



Reflections on the OECD guidelines for in vitro skin absorption studies

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Reflections on the OECD guidelines for *in vitro* skin absorption studies

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ABSTRACT

At the 8th conference of Occupational and Environmental Exposure of the Skin to Chemicals (OEESC) (16–18 September 2019) in Dublin, Ireland, several researchers performing skin permeation assays convened to discuss *in vitro* skin permeability experiments. We, along with other colleagues, all of us hands-on skin permeation researchers, present here the results from our discussions on the available OECD guidelines. The discussions were especially focused on three OECD skin absorption documents, including a recent revision of one: i) OECD Guidance Document 28 (GD28) for the conduct of skin absorption studies (OECD, 2004), ii) Test Guideline 428 (TGD428) for measuring skin absorption of chemical *in vitro* (OECD, 2004), and iii) OECD Guidance Notes 156 (GN156) on dermal absorption issued in 2011 (OECD, 2011). GN156 (OECD, 2019) is currently under review but not finalized. A mutual concern was that these guidance documents do not comprehensively address methodological issues or the performance of the test, which might be partially due to the years needed to finalize and update OECD documents with new skin research evidence. Here, we summarize the numerous factors that can influence skin permeation and its measurement, and where guidance on several of these are omitted and often not discussed in published articles. We propose several improvements of these guidelines, which would contribute in harmonizing future *in vitro* skin permeation experiments.

1. Introduction

At the 8th conference of Occupational and Environmental Exposure of the Skin to Chemicals (OEESC) (16–18 September 2019) in Dublin, Ireland, several researchers performing skin permeation assays

convened to discuss *in vitro* skin permeability experiments. The Organization for Economic co-operation and Development (OECD) produces internationally accepted guidelines, which give specifications for testing of chemicals. These OECD Test Guidelines are under constant review, with guidelines being periodically updated and new guidelines being

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adopted. The discussions were especially focused on three OECD skin absorption documents, including a recent revision of one:

- i) OECD Guidance Document 28 (GD28) for the conduct of skin absorption studies (OECD, 2004),
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- iii) OECD Guidance Notes 156 (GN156) on dermal absorption issued in 2011 (OECD 2011). GN156 (OECD, 2019) is currently under review but not finalized. Citations to GN156 refer to GN156 issued in 2011, except when designated specifically as GN156–2011 or GN156-2019 because there are differences.

A mutual concern was that these guidance documents do not comprehensively address methodological issues or the performance of the test, which might be partially due to the years needed to finalize and update OECD documents with new skin research evidence. Numerous factors can influence skin permeation and its measurement (Law et al., 2019; Sullivan et al., 2017, IPCS, 2006) but guidance on several of these are omitted and often not discussed in published articles. We, along with other colleagues, all of us hands-on skin permeation researchers, present here the results from our discussions on the available OECD guidelines. We aim to (1) assist other skin absorption laboratories in planning their experiments considering factors that are currently not defined in the OECD documents; and (2) provide some practical insights to the OECD expert group that currently is revising the GN156-2019 guideline. The overall goal is to contribute to the harmonization of skin permeation experiments and of what is reported about the skin permeation experiments.

We have divided the discussion of the OECD guidance documents into the following overarching themes: terminology, interpretation of *in vitro* data, scope, diffusion cell, receptor fluid, sampling, skin procurement and preparation, skin thickness, skin integrity, skin viability, test substance concentrations and formulations, application to the skin, temperature, recovery procedures, chemical analysis, data, replicates, and test report. When applicable, these themes have been further subdivided into factors affecting the experimental set-up.

2. Terminology

Terminology such as penetration, permeation, absorption should be defined in each guidance document and used consistently across the guidance documents. Skin absorption is a process that describes the passage of compounds across the skin and includes penetration - the entry of a substance into the stratum corneum and permeation through epidermis. In GN156 paragraph number 30 (par. 30), the definition of dermal penetration is the mass of the test substance that enters the skin, and dermal permeation is the mass that has transferred from the skin to the reservoir compartment fluid. Dermal permeation is also described as dermal absorption (TGD428, GD28, GN156). The absorbed dose is the amount absorbed into the receptor fluid or systemic circulation within a specified period of time (TGD428, GD28, GN156), whereas the absorbable dose (TG428, GD28, GN156) is the test substance mass in and on the skin after washing at the same specified time (indicating that some or all of it would transfer to the receptor fluid or systemic circulation if the experiment had continued). Finally, dermal delivery is the sum of the absorbed and absorbable dose.

