin is much more permeable than human skin and should

not be prioritized in initial studies (when the permeation of a certain molecule is not yet known). Human skin and pig skin are more suitable in these situations. Mouse/mice skin can be useful, for example, for comparing the performance of different formulations and *in vivo* distribution studies. Likewise, the TST permeation results obtained with artificial membranes are quite different those with human skin.

Although various cutaneous permeation studies with TST were presented in this review, few studies evaluating its retention in the dermis and epidermis are reported. As already mentioned, the skin presents specific receptors for TST, which makes this type of study even more important. This fact could be justified by the complexity of separating the epidermis from the dermis. As already mentioned, the currently available methods that use heating or chemical treatment can affect tissue integrity. For this reason, manual tissue separation has been proposed, but poorly reproducible results are usually found.

Although traditional methods in the pharmaceutical field have been considered for the quantification of TST in biological matrices, in-depth studies of sample preparation are still scarce. Improvements in terms of the method sensitivity could be achieved with an adequate processing of the samples, reducing the experimental time and the data generated would also be more reliable.

In summary, this review contributes to define more properly the experimental variables of cutaneous permeation assays with poorly soluble molecules such as the TST, creating new discussions for future revisions of official guidelines.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

ACKNOWLEDGEMENTS

We gratefully acknowledge the Brazilian governmental agencies CNPq/MCTI (Universal 01/2016 - Grant number: 408229/2016-0) and CAPES for financial support and student scholarships, respectively.

REFERENCES

Abd, E., Yousef, S.A., Pastore, M.N., Telaprolu, K., Mohammed, Y.H., Namjoshi, S., Grice, J.E., Roberts, M.S., 2016. Skin models for the testing of transdermal drugs. Clin. Pharmacol. Adv. Appl. 8, 163–176. https://doi.org/10.2147/CPAA.S64788

Ashcroft, G.S., Mills, S.J., 2002. Androgen receptor–mediated inhibition of cutaneous wound healing. J. Clin. Invest. 110, 615–624.

Baert, B., Roche, N., Burvenich, C., De Spiegeleer, B., 2012. Increase of the transdermal penetration of testosterone by miconazole nitrate. Arch. Pharm. Res. 35, 2163–2170. https://doi.org/10.1007/s12272-012-1214-4

Barbero, A.M., Frasch, H.F., 2016. Effect of frozen human epidermis storage duration and cryoprotectant on barrier function using two model compounds. Skin Pharmacol. Physiol. 29, 31–40. https://doi.org/10.1159/000441038

Bartosova, L., Bajgar, J., 2012. Transdermal drug delivery *in vitro* using diffusion cells. Curr. Med. Chem. 19, 4671–4677. https://doi.org/10.2174/092986712803306358

Baumann, G., 2002. Growth hormone binding protein. The soluble growth hormone receptor. Minerva Endocrinol. 27, 265–276.

Bennion, B.J., Be, N.A., McNerney, M.W., Lao, V., Carlson, E.M., Valdez, C.A., Malfatti, M.A., Enright, H.A., Nguyen, T.H., Lightstone, F.C., Carpenter, T.S., 2017. Predicting a drug's membrane permeability: a computational model validated with *in vitro* permeability assay data. J. Phys. Chem. B 121, 5228–5237. https://doi.org/10.1021/acs.jpcb.7b02914

Bernard, P., Scior, T., Do, Q.T., 2012. Modulating testosterone pathway: a new strategy to tackle male skin aging? Clin. Interv. Aging 7, 351-361.

Blythe, L. Lou, Gannon, J.R., Craig, A.M., 1994. Care of the racing greyhound. American Greyhound Council.

Boer, M., Duchnik, E., Maleszka, R., Marchlewicz, M., 2016. Structural and biophysical characteristics of human skin in maintaining proper epidermal barrier function. Adv. Dermatology Allergol. 1, 1–5. https://doi.org/10.5114/pdia.2015.48037

Bronaugh, R.L., Stewart, R.F., 1985. Methods for *in vitro* percutaneous absorption studies IV: The flow-through diffusion cell. J. Pharm. Sci. 74, 64–67.

Carrer, V., Alonso, C., Pont, M., Zanuy, M., Córdoba, M., Espinosa, S., Barba, C., Oliver, M.A., Martí, M., Coderch, L., 2020. Effect of propylene glycol on the skin penetration of drugs. Arch. Dermatol. Res. 312, 337–352.

Caussin, J., Gooris, G.S., Janssens, M., Bouwstra, J.A., 2008. Lipid organization in human and porcine stratum corneum differs widely, while lipid mixtures with porcine ceramides model human stratum corneum lipid organization very closely. Biochim. Biophys. Acta - Biomembr. 1778, 1472–1482. https://doi.org/10.1016/j.bbamem.2008.03.003

Ceschel, G., Bergamante, V., Maffei, P., Borgia, S.L., Calabrese, V., Biserni, S., Ronchi, C., 2005. Solubility and transdermal permeation properties of a

dehydroepiandrosterone cyclodextrin complex from hydrophilic and lipophilic vehicles. Drug Deliv. 12, 275–280. https://doi.org/10.1080/10717540500176563

Choudhry, R., Hodgins, M.B., Van der Kwast, T.H., Brinkmann, A.O., Boersma, W.J.A., 1992. Localization of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands. J. Endocrinol. 133, 467-NP.

Cilurzo, F., Musazzi, U.M., Franzé, S., Fedele, G., Minghetti, P., 2018. Design of *in vitro* skin permeation studies according to the EMA guideline on quality of transdermal patches. Eur. J. Pharm. Sci. 125, 86–92. https://doi.org/10.1016/j.ejps.2018.09.014

Clowes, H.M., Scott, R.C., Heylings, J.R., 1994. Skin absorption: flow-through or static diffusion cells. Toxicol. Vitr. 8, 827–830.

Couto, A., Fernandes, R., Cordeiro, M.N.S., Reis, S.S., Ribeiro, R.T., Pessoa, A.M., 2014. Dermic diffusion and stratum corneum: a state of the art review of mathematical models. J. Control. Release 177, 74–83. https://doi.org/10.1016/j.jconrel.2013.12.005

Davies, D.J., Ward, R.J., Heylings, J.R., 2004. Multi-species assessment of electrical resistance as a skin integrity marker for *in vitro* percutaneous absorption studies. Toxicol. Vitr. 18, 351–358. https://doi.org/10.1016/j.tiv.2003.10.004

Drabik, A., Bodzoń-Kułakowska, A., Silberring, J., 2016. 7 - Gel electrophoresis, In: Ciborowski, P., Silberring, J.B.T.-P.P. and A.C. (Second E. (Eds.), . Elsevier, Boston, pp. 115–143. https://doi.org/https://doi.org/10.1016/B978-0-444-63688-1.00007-0

Fabian, E., Oesch, F., Ott, K., Landsiedel, R., Van Ravenzwaay, B., 2017. A protocol to determine dermal absorption of xenobiotica through human skin *in vitro*. Arch. Toxicol. 91, 1497–1511.

Fahmy, F.S., Navsaria, H.A., Frame, J.D., Jones, C.R., Leigh, I.M., 1993. Skin graft storage and keratinocyte viability. Br. J. Plast. Surg. 46, 292–295.

Fang, J.-Y., Hwang, T.-L., Fang, C.-L., Chiu, H.-C., 2003. *In vitro* and *in vivo* evaluations of the efficacy and safety of skin permeation enhancers using flurbiprofen as a model drug. Int. J. Pharm. 255, 153–166.

Finnin, B., Walters, K.A., Franz, T.J., 2012. *In vitro* skin permeation methodology, In: Benson, A.E., H., Watkinson, A.C. (Eds.), Transdermal and topical drug delivery: principles and practice. John Wiley & Sons, Inc, pp. 85–108.

Flynn, G.L., Yalkowsky, S.H., Roseman, T.J., 1974. Mass transport phenomena and models: theoretical concepts. J. Pharm. Sci. 63, 479–510.

Guth, K., Schäfer-Korting, M., Fabian, E., Landsiedel, R., van Ravenzwaay, B., 2015. Suitability of skin integrity tests for dermal absorption studies *in vitro*. Toxicol. Vitr. 29, 113–123. https://doi.org/10.1016/j.tiv.2014.09.007

Haq, A., Michniak-Kohn, B., 2018. Effects of solvents and penetration enhancers on transdermal delivery of thymoquinone: permeability and skin deposition study. Drug Deliv. 25, 1943–1949.

Hewitt, N.J., Grégoire, S., Cubberley, R., Duplan, H., Eilstein, J., Ellison, C., Lester, C., Fabian, E., Fernandez, J., Géniès, C., Jacques-Jamin, C., Klaric, M., Rothe, H., Sorrell, I., Lange, D., Schepky, A., 2020. Measurement of the penetration of 56 cosmetic relevant chemicals into and through human skin using a standardized protocol. J. Appl. Toxicol. 40, 403–415. https://doi.org/10.1002/jat.3913

Heylings, J.R, Davies, D.J., Burton, R., 2018. Dermal absorption of testosterone in human and pig skin *in vitro*. Toxicol. Vitr. 48, 71–77. https://doi.org/10.1016/j.tiv.2017.12.014

Heylings, Jon R., Davies, D.J., Burton, R., 2018. Dermal absorption of testosterone in human and pig skin *in vitro*. Toxicol. Vitr. 48, 71–77. https://doi.org/10.1016/j.tiv.2017.12.014

Horita, D., Todo, H., Sugibayashi, K., 2012. Effect of ethanol pretreatment on skin permeation of drugs. Biol. Pharm. Bull. 35, 1343–1348.

Houghton, E., Maynard, S., 2010. Some aspects of doping and medication control in equine sports. Doping Sport. Biochem. Princ. Eff. Anal. 369–409.

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

Hueber, F., Wepierre, J., Schaefer, H., 1992. Role of transepidermal and transfollicular routes in percutaneous absorption of hydrocortisone and testosterone: *in vivo* study in the hairless rat. Skin Pharmacol. Physiol. 5, 99–107.

Ishii, H., Todo, H., Sugibayashi, K., 2010. Effect of thermodynamic activity on skin permeation and skin concentration of triamcinolone acetonide. Chem. Pharm. Bull. (Tokyo). 58, 556–561. https://doi.org/10.1248/cpb.58.556

Jacobi, U., Kaiser, M., Toll, R., Mangelsdorf, S., Audring, H., Otberg, N., Sterry, W., Lademann, J., 2007. Porcine ear skin: an *in vitro* model for human skin. Ski. Res. Technol. 13, 19–24. https://doi.org/10.1111/j.1600-0846.2006.00179.x

Kaminetsky, J., Wynia, B., 2015. Current and emerging testosterone therapies for male hypogonadism. Res. Reports Endocr. Disord. 5, 59-69. https://doi.org/10.2147/RRED.S46193

Kao, J.S., Garg, A., Mao-Qiang, M., Crumrine, D., Ghadially, R., Feingold, K.R., Elias, P.M., 2001. Testosterone perturbs epidermal permeability barrier homeostasis. J. Invest. Dermatol. 116, 443–451. https://doi.org/10.1046/j.1523-1747.2001.01281.x

Kaufmann, K., Dohmen, P., 2016. Adaption of a dermal *in vitro* method to investigate the uptake of chemicals across amphibian skin. Environ. Sci. Eur. 28. https://doi.org/10.1186/s12302-016-0080-y

Kim, M.K., Zhao, H., Lee, C.H., Kim, D.D., 2001. Formulation of a reservoir-type testosterone transdermal delivery system. Int. J. Pharm. 219, 51–59. https://doi.org/10.1016/S0378-5173(01)00631-7

Knorr, F., Lademann, J., Patzelt, A., Sterry, W., Blume-Peytavi, U., Vogt, A., 2009. Follicular transport route–research progress and future perspectives. Eur. J. Pharm. Biopharm. 71, 173–180.

Kretsos, K., Miller, M.A., Zamora-Estrada, G., Kasting, G.B., 2008. Partitioning, diffusivity and clearance of skin permeants in mammalian dermis. Int. J. Pharm. 346, 64–79. https://doi.org/10.1016/j.ijpharm.2007.06.020

Krugluger, W., Rohrbacher, W., Laciak, K., Moser, K., Moser, C., Hugeneck, J., 2005. Reorganization of hair follicles in human skin organ culture induced by cultured human follicle-derived cells. Exp. Dermatol. 14, 580–585.

Lauterbach, A., Müller-Goymann, C.C., 2015. Applications and limitations of lipid nanoparticles in dermal and transdermal drug delivery via the follicular route. Eur. J. Pharm. Biopharm. 97, 152–163.

Leichtnam, M.-L., Rolland, H., Wüthrich, P., Guy, R.H., 2006. Enhancement of transdermal testosterone delivery by supersaturation. J. Pharm. Sci. 95, 2373–2379. https://doi.org/10.1002/jps.20669

Lillywhite, H.B., 2006. Water relations of tetrapod integument. J. Exp. Biol. 209, 202–226. https://doi.org/10.1242/jeb.02007

Llewelyn, V.K., Berger, L., Glass, B.D., 2019. Permeability of frog skin to chemicals: effect of penetration enhancers. Heliyon 5, e02127.

Magnusson, B.M., Cross, S.E., Winckle, G., Roberts, M.S., 2006. Percutaneous absorption of steroids: determination of *in vitro* permeability and tissue reservoir characteristics in human skin layers. Skin Pharmacol. Physiol. 19, 336–342. https://doi.org/10.1159/000095254

Makin, H.L.J., Honour, J.W., Shackleton, C.H.L., Griffiths, W.J., 2010. General methods for the extraction, purification, and measurement of steroids by chromatography and mass spectrometry BT - steroid analysis, In: Makin, H.L.J., Gower, D.B. (Eds.). Springer Netherlands, Dordrecht, pp. 163–282. https://doi.org/10.1023/b135931_3

Markova, M.S., Zeskand, J., McEntee, B., Rothstein, J., Jimenez, S.A., Siracusa, L.D., 2004. A role for the androgen receptor in collagen content of the skin. J. Invest. Dermatol. 123, 1052–1056.

Mazer, N.A., 2000. New clinical applications of transdermal testosterone delivery in men and women. J. Control. Release 65, 303–315.

McFarlane, R.M., DeYoung, G., Henry, R.A., McFarlane, R.M., 1965. The design of a pedicle flap in the rat to study necrosis and its prevention. Plast. Reconstr. Surg. 35, 177–182.