Care should be taken to avoid ambiguous, incomplete or inconsistent descriptions. For example, the definition of the “absorbed dose (*in vitro*)” in TGD428, GD28 and GN156 as “the mass of test substance reaching the receptor or systemic circulation” is actually the definition of the absorbed dose *in vitro* and *in vivo*. Another example is the definition of “infinite dose” (GD28, par. 57 and p. 30 and GN156, p. 63) as the amount of test preparation applied to the skin where a maximum absorption rate of the test substance is achieved and maintained. This definition needs expanding to include infinite dose experiments of test

substances with such long lag times that the maximum (and steady state) absorption rate is not reached within a reasonable time for the *in vitro* diffusion experiment. In GN156 (p. 63) dermal absorption is defined as “the process by which a substance is transported across the skin and taken up into the living tissue of the body”, which is confusing because skin itself is a living tissue of the body. This inconsistency has been resolved in GN156-2019 (p. 56), which defines absorption as “the movement of a chemical from the outer surface of the skin into the circulatory system, eventually leading to systemic exposure towards the chemical (dermal bioavailability) and its metabolites”. Other examples of improvements that have been made in more recent documents include the change from “terminal procedures” used in the 2004 documents (TGD428, GD28) to “mass balance” in GN156, and from “recovery” to “mass balance recovery”.

We recommend that when revising the guidelines, a particular effort be made to harmonize the definition of terms. With respect to accuracy, the definitions in GD28 (pp. 30–31) and GN156-2019 (pp. 56–57) should be updated to specify that the permeability coefficient (K_p) is defined at steady state, but flux measurements need not be at steady state except when used to calculate K_p (see discussion in the next section).

3. Interpretation of *in vitro* data

In vitro experiments are used to characterize skin permeation in a laboratory setting. Chemicals in the receptor fluid have crossed the skin barrier and are systemically available to circulate to other organs, while chemicals retained in the skin have the potential for systemic availability. Commonly measured quantities include the test substance permeation across the skin into the receptor fluid as a function of time ($\Delta m(t_i)$, $i = 1, 2, 3, \dots$), the quantity retained in the skin following a wash (sometimes including analyses of tape strips and/or separate analyses of epidermis and dermis), and the total mass balance including amounts in receptor solution, wash, skin and a final cell wash. The total mass balance is to be within the range 90–110% (TGD 428, par. 20; GD28, par 77; GN156, par. 95) with a few exceptions. Unlabeled substances or volatile substances have an acceptable range 80–120% (GD28, par. 77; GN156, par. 95), while pesticides have a more stringent range 95–105% (GN156-2019, par. 95). We note that the total mass balance may not be meaningful for infinite dose experiments, in which the amount of test substance in the applied dose far exceeds the amounts in the other compartments. This point is noted even more strongly in GD28 (par. 79), which states: “For infinite dose experiments ... recovery determination is not relevant”.

Permeation data are most often displayed in one of two ways:

$$M(t_i) = \frac{1}{A} \sum_{i=1}^n \Delta m(t_i) \quad \text{cumulative amount permeated at time } t_i, i = 1, 2, 3, \dots \quad (1)$$

$$J \left(t_{i-\frac{1}{2}} \right) = \frac{M(t_i) - M(t_{i-1})}{t_i - t_{i-1}} \quad \text{permeation rate or flux over the interval } t_{i-1} \text{ to } t_i \quad (2)$$

In Eq. (1), A is the cross-sectional area of the diffusion cell. Thus, both M and J are normalized per unit area. The time $t_{i-1/2}$ is the midpoint of the i th time interval, i.e. $t_{i-1/2} = (t_{i-1} + t_i)/2$ (Sclafani et al., 1993). With sufficient sampling points, these discrete functions can be interpolated to yield continuous functions, $M(t)$ and $J(t)$. These functions may be divided by the applied dose per unit area and multiplied by 100% to convert to % of dose permeated and the permeation rate %, respectively.