Michel, M., L'Heureux, N., Pouliot, R., Xu, W., Auger, F.A., Germain, L., 1999. Characterization of a new tissue-engineered human skin equivalent with hair. Vitr. Cell. Dev. Biol. 35, 318.

Mills, P.C., 2007. Vehicle effects on the In vitro penetration of testosterone through equine skin. Vet. Res. Commun. 31, 227–233. https://doi.org/10.1007/s11259-006-3446-6

Mills, P.C., Magnusson, B.M., Cross, S.E., 2006. The effects of vehicle and region of application on *in vitro* penetration of testosterone through canine skin. Vet. J. 171, 276–280. https://doi.org/10.1016/j.tvjl.2004.11.013

Münster, U., Hammer, S., Blume-Peytavi, U., Schäfer-Korting, M., 2003. Testosterone metabolism in human skin cells *in vitro* and its interaction with estradiol and dutasteride. Skin Pharmacol. Physiol. 16, 356–366.

Netzlaff, F., Schaefer, U.F., Lehr, C.-M., Meiers, P., Stahl, J., Kietzmann, M., Niedorf, F., 2006. Comparison of bovine udder skin with human and porcine skin in percutaneous permeation experiments. ATLA Altern. to Lab. Anim. 34, 499–513.

Neupane, R., Boddu, S.H.S., Renukuntla, J., Babu, R.J., Tiwari, A.K., 2020. Alternatives to biological skin in permeation studies: current trends and possibilities. Pharmaceutics 12, 152. https://doi.org/10.3390/pharmaceutics12020152

Ng, S.F., Rouse, J.J., Sanderson, F.D., Meidan, V., Eccleston, G.M., 2010. Validation of a static Franz diffusion cell system for *in vitro* permeation studies. AAPS PharmSciTech 11, 1432–1441. https://doi.org/10.1208/s12249-010-9522-9

Norman, A.W., Litwack, G., 1997. Hormones. Academic Press.

OECD, 2004. Guidance document for the conduct of skin absorption studies. OECD series on testing and assessment. Available at: https://doi.org/10.1787/9789264078796-en

Pelletier, G., Ren, L., 2004. Localization of sex steroid receptors in human skin. Histol. Histopathol. 19, 629–636. https://doi.org/10.14670/HH-19.629

Plumb, D.C., 2002. Meclizine. Veterinary Drug Book, 4th. ed. Ames.

Polson, C., Sarkar, P., Incledon, B., Raguvaran, V., Grant, R., 2003. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry. J. Chromatogr. B 785, 263–275. https://doi.org/10.1016/S1570-0232(02)00914-5

Qvist, M.H., Hoeck, U., Kreilgaard, B., Madsen, F., Frokjaer, S., 2000. Evaluation of Gottingen minipig skin for transdermal *in vitro* permeation studies. Eur. J. Pharm. Sci. 11, 59–68. https://doi.org/10.1016/S0928-0987(00)00091-9

Riggs, B.L., Khosla, S., Melton III, L.J., 2002. Sex steroids and the construction and conservation of the adult skeleton. Endocr. Rev. 23, 279–302.

Rodger, A., Sanders, K., 2017. UV-visible absorption spectroscopy, biomacromolecular applications, In: Encyclopedia of Spectroscopy and Spectrometry. Elsevier, pp. 495–502.

SCCS, 2010. Basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients. Eur. Comm 1–14.

Schlupp, P., Weber, M., Schmidts, T., Geiger, K., Runkel, F., 2014. Development and validation of an alternative disturbed skin model by mechanical abrasion to study drug penetration. Results Pharma Sci. 4, 26–33. https://doi.org/10.1016/j.rinphs.2014.09.002

Schreiber, S., Mahmoud, A., Vuia, A., Rübbelke, M.K., Schmidt, E., Schaller, M., Kandárová, H., Haberland, A., Schäfer, U.F., Bock, U., Korting, H.C., Liebsch, M., Schäfer-Korting, M., 2005. Reconstructed epidermis versus human and animal skin in skin absorption studies. Toxicol. Vitr. 19, 813–822.

https://doi.org/10.1016/j.tiv.2005.04.004

Schwarb, F.P., Imanidis, G., Smith, E.W., Haigh, J., Surber, C., 1999. Effect of concentration and degree of saturation of topical fluocinonide formulations on *in vitro* membrane transport and *in vivo* availability on human skin. Pharm. Res. 16, 909–15. https://doi.org/10.1023/A:1018890422825

Shapiro, J., Price, V.H., 1998. Hair regrowth: therapeutic agents. Dermatol. Clin. 16, 341–356.

Simard, M., Julien, P., Fradette, J., Pouliot, R., 2019. Modulation of the lipid profile of reconstructed skin substitutes after essential fatty acid supplementation affects testosterone permeability. Cells 8, 1142. https://doi.org/10.3390/cells8101142

Snow, D.H., 1993. Anabolic steroids. Vet. Clin. North Am. Equine Pract. 9, 563–576.

Srinivasan, B., Kolli, A.R., Esch, M.B., Abaci, H.E., Shuler, M.L., Hickman, J.J., 2015. TEER measurement techniques for *in vitro* barrier model systems. J. Lab. Autom. 20, 107–126. https://doi.org/10.1177/2211068214561025

Swarbrick, J., Lee, G., Brom, J., 1982. Drug permeation through human skin: I. Effects of storage conditions of skin. J. Invest. Dermatol. 78, 63–66.

Thomas, N.S., Panchagnula, R., 2003. Transdermal delivery of zidovudine: effect of vehicles on permeation across rat skin and their mechanism of action. Eur. J. Pharm. Sci. 18, 71–79.

Van de Sandt, J.J.M., Van Burgsteden, J.A., Cage, S., Carmichael, P.L., Dick, I., Kenyon, S., Korinth, G., Larese, F., Limasset, J.C., Maas, W.J.M., 2004. *In vitro* predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study. Regul. Toxicol. Pharmacol. 39, 271–281.

Veryser, L., Boonen, J., Taevernier, L., Guillaume, J., Risseeuw, M., Shah, S.N.H., Roche, N., Van Calenbergh, S., De Spiegeleer, B., 2015. The influence of the acyl chain on the transdermal penetration-enhancing effect of synthetic phytoceramides. Skin Pharmacol. Physiol. 28, 124–136. https://doi.org/10.1159/000365730

Wei, J.C.J., Edwards, G.A., Martin, D.J., Huang, H., Crichton, M.L., Kendall, M.A.F., 2017. Allometric scaling of skin thickness, elasticity, viscoelasticity to mass for micro-medical device translation: from mice, rats, rabbits, pigs to humans. Sci. Rep. 7, 15885. https://doi.org/10.1038/s41598-017-15830-7

WHO, 2006. Dermal absorption. World Health Organization.

Wilkinson, S.C., Maas, W.J.M., Nielsen, J.B., Greaves, L.C., Van de Sandt, J.J.M., Williams, F.M., 2006. Interactions of skin thickness and physicochemical properties of test compounds in percutaneous penetration studies. Int. Arch. Occup. Environ. Health 79, 405–413.

Williams, A.C., Barry, B.W., 2012. Penetration enhancers. Adv. Drug Deliv. Rev. 64, 128–137.

Zhou, L., Yang, L., Tilton, S., Wang, J., 2007. Development of a high throughput equilibrium solubility assay using miniaturized shake-flask method in early drug discovery. J. Pharm. Sci. 96, 3052–3071. https://doi.org/10.1002/jps.20913

Zsikó, S., Csányi, E., Kovács, A., Budai-Szűcs, M., Gácsi, A., Berkó, S., 2019. Methods

to evaluate skin penetration *in vitro*. Sci. Pharm. 87, 19.

Table captions

Table 1. Testosterone solubility in different solvents and experimental conditions.

Table 2. Experimental conditions used in skin permeation studies with testosterone.

CRediT author statement

Nicole Esposto Biondo: Writing - Original Draft.

Débora Fretes Argenta: Conceptualization; Writing - Review & Editing.

Gabriela Schneider Rauber: Writing - Review & Editing.

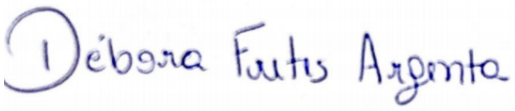
Thiago Caon: Conceptualization; Writing - Review & Editing; Supervision; Project Administration.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Nicole Esposto Biondo



Débora Fretes Argenta

Gabriela Schneider Rauber

Thiago Caon

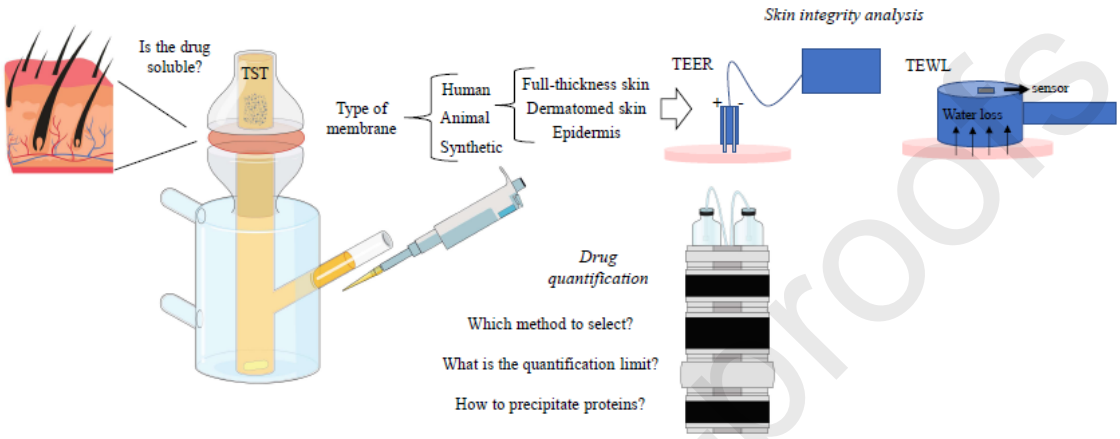


Table 1. Testosterone solubility in different solvents and experimental conditions.

Medium/solvent	*pH*	*Temperature*	*Incubation time*	*Solubility*	*Reference*
HEPES (10 mM + NaCl 0,154 M)	7.4	25 ºC	18 h	0.02 mg/mL	(Schwarz et al., 2017)
HEPES + HSA (4% w/v)	7.4	25 ºC	18 h	0.21 mg/L	
HEPES + *β*-CD (1,5% w/v)	7.4	25 ºC	18 h	0.06 mg/mL	
HEPES + HP-*β*-CD (4% w/v)	7.4	25 ºC	18 h	3.90 mg/mL	
HEPES + SBE7-*β*-CD (4% w/v)	7.4	25 ºC	18 h	3.23 mg/mL	
HEPES + HSES (1,5% w/v)	7.4	25 ºC	18 h	5.72 mg/mL	
HEPES + HTMT (1,5% w/v)	7.4	25 ºC	18 h	2.23 mg/mL	
HEPES + HTG (1,5% w/v)	7.4	25 ºC	18 h	0.07 mg/mL	
Ethanol/PG/Water (4:1:1)	-	25 ºC	12 h	26.99 mg/mL	(Leichtnam et al., 2006)
Isopropyl myristate	-	25 ºC	Several days	0.03 mg/mL	(Imai et al., 2016)
Water	-	25 ± 1 ºC	72 h	0.02 mg/mL	(Lu et al., 2013)
PBS	7.4	25 ± 1 ºC	72 h	0.02 mg/mL	
PEG 200 water solution (40%)		25 ± 1 ºC	72 h	0.32 mg/mL	
Azone®	-	25 ± 1 ºC	72 h	92.38 mg/mL	
N-methyl-2-pyrrolidone (NMP)	-	25 ± 1 ºC	72 h	518.89 mg/mL	
PG	-	25 ± 1ºC	72 h	103.00 mg/mL	
Isopropyl myristate	-	25 ± 1ºC	72 h	6.83 mg/mL	
Ethanol/water (70:30)	-	37 ºC	48 h	68.31 mg/mL	(Kim et al., 2001)
Ethanol/water (70:30) +Dodecylamine 1% (w/v)	-	37 ºC	48 h	66.71 mg/mL	
Ethanol/water (70:30) + oleic acid 1% (w/v)	-	37 ºC	48 h	68.71 mg/mL	
Ethanol/water (70:30) + lauric acid 1% (w/v)	-	37 ºC	48 h	66.35 mg/mL	
Ethanol/water (70:30) + HPE-101 1% (w/v)	-	37 ºC	48 h	64.11 mg/mL	
Ethanol/water (70:30) + transcutol® 1% (w/v)	-	37 ºC	48 h	69.05 mg/mL	
PBS	-	3 ºC	24 h	0.06 mg/mL	(Mills et al., 2006)
PBS/Ethanol (50:50; w/w)	-	3 ºC	24 h	1.85 mg/mL	
PBS/ PG (50:50; w/w)	-	3 ºC	24 h	1.50 mg/mL	
PBS	-	30 ºC	24 h	0.06 mg/mL	(Mills, 2007)
PBS/Ethanol (50:50; w/w)	-	30 ºC	24 h	1.82 mg/mL	
PBS/ PG (50:50; w/w)	-	30 ºC	24 h	1.48 mg/mL	
PG	-	30 ºC	48-72 h	75.90 mg/mL	(Bonina et al., 1993)
Liquid petrolatum	-	30 ºC	48-72 h	0.43 mg/mL	
Water	-	30 ºC	48-72 h	0.03 mg/mL	
Transcutol®	-	30 ºC	48-72 h	104.00 mg/mL	
Labrasol®	-	30 ºC	48-72 h	46.10 mg/mL	
Labrafil®	-	30 ºC	48-72 h	20.90 mg/mL	
DPPG	-	30 ºC	48-72 h	13.20 mg/mL	
Water	-	32 ºC	1 or 2 weeks	0.03 mg/mL	(Binks et al., 2012)
PBS	-	32 ºC	1 or 2 weeks	0.04 mg/mL	
Isopropyl myristate	-	32 ºC	1 or 2 weeks	8.08 mg/mL	
20% hydroethanolic solution	-	20 ºC	48 h	0.25 mg/mL	