In an infinite dose experiment, the permeant concentration is constant over a long enough experimental duration to establish steady state characterized by the flux J reaching a constant (i.e., steady state) value,

designated J_{ss} , and the curve $M(t)$ becoming linear. The ratio of J_{ss} (i.e., the slope of the linear portion of $M(t)$) to the external concentration difference ΔC_{ext} is defined as the permeability coefficient, K_p . If sink conditions are maintained in the receptor fluid, then ΔC_{ext} is equal to the test compound concentration in the vehicle, C_v . In this case,

$$J_{ss} = K_p \cdot C_v \quad (3)$$

The intercept of the linear (i.e., steady state) portion of $M(t)$ with the time axis is defined as the time lag, t_L . The quantities K_p and t_L are thus experimentally determined and are model-independent. Their values can be interpreted in terms of membrane properties by the use of an appropriate mathematical model. In the simplest case of Fickian diffusion through a homogeneous membrane of thickness h , these values are given by

$$K_p = \frac{DK_{mv}}{h} \quad (4)$$

and

$$t_L = \frac{h^2}{6D} \quad (5)$$

where K_{mv} is the membrane/vehicle partition coefficient of the permeant and D is its diffusivity within the membrane. For permeation of highly polar or highly lipophilic compounds through skin, more complex models are often appropriate. A comprehensive discussion is given by Mitragotri et al., (2011).

Depending on the selected measurement times and data variability, plots of $M(t)$ versus t may appear to be linear before they in fact are (i.e., before steady state is achieved). Including measurements collected at times before steady state leads to underestimates of both K_p and t_L (Barry, 1983; Bunge et al., 1995; Shah, 1993). Based on mathematical modeling of membrane permeation from an infinite dose, linear regression of $M(t)$ versus time data should exclude measurements collected at times less than approximately $2.4 t_L$, which are not at steady state (Bunge et al., 1995). An iterative process may be required in which data points are added or excluded from the linear regression based on the derived t_L . Values of K_p and t_L can be derived from infinite dose experiments that do not reach steady state (e.g., because t_L is large), but this requires a mathematical model (e.g., Bunge et al., 1995; Piroot et al., 1997; Selzer et al., 2013) making the parameter values model dependent.

In finite dose experiments, the amount of permeant is too small to maintain a constant concentration in the vehicle over the course of the experiment causing the flux to exhibit a peak value J_{peak} at time t_{peak} . A peak flux may also be observed if the vehicle volume is too small to keep the application area on the skin surface covered over the experimental duration, or if there are changes in the vehicle composition that cause the rate of permeant penetration into the skin to decrease (e.g., evaporation and/or absorption of a component that enhances permeation or permeant precipitation arising from solvent loss). For finite dose experiments, the total loading on skin, which depends on both the concentration of the test chemical concentration and the vehicle volume, becomes a key variable as described in Frasch et al., (2014b). GN156 (pars. 8 and 63) recommends using the percentage absorbed rather than flux for finite dose applications. A problem with this recommendation is that the percentage absorbed is not always independent of loading, is not a constant for a given chemical, and is easily manipulated (e.g., by changing the vehicle volume while keeping the test chemical loading constant) to give misleading results (e.g., see Kissel, 2011 and Figure2 and Case Study 1 in Frasch et al., 2014a). We recommend that results reported as percentage absorbed should list the amounts of both the test substance and formulation applied per skin area along with information on other factors that can affect the results.

In some cases values for K_p , normally determined in an infinite dose experiment can be derived from finite dose experiment by fitting an

appropriate mathematical model to the finite dose data provided that the different exposure durations of the finite and infinite dose experiments do not affect the skin differently (i.e., the values of K_p and t_L are the same in both) (Franz et al., 1993). Generally, K_p estimates derived from apparently linear portions of the $M(t)$ versus time curve in a finite dose experiment underestimate the true value. Guidance describing what information should be provided to judge the quality of K_p values derived from finite doses would be helpful.