20% hydroethanolic solution	-	37 ºC	48 h	0.34 mg/mL	(Ainbinder and Touitou, 2005)
20% ethanol in PBS	-	20 ºC	48 h	0.22 mg/mL	
20% ethanol in PBS	-	37 ºC	48 h	0.30 mg/mL	
50% hydroethanolic solution	-	20 ºC	48 h	6.76 mg/mL	
50% hydroethanolic solution	-	37 ºC	48 h	10.77 mg/mL	
0.5% Brij® 98 aqueous solution	-	20 ºC	48 h	0.06 mg/mL	
0.5% Brij® 98 aqueous solution	-	37 ºC	48 h	0.08 mg/mL	
6.0 % Brij® 98 aqueous solution	-	20 ºC	48 h	0.17 mg/mL	
6.0 % Brij® 98 aqueous solution	-	37 ºC	48 h	0.19 mg/mL	
Hydrated soybean oil	-	27 ºC	-	6.00 mg/g	(Land et al., 2005)
Desiccated soybean oil	-	27 ºC	-	8.60 mg/g	
Hydrated olive oil	-	27 ºC	-	5.20 mg/g	
Desiccated olive oil	-	27 ºC	-	8.00 mg/g	
Hydrated miglyol 812 oil	-	27 ºC	-	8.90 mg/g	
Desiccated miglyol 812 oil	-	27 ºC	-	14.30 mg/g	
Ethanol	-	25 ºC	One whole day and night	209.99 mg/mL	(Araya et al., 2005)
Water	-	25 ºC	One whole day and night	0.02 mg/mL	
PBS	-	30 ºC	24 h	0.07 mg/mL	(Mills, 2007)
Ethanol/PBS (50:50; w/w)	-	30 ºC	24 h	6.31 mg/mL	
PG/PBS (50:50; w/w)	-	30 ºC	24 h	5.14 mg/mL	

HSA = human serum albumin ; *β*-CD = *β*-cyclodextrin; HP-*β*-CD = hydroxypropyl-*β*-cyclodextrin; SBE7-*β*-CD = sulfobutyl-*β*-cyclodextrin; HSES = heptakis-6-sulfoethylsulfanyl6-deoxy- *β*-cyclodextrin; HTMT = eptakis-6-methylsulfanyl-6-deoxy-2-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)]-*β*-cyclodextrin; HTG = heptakis-6-thioglyceryl-6-deoxy-*β*-cyclodextrin; LBS = glycolysed ethoxylated C_8/C_{10} glycerides; LBF = glycolysed ethoxylated glycerides; TSC = diethylene glycol monoethyl ether; DPPG = propylene glycol dipelargonate; PBS = phosphate-buffered saline; PG = propylene glycol.

Table 2. Experimental conditions used in skin permeation studies with testosterone.

Animal model	Thickness of tissue	Section of tissue	Tissue preparation	Composition of donor chamber	Volume of donor solution	Drug concentration in the donor chamber	Composition of receptor chamber	Volume of receptor medium	Experimental total time	Permeation parameters (lag time, permeation coefficient and flux)	Retained drug amount or information on TST retention	Protein precipitation solvent (Y or N) / freezing samples? (Y or N)	Quantification method / Injected amount (HPLC)	Area for permeation	Reference
Human cadaver skin	300 µm	Dermis	Dermatomed tissue, following by heating at 55°C for 2min	TST solubilized in ethanol	100 µL	50Ci/mL of ^{3}H-TST	PBS, PBS + 2% BSA or PBS + 2% HSA	6 mL	-	3.3 x 10^{-3} cm/h	NF	N N	Liquid scintillation counting	1.77 cm²	(Kretsos et al., 2008)
Neonatal piglet skin	-	Epidermis and dermis	Surgical scissors	TST (4% w/v) solubilized in ethanol 95% (V/V)	5 µL	4% of TST	Ethanol 20% (v/v) and sodium azide 0,1% (m/v) in water	NF	24 h	0.059 µg/cm².h	NF	N N	HPLC 20 µL	0.79 cm²	(Niolazzo et al., 2005)
Neonatal piglet skin	-	Epidermis and dermis	Surgical scissors	TST (4% w/v) solubilized in ethanol 95% (V/V) and oleic acid 5% (m/V)	5 µL	4% of TST	Ethanol 20% (v/v) and sodium azide 0,1% (m/v) in water	NF	24 h	0.186 µg/cm².h	NF	N N	HPLC 20 µL	0.79 cm²	
Neonatal piglet skin	-	Epidermis and dermis	Surgical scissors	TST (4% w/v) solubilized in ethanol 95% (V/V), followed by application of 400 µL of PG 20%	5 µL	4% of TST	Ethanol 20% (v/v) and sodium azide 0,1% (m/v) in water	NF	24 h	0.515 µg/cm².h	NF	N N	HPLC 20 µL	0.79 cm²	
Nude mouse skin	-	Full-thickness skin	NF	TST in 50% aqueous ethanol solution	3.1 mL	27 mg/mL (TST was at 71% saturation)	Hydroalcoholic solution (50% ethanol)	3.1 mL	25 h	2.5 x 10^{-3} cm/h	NF	N N	Scintillation counting	NF	(Kalpun-Frischoff and Touitou, 1997)
Nude mouse skin	-	Full-thickness skin	NF	Inclusion of a chemical absorption enhancer Eutectic mixture of menthol:TST (4:1) in 50% aqueous ethanol solution	3.1 mL	76.8 mg/mL	Hydroalcoholic solution (50% ethanol)	3.1 mL	25 h	6.6 x 10^{-3} cm/h	NF	N N	Scintillation counting	NF	
Silastic membrane (silicone membrane)	-	-	NF	TST in 50% aqueous ethanol solution	3.1 mL	27 mg/mL (TST was at 71% saturation)	Hydroalcoholic solution (50% ethanol)	3.1 mL	25 h	9.95 x 10^{-3} cm/h	NF	N N	Scintillation counting	NF	
Silastic	-	-	NF	Inclusion of a	3.1 mL	76.8 mg/mL	Hydroalcoholic	3.1 mL	25 h	10.46 x 10^{-3} cm/h	NF	N N	Scintillation	NF	

membrane (silicone membrane)				chemical absorption enhancer Eutectic mixture of menthol:TST (4:1) in 50% aqueous ethanol solution			solution (50% ethanol)						counting		
Rat skin	-	Epidermis+dermis	Surgical materials	Ethanol/water (70/30)	3 mL	Saturated solution of TST	Saline solution containing 40% (v/v) polyethylene glycol 400	12 mL	24 h	0.04 x 10^{-3} cm/h (lag time = 2.88 h)	NF	N Y(-20°C)	HPLC 20 µL	2.14 cm^2	(KIM et al., 2000)
Rat skin	-	Epidermis+dermis	Surgical materials	Ethanol/water (70/30) plus 1% (m/V) transcutol	3 mL	Saturated solution of TST	Saline solution containing 40% (v/v) polyethylene glycol 400	12 mL	24 h	0.04 x 10^{-3} cm/h (lag time = 1.96 h)	NF	N Y(-20°C)	HPLC 20 µL	2.14 cm^2	
Rat skin	-	Epidermis+dermis	Surgical materials	Ethanol/water (70/30) plus 1% (m/V) lauric acid	3 mL	Saturated solution of TST	Saline solution containing 40% (v/v) polyethylene glycol 400	12 mL	24 h	0.09 x 10^{-3} cm/h (lag time = 5.5 h)	NF	N Y(-20°C)	HPLC 20 µL	2.14 cm^2	
Rat skin	-	Epidermis+dermis	Surgical materials	Ethanol/water (70/30) plus 1% (m/V) oleic acid	3 mL	Saturated solution of TST	Saline solution containing 40% (v/v) polyethylene glycol 400	12 mL	24 h	0.20 x 10^{-3} cm/h (lag time = 7.04 h)	NF	N Y(-20°C)	HPLC 20 µL	2.14 cm^2	
Rat skin	-	Epidermis+dermis	Surgical materials	Ethanol/water (70/30) plus 1% (m/V) dodecylamine	3 mL	Saturated solution of TST	Saline solution containing 40% (v/v) polyethylene glycol 400	12 mL	24 h	0.72 x 10^{-3} cm/h (lag time = 7.09 h)	NF	N Y(-20°C)	HPLC 20 µL	2.14 cm^2	
Human skin	400 µm	Epidermis+dermis	Dermatomed tissue	[^{14}C]-TST was dissolved in 40% ethanol in water	10 µL/cm^2	10 µL/cm^2 (from a solution with 1 mg/mL of TST)	Physiological saline + 5% bovine serum albumine + 0.1% sodium azide	4.5 mL	24h	0.005 µg/cm^2.h	Stratum corneum (0.16 %); remaining epidermis (0.41 %); dermis (0.26 %)	N N	Scintillation Tissue digestion	2.54c m^2	(J R Heylings et al., 2018)
Pig skin	400 µm	Epidermis+dermis	Dermatomed tissued	[^{14}C]-TST was dissolved in 40% ethanol in water	10 µL/cm^2	10 µL/cm^2 (from a solution with 1 mg/mL of TST)	Physiological saline + 5% bovine serum albumine + 0.1% sodium azide	4.5 mL	24h	0.055 µg/cm^2.h	Stratum corneum (0.23 %); remaining epidermis (0.89 %);	N N	Scintillation Tissue digestion	2.54c m^2	

											dermis (1.17 %)				
Human skin	400 µm	Epidermis+dermis	Dermatomed tissue	Ethanol/water 50/50 (V/V)	500 µL	32 mM	PBS + 5% (m/V) bovine serum albumin	5 mL	24 h	2.08 to 6.79 x 10^{-4} cm/h (range)	NF	Y (acetonitrile) N	HPLC Limit of quantification = 0.17 µg/mL	0.64 cm^2	(Baert et al., 2012)
Equine Skin	-	Epidermis+dermis	Surgical materials	PBS	1 mL	Saturated TST solution (0.22 mmol/L)	PBS + 4% (m/V) bovine serum albumin	3.5 mL	24h	6.82 x 10^{-3} cm/h	PBS > EtOH = PG	N N	Liquid Scintillation Analyzer	-	(Mills, 2007)
Equine Skin	-	Epidermis+dermis	Surgical materials	Ethanol/PBS 50/50 (m/m)	1 mL	Saturated TST solution (6.31 mmol/L)	PBS + 4% (m/V) bovine serum albumin	3.5 mL	24h	1.59 x 10^{-3} cm/h	PBS > EtOH = PG	N N	Liquid Scintillation Analyzer	-	
Equine Skin	-	Epidermis+dermis	Surgical materials	Propylene glycol (PG)/PBS 50/50 (m/m)	1 mL	Saturated TST solution (5.14 mmol/L)	PBS + 4% (m/V) bovine serum albumin	3.5 mL	24h	2.04 x 10^{-3} cm/h	PBS > EtOH = PG	N N	Liquid Scintillation Analyzer	-	
Human skin	-	Stratum corneum + epidermis	Forceps	TST solubilized in 2% of Igepal®	0.5 mL	40 µg/mL	Phosphate buffered saline (PBS)	12.1 mL	30 h	2.31 x 10^{-7} cm/s	-	NF NF	HPLC 50 µL	1.767 cm^2	(Netzlaff et al., 2006)
Porcine skin	1000 µm	Epidermis+dermis	Dermatomed tissue	TST solubilized in 2% of Igepal®	0.5 mL	40 µg/mL	Phosphate buffered saline (PBS)	12.1 mL	30 h	1.29 x 10^{-7} cm/s	-	NF NF	HPLC 50 µL	1.767 cm^2	
Bovine skin	1000 µm	Epidermis+dermis	Dermatomed tissue	TST solubilized in 2% of Igepal®	0.5 mL	40 µg/mL	Phosphate buffered saline (PBS)	12.1 mL	30 h	5.42 x 10^{-7} cm/s	-	NF NF	HPLC 50 µL	1.767 cm^2	
Canine skin (thorax)	-	Epidermis+dermis	Forceps and scissors	Phosphate buffered saline (PBS)	1 mL	Saturated solution (0.21 mmol/L)	PBS pH 7.4 + 4% bovine serum albumin (BSA)	3.5 mL	24h	5.53 x 10^{-4} cm/h	Neck > groin > thorax PBS>PG>EtOH	N N	Scintillation Radiolabeled (14C) TST	-	(Mills et al., 2006)
Canine skin (neck)	-	Epidermis+dermis	Forceps and scissors	Phosphate buffered saline (PBS)	1 mL	Saturated solution (0.21 mmol/L)	PBS pH 7.4 +4% BSA	3.5 mL	24h	4.32 x 10^{-4} cm/h		N N	Scintillation Radiolabeled (14C) TST	-	
Canine skin (groin)	-	Epidermis+dermis	Forceps and scissors	Phosphate buffered saline (PBS)	1 mL	Saturated solution (0.21 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	3.85 x 10^{-4} cm/h		N N	Scintillation Radiolabeled (14C) TST	-	
Canine skin (thorax)	-	Epidermis+dermis	Forceps and scissors	Ethanol/PBS 50/50 (w/w)	1 mL	Saturated solution (6.26 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	1.12 x 10^{-4} cm/h	Neck = thorax > groin PBS>PG>EtOH	N N	Scintillation Radiolabeled (14C) TST	-	
Canine skin (neck)	-	Epidermis+dermis	Forceps and scissors	Ethanol/PBS 50/50 (w/w)	1 mL	Saturated solution (6.26 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	1.21 x 10^{-4} cm/h		N N	Scintillation Radiolabeled (14C) TST	-	
Canine skin (groin)	-	Epidermis+dermis	Forceps and scissors	Ethanol/PBS 50/50 (w/w)	1 mL	Saturated solution (6.26 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	0.73 x 10^{-4} cm/h		N N	Scintillation Radiolabeled (14C) TST	-	