It is evident from Eq. (4) that permeation experiments conducted using different vehicles will yield different K_p values, since K_p depends on K_{mv} . A particularly useful extension of Eqs. (3) and (4) is to consider the flux from a saturated solution of the test compound. In this case, C_v is the solubility of the test compound in the vehicle (S_v) and the flux is defined as the maximum flux, J_{max} . Thus,

$$J_{max} = K_p \cdot S_v \quad (6)$$

Maximum flux is independent of the choice of vehicle for vehicles that do not interact with the skin. However, maximum flux from an aqueous solution is often a useful starting point for risk assessment because methods of estimating or measuring K_p from aqueous solution and water solubility S_v are widely available. Complications arise when the test compound is either very soluble or very insoluble in water, or when it has a significant impact on skin permeability at high concentrations. Examples of the former include ethanol, 2-butoxyethanol and 2-hydroxypropyl acrylate, water-miscible solvents where permeation has a maximum at intermediate aqueous concentrations (Berner et al. 1989a, 1989b; Bunge et al., 2012; Frasch et al., 2014a). Examples of the latter include polycyclic aromatic hydrocarbons and many pesticides. For these compounds, our recommendation is to not rely on aqueous K_p values for risk assessment, but rather to conduct experiments that best represent the exposure scenarios which individuals may encounter.

We recommend that the OECD guidelines include the theoretical basis for calculating dermal absorption parameters listed above, as well as the information about determining K_p , t_L and J , and about identifying data that are (and are not) at steady state. The definitions should be revised for flux (GD28, p. 30 and GN156-2011, p. 63, GN156-2019, p. 56), which should not be restricted to steady state, and for K_p (GD28, p. 31 and GN156-2011, p. 64, GN156-2019, p. 57), which is defined at steady state whether it was derived from steady state or unsteady state measurements. Reported flux values should be identified as steady state, unsteady state or peak measurements.

4. Scope of the OECD skin absorption guidance documents

The focus of GN156 is dermal absorption for occupational and public health risk assessment of pesticides and biocides with only a mention that the guidelines may also be relevant for other groups of chemicals such as veterinary medicines, pharmaceuticals, and industrial chemicals. The other OECD guidelines (TG428 and GD28) do not have this restriction in scope. Guidance like that in GN156 should characterize skin permeation more broadly and consider chemicals in any type of use including finite dose as well as infinite dose scenarios at both steady state and unsteady state. Guidelines with a wider scope would be useful to skin scientists in general along with risk assessors specifically, who could then estimate skin permeation to evaluate both public and occupational health risks from exposure by any chemical.

5. Diffusion cell

Material. In all diffusion cells, the skin sample separates the donor and receptor compartments, and appearance of the test chemical and/or its metabolites in the receptor fluid is determined as a function of time. Diffusion cells are made of materials that are minimally reactive with many chemicals (e.g., glass, Teflon or Neoflon®) to minimize interferences with the test substance. Receptor compartments are configured as static (no flow) systems with periodic sampling of the receptor

fluid, or as flow-through systems, in which the receptor fluid is continuously replaced and collected over set sampling intervals. Commonly, a multi-channel peristaltic pump with flexible tubing (e.g. silicone rubber or plastic) transfers fresh fluid from a reservoir to the receptor compartments of multiple cells simultaneously. The choice of tubing material needs to be stated as these might interfere with the experimental results by contamination (e.g. using vinyl tubing containing di-ethyl hexyl phthalate as a plasticizer when testing skin absorption of phthalates (Hopf et al., 2014)) or by absorption (e.g. when testing organic solvents). In such cases, it might be better to run the diffusion experiment in static mode without tubing (Schenk et al., 2018). Generally, static cells are preferred for measuring permeation of volatile chemicals, which might be lost during transfer from the flow-through receptor to the sample vial. Information related to the parts that deteriorate over time, such as the tubing replacement schedule, should be reported.