Canine skin (thorax)	-	Epidermis+dermis	Forceps and scissors	Propylene glycol/PBS 50/50 (w/w)	1 mL	Saturated solution (5.21 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	1.63 x 10^{-4} cm/h	Neck = thorax > groin PBS>PG>EtOH	NN	Scintillation Radiolabeled (14C) TST	-	
Canine skin (neck)	-	Epidermis+dermis	Forceps and scissors	Propylene glycol/PBS 50/50 (w/w)	1 mL	Saturated solution (5.21 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	3.14 x 10^{-4} cm/h		NN	Scintillation Radiolabeled (14C) TST	-	
Canine skin (groin)	-	Epidermis+dermis	Forceps and scissors	Propylene glycol/PBS 50/50 (w/w)	1 mL	Saturated solution (5.21 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	0.81 x 10^{-4} cm/h		NN	Scintillation Radiolabeled (14C) TST	-	
Human skin	< 1000 µm	Epidermis Full-thickness skin	Forceps and scissors	PBS + 2% Igepal®	-	0.004%	PBS	12 mL	24h	9.4 x 10^{-4} cm/h (lag time = 0.03 h)	NF	NN	HPLC	1.768 cm^2	(Schreiber et al., 2005)
Reconstructed epiderm EpiDerm™	-	Epidermis	Purchased by LaboratoireMatTek Corp.	PBS + 2% Igepal®	-	0.004%	PBS	12 mL	24h	122.4 x 10^{-4} cm/h (lag time = 0 h)	NF	NN	HPLC	1.768 cm^2	
Reconstructed epiderm SkinEthic®	-	Epidermis	Purchased by LaboratoireSkinEthic	PBS + 2% Igepal®	-	0.004%	PBS	12 mL	24h	212.4 x 10^{-4} cm/h (lag time = 0.01 h)	NF	NN	HPLC	1.768 cm^2	
Porcine Skin	< 1000 µm	Epidermis	Forceps and scissors	PBS + 2% Igepal®	-	0.004%	PBS	12 mL	24h	11.5 x 10^{-4} cm/h (lag time = 7.63 h)	NF	NN	HPLC	1.768 cm^2	
Rat skin	-	Full-thickness skin	Dermatomed skin	EtOH/propylene glycol /water (4:1:1)	250 µL	Saturated solution	PBS	Perfusion (0.6 mL/min)	6 h	2.1 µg/cm^2.h	NF	NN	HPLC	0.785 cm^2	(M.-L. Leichtnam et al., 2006)
Rat skin	-	Full-thickness skin	Dermatomed skin	EtOH/propylene glycol /water (4:1:1)	250 µL	Supersaturated solution (supersaturation degree = 1.4)	PBS	Perfusion (0.6 mL/min)	6 h	3.1 µg/cm^2.h	NF	NN	HPLC	0.785 cm^2	
Rat skin	-	Full-thickness skin	Dermatomed skin	EtOH/propylene glycol /water (4:1:1)	250 µL	Saturated solution (supersaturation degree = 2.1)	PBS	Perfusion (0.6 mL/min)	6 h	2.2 µg/cm^2.h (drug crystallization)	NF	NN	HPLC	0.785 cm^2	
Rat skin	-	Full-thickness skin	Dermatomed skin	EtOH/propylene glycol /water (4:1:1)	250 µL	Saturated solution (supersaturation degree = 2.6)	PBS	Perfusion (0.6 mL/min)	6 h	2.2 µg/cm^2.h (drug crystallization)	NF	NN	HPLC	0.785 cm^2	
Mice skin	-	Full-thickness skin	NF	TST solubilized in isopropyl myristate (1/99)	1 g	1%	PBS	7-7.3 mL	10 h	2.0 µg/cm^2.h (lag time<0.5 h)	NF	N	HPLC	1.74 cm^2	(Imai et al., 2016)
Mice skin	-	Full-thickness skin	NF	TST in isopropyl myristate/l	1 g	1%	PBS	7-7.3 mL	10 h	2.6 µg/cm^2.h (lag	NF	N	HPLC	1.74 cm^2	

				ecithin (1/69/30)						time<2h)					
Mice skin	-	Full-thickness skin	NF	TST in isopropyl myristate/lecithin/water (1/65.6/30/3.4)	1 g	1%	PBS	7-7.3 mL	10 h	3.98 µg/cm^2.h (lag time=1.06h)	NF	N	HPLC	1.74 cm^2	
Mice skin	-	Full-thickness skin	NF	TST in water/lecithin/D-ribose (1/62.8/30/6.2)	1 g	1%	PBS	7-7.3 mL	10 h	2.69 µg/cm^2.h (lag time=0.77h)	NF	N	HPLC	1.74 cm^2	
Mice skin	-	Full-thickness skin	NF	TST in lecithin/D-ribose/tetraglycerol (1/30/55.6/13.4)	1 g	1%	PBS	7-7.3 mL	10 h	2.35 µg/cm^2.h (lag time=1.02h)	NF	N	HPLC	1.74 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (20:80)	3 mL	Saturated solution of TST (0.45 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	1.8 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 µL	2.01 cm^2	(Kim et al., 2001)
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (40:60)	3 mL	Saturated solution of TST (8.53 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.13 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 µL	2.01 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (50:50)	3 mL	Saturated solution of TST (18.52 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.07 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 µL	2.01 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (60:40)	3 mL	Saturated solution of TST (38.95 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.05 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 µL	2.01 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (70:30)	3 mL	Saturated solution of TST (68.32 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.04 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 µL	2.01 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (80:20)	3 mL	Saturated solution of TST (140.01 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.02 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 µL	2.01 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol 100%	3 mL	Saturated solution of TST (334.03	Saline solution containing 40%	12 mL	24 h	0.00 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 µL	2.01 cm^2	

						mg/mL)	(V/V) polyethylene glycol 400								
Mice skin	-	Epidermis+dermis	Surgical materials	TST solubilized in ethanol with and without chemical absorption enhancer (Azone®, isopropyl myristate, *N*-methyl-2-pyrrolidone and propylene glycol)	100 µL	5% (m/V)	40% PEG 200	7 mL	24 h	Azone>IPM>PG>NMP	NF	NN	HPLC	3.14 cm^2	(Lu et al., 2013)
Amphibian skin (fresh dorsal)	-	Full-thickness skin	Surgical materials	TST dissolved in ethanol/water 1/1 (v/v)	10µL/cm^2	40 µg/cm^2 (dose solution 4 mg/mL)	5% BSA in amphibian Ringer's solution	12.5 mL	8h	J = 5.3 µg/cm^2.h P = 1.3 x 10^{-3} cm/h	NF	NN	Liquid scintillation counting	1.85 cm^2	(Kaufmann and Dohmen, 2016)
Amphibian skin (fresh ventral tissue)	-	Full-thickness skin	Surgical materials	TST dissolved in ethanol/water 1/1 (v/v)	10µL/cm^2	40 µg/cm^2 (dose solution 4 mg/mL)	5% BSA in amphibian Ringer's solution	12.5 mL	8h	J = 12.0 µg/cm^2.h P = 3.0 x 10^{-3} cm/h	NF	NN	Liquid scintillation counting	1.85 cm^2	
Amphibian skin (frozen dorsal tissue)	-	Full-thickness skin	Surgical materials	TST dissolved in ethanol/water 1/1 (v/v)	10µL/cm^2	40 µg/cm^2 (dose solution 4 mg/mL)	5% BSA in amphibian Ringer's solution	12.5 mL	8h	J = 7.4 µg/cm^2.h P = 1.9 x 10^{-3} cm/h	NF	NN	Liquid scintillation counting	1.85 cm^2	
Amphibian skin (frozen ventral tissue)	-	Full-thickness skin	Surgical materials	TST dissolved in ethanol/water 1/1 (v/v)	10µL/cm^2	40 µg/cm^2 (dose solution 4 mg/mL)	5% BSA in amphibian Ringer's solution	12.5 mL	8h	J = 11.4 µg/cm^2.h P = 2.9 x 10^{-3} cm/h	NF	NN	Liquid scintillation counting	1.85 cm^2	
Gottingen Minipigs (1.5 months)	765 µm	Epidermis+dermis	Dermatomed skin	20% ethanol in 0.05 M phosphate buffer (pH=7.4)	NF	0.1 mg/ml	0.05 M phosphate buffer	12.1 mL	28 h	J = 0.223 µg/cm^2.h Lag time: 3 to 5 h	NF	NN	Liquid scintillation counting	1.77 cm^2	(Qvist et al., 2000)
Gottingen Minipigs (3 months)	765 µm	Epidermis+dermis	Dermatomed skin	20% ethanol in 0.05 M phosphate buffer (pH=7.4)	NF	0.1 mg/ml	0.05 M phosphate buffer	12.1 mL	28 h	J = 0.361 µg/cm^2.h Lag time: 3 to 5 h	NF	NN	Liquid scintillation counting	1.77 cm^2	
Gottingen Minipigs (6 months)	765 µm	Epidermis+dermis	Dermatomed skin	20% ethanol in 0.05 M phosphate buffer (pH=7.4)	NF	0.1 mg/ml	0.05 M phosphate buffer	12.1 mL	28 h	J = 0.538 µg/cm^2.h Lag time: 3 to 5 h	NF	NN	Liquid scintillation counting	1.77 cm^2	
Domestic pigs	765 µm	Epidermis+dermis	Dermatomed skin	20% ethanol in 0.05 M phosphate buffer (pH=7.4)	NF	0.1 mg/ml	0.05 M phosphate buffer	12.1 mL	28 h	J = 0.792 µg/cm^2.h Lag time: 3 to 5 h	NF	NN	Liquid scintillation counting	1.77 cm^2	
Human skin (abdominal and breast skin)	408 µm	Epidermis+dermis	Dermatomed skin	20% ethanol in 0.05 M phosphate buffer (pH=7.4)	NF	0.1 mg/ml	0.05 M phosphate buffer	12.1 mL	28 h	J = 0.501 µg/cm^2.h Lag time: 3 to 5 h	NF	NN	Liquid scintillation counting	1.77 cm^2	
Mice skin	-	Full-thickness skin	Surgical materials	PEG 400 (5%, w/v) in saline	5 mL (containing 5 mg TST)	1 mg/mL	PBS/PEG 400 (95/5, V/V)	17.5 mL	12 h	J = 0.25 µg/cm^2.h P = 2.49 x 10^{-4} cm/h	NF	NN	HPLC 10µL	1.33 cm^2	(Zhang et al., 2017)
Mice skin	-	Full-thickness	Surgical	Imidazolium ionic	5 mL	1 mg/mL	PBS/PEG 400	17.5 mL	12 h	J = 0.42 to 0.9	NF	NN	HPLC 10µL	1.33 cm^2	