Flow through diffusion cells. Flow-through cells differ from static cells in two important ways that can affect results. First, flow-through cells introduce an additional lag time that is approximately equal to the volume of the receptor compartment and the outlet tubing (i.e. π (inside diameter)²/4 multiplied by the tube length from the receptor chamber to the sample vial) divided by the flow rate (e.g., 1 h if the volume is 1 mL and the flowrate is 1 mL/h). Second, concentrations in static cell samples represent the mass in the receptor at each sampling time (e.g., at each hour if sampling is hourly), whereas concentrations in flow-through samples are averages of the sampling interval (e.g., the average over an hour if sampling is hourly). These differences are minimized if the receptor chamber and outlet tubing are flushed multiple times each hour (e.g., >10 times in the study by Bronaugh and Stewart, 1985) and the sampling intervals are not too long (Sclafani et al., 1993).

Test chemical permeability combined with analytical limitations may require lower flow rates that produce larger concentrations; this may also necessitate a longer sampling interval. In infinite dose experiments, flow-through and static diffusion cells will still give comparable steady state flux results, although steady state will be reached later in flow-through cells (Bronaugh and Stewart, 1985) by an amount equal approximately to the lag time for flow through the receptor compartment and outlet tubing. For finite dose experiments, the added lag time and concentration averaging over the sampling interval in the flow-through cell experiment can produce significantly different values for the peak flux and time of the peak flux compared with static cells or with flow-through cell systems operated at different conditions (e.g., flow rates, sampling intervals) even if the same cells are used (Harrison and Knutson, 1995; Reifenrath et al., 1994; Sclafani et al., 1993). Theoretically, peak flux is lower and the time to peak flux longer than observed in static cells by amounts that depend on the flow rate and volumes of the receptor compartment and outlet tubing (Harrison and Knutson, 1995; Sclafani et al., 1993). Mathematical methods can be applied to derive the equivalent static cell results from flow-through cell measurements (Anissimov and Roberts 1999, 2000, 2001; Harrison and Knutson, 1995; Sclafani et al., 1993). Results from flow-through cell experiments should specify the receptor flow rate, the sampling interval, and the receptor compartment and outlet tubing volumes. For both infinite and finite dose experiments, the total amount permeated does not depend on the cell volume or flow rate as long as the sampling interval and diffusion cell lag time (i.e., the ratio of the receptor compartment and outlet tubing volumes to the flow rate) is small relative to the time for concentration change. The appropriateness of comparing flow-through experiments performed at different conditions or flow-through and static cell experiments should be discussed.

In flow-through cell experiments, the mass of test chemical permeated depends on the collected sample volume. The volume delivered by multi-channel peristaltic pumps can vary cell-to-cell, especially when sampling volumes are small (e.g., 1 mL or less), as well as day-to-day as the tubing ages. Methods for monitoring sample volumes should be described. If average values are used rather than measurements of

individual sample volumes, the expected variability should be reported.

Occlusion. The guidelines recommend exposing the skin without occlusion as this corresponds to the most common exposure scenarios. However, it may be necessary to work under occlusion in some cases, for example, when the test substance is volatile or to mimic a scenario where the skin is contaminated and covered, e.g. with protective gloves. It is important to be aware that occlusion influences certain parameters that may affect the skin permeation (skin temperature and skin barrier properties such as transepidermal water loss (TEWL), hydration and skin surface pH) (du Plessis et al., 2013; Stefaniak et al., 2013). Also, materials that are good barriers to water vapor (e.g., Parafilm) are not always effective barriers to volatile or semi-volatile compounds. The guidelines should be more detailed on how to perform experiments under occlusion (type of membrane for example) to enable comparisons of results between laboratories.

Size. The diffusion cells come in different sizes with cell surface areas ranging from 0.3 to >2 cm². The choice of cell depends on the chemical to be tested and amount of available skin, in addition to various practical and experimental considerations. Diffusion cells with a small receptor volume may allow better measurement of the permeation at low quantities, thereby avoiding dilution of the substance, which improve analytical detection. In some cases, larger volumes or skin area are necessary to accommodate the diffusion cell operation and analytical requirements. One such example is a system where the receptor medium recirculates between the diffusion cells and an Inductively Coupled Plasma Mass Spectrometry ICP-MS instrument, where 4500 readings were collected during 2 h of exposure (Midander et al., 2020). We recommend that the OECD Guidelines indicate the rationale for choosing a particular diffusion cell. Moreover, a selection guide to match the best cells for optimal experimental set-up would be welcomed.