		s skin	materials	liquids and PEG 400 (5%, w/v) in water *Assay were performed with 20 different types of imidazolium ionic liquids	(containing 5 mg TST)		(95/5, V/V)			µg/cm².h (range) P= 4.18 to 8.95 x 10^{-4} cm/h					
Human skin (abdominal)	400 ± 50 µm	Edpiermis+dermis	Dermatomed	Pure ethanol	10 µL	1.64 µg/cm²	0.9% NaCl in water + 1% (m/V) BSA + 0.05% (V/V) gentamycin sulfate	NF	24 h	$Q_{permeated}$ = 4.7% of applied dose	NF	N N	Scintillation counting	1.00 cm²	(Hewitt et al., 2020)
Reconstructed skin model (*in vitro*)	-	Epidermis+dermis	Cell cultivation without alpha-linolenic acid and linoleic acid	TST solubilized in Ethanol/water (1/1, V/V)	100 µL (400 µg TST)	4 mg/mL	PBS + 5% bovine serum albumin	5 mL	24 h	J = 78.3 and 52.9 µg/cm².h (up to 2 h – two different experimental groups)	NF	N Y (4°C)	UPLC 5 µL	0.63 cm²	(Simard et al., 2019)
Reconstructed skin model (*in vitro*)	-	Epidermis+dermis	Cell cultivation with alpha-linolenic acid (ALA)	TST solubilized in Ethanol/water (1/1, V/V)	100 µL (400 µg TST)	4 mg/mL	PBS + 5% bovine serum albumin	5 mL	24 h	J = 46.8 µg/cm².h (up to 2 h)	NF	N Y (4°C)	UPLC 5 µL	0.63 cm²	
Reconstructed skin model (*in vitro*)	-	Epidermis+dermis	Cell cultivation with linolenic acid (ALA)	TST solubilized in Ethanol/water (1/1, V/V)	100 µL (400 µg TST)	4 mg/mL	PBS + 5% bovine serum albumin	5 mL	24 h	J = 48.8 µg/cm².h (up to 2 h)	NF	N Y (4°C)	UPLC 5 µL	0.63 cm²	
Silicone membrane	0.05 cm	HT-6240 BISCO® transparent membrane	-	TST solubilized in EtOH:PBS (50: 50)	900 µL	7.07 x 10^3 µg/mL	EtOH: PBS (50: 50)	5 mL	8 h	J = 64 µg/cm² P = 9.2 x 10^{-3} cm/h Lag time = 0.22 h	NF	N N	UV spectroscopy (245 nm)	0.64 cm²	(Alberti et al., 2017)
Human skin (abdominal)	1000 µm	Full-thickness skin	Dermatomed skin	TST solubilized in ethanol/water 1/1 (v/v)	25 µL/cm²	4 mg/mL	5% BSA in water	4 mL	24 h	P = 69.3 x10^{-5} cm/h Lag time = 4.4	1.7%	Y (ethanol) N	Liquid scintillation counting	1 cm²	(Guth et al., 2015)
Human skin	400 µm	Epidermis+dermis	Dermatomed skin	80% of the maximal solubility in a 50/50 ethanol/H2O (% V/V)	500 µL	9.71 mg/mL	PBS + 5% (m/V) bovine serum albumin	5 mL	24 h	3.92 x 10^{-4} cm/h	NF	Y (acetonitrile) N	HPLC 25 µL	0.64 cm²	(Verysert et al., 2015)
Human skin	400 µm	Epidermis+dermis	Dermatomed skin	80% of the maximal solubility in a 50/50 ethanol/H2O (% V/V) + Azone (1%, W/V)	500 µL	9.71 mg/mL	PBS + 5% (m/V) bovine serum albumin	5 mL	24 h	25.5 x 10^{-4} cm/h	NF	Y (acetonitrile) N	HPLC 25 µL	0.64 cm²	
Human skin	400 µm	Epidermis+dermis	Dermatomed skin	80% of the maximal solubility in a 50/50 ethanol/H2O (% V/V) + phytoceramides (1%, W/V)	500 µL	9.71 mg/mL	PBS + 5% (m/V) bovine serum albumin	5 mL	24 h	2.05 to 7.02 x 10^{-4} cm/h (range) 10 phytoceramides were tested as chemical absorptio	NF	Y (acetonitrile) N	HPLC 25 µL	0.64 cm²	

										n enhancers					
Human skin	1) 500 µm 2) 900-1100 µm	Epidermis+dermis	1) Dermatomed skin 2) Full-thickness skin	TST solubilized in ethanol 50% (V/V)	16 µL	4 mg/mL	Saline + 5% (w/v) bovine serum albumin, pH 7.4	0.4 mL		1) 1.82 µg/cm^2.h (lag time = 0.6 h) 2) 0.18 µg/cm^2.h (lag time = 2.2 h)	1) 136 µg /mL 2) 112 µg/mL	N	Liquid scintillation counting	0.64 cm^2	(Wilkinson et al., 2006)