6. Receptor fluid

The TGD428 (par. 10) describes the property for the receptor fluid as "... physiologically conducive receptor fluid.". The choice of the receptor fluid depends on the characteristics of the skin used and on the compounds tested. The receptor fluid should mimic the intercellular fluid in the skin. GD28 recommends saline solution for non-viable skin and water-soluble compounds, and physiological based receptor fluid (e.g. tissue culture medium) for metabolically active skin. In any case, the solubility of the compound in the receptor fluid is critical, which is why it must sometimes be supplemented or replenished (or both) (GN156, pars. 39–44). We recommend that the guidelines specify the composition of the physiologically relevant media.

In TGD428 (par. 10) it is stated that "adequate solubility of the test chemical in the receptor fluid should be demonstrated so that it does not act as a barrier to absorption." According to GD28 (par. 28), solubility that is 10 times greater than the estimated maximum concentration of the test substance in the receptor solution is considered adequate. It is, however, difficult to anticipate the maximum concentration of the test chemical with only a few pilot scale permeability experiments. We recommend that the guidelines provide instructions on how to estimate this.

It would be helpful if examples of appropriate receptor fluids for various types of poorly soluble analytes could be provided. This is especially valuable if multiple concentrations of toxic industrial chemicals are being investigated.

Receptor fluids that "support skin viability throughout the experiment" (TGD428, par. 10) include a culture medium such as RPMI, which has been shown to keep the skin metabolically active for over 24 h (Sanchez et al., 2010) and ensures physiological osmolality. Alternatively, one might consider using Ringer's solution in case of metabolic (or prolonged) studies as it contains several types of ions/salts. However, Gaucher et al., (2012) suggest that skin retains more viability with RPMI than with Ringer's solution. Studies are thus necessary to validate

that Ringer's solution is equivalent to cell culture media in supporting skin viability as a receptor fluid in percutaneous experiments. Usually it is sufficient to ensure that the initial glucose concentration is 1 g/L (Tarnowska et al., 2019). Apart from composition, receptor fluid pH should mimic the body fluid (close to 7.35) and have sufficient buffering capacity to counter the skin's high lactate production and maintain the skin's metabolic activity (Tarnowska et al., 2019). We recommend that the guidelines specify acceptable composition as well as the pH of the receptor fluids.

7. Sampling

Sampling frequency is another experimental parameter that needs to be defined based on the anticipated skin permeation rate of the test substance, i.e. the faster the permeation of the test substance the less time between sampling points. GD28 (par. 71) recommends 6–12 sampling points over 24 h. Sampling frequency depends on how many points you need to obtain a satisfactory curve that can describe the kinetic absorption profile, which can probably be discussed at length as this is related to what the researcher accepts in terms of variability. The problem often solves itself in the laboratory based on available equipment and resolution of practical issues related to experimental feasibility. The first sampling time is often determined by the detection limit of the analytical method for the test chemical, and the number of sampling points may be influenced by various factors such as analysis costs. Consequently, this may lead to judgement of sampling rate requirements based on previous experience resulting in incongruent skin permeation characteristics. For example, many studies investigating metals and metal powders set their first sampling point at 2 h (Crosera et al., 2018; Franken et al., 2015a; Larese Filon et al., 2013); meanwhile, a continuous sampling experiment with a highly sensitive analytical method showed that permeation occurred much earlier (Midander et al., 2020). Sampling frequencies required to characterize rapidly permeating organic solvents are often greater than 12 points that may benefit from increasing sampling intervals (e.g., every 10 min for the first hour, then every 20 min, and towards the end every 30 min (Schenk et al., 2018)). We recommend that sampling frequency be determined in a pilot study. For derivation of K_p from steady state measurements in an infinite dose experiment, several samples should be collected at times longer than $\sim 2.4 t_L$, which theoretically represents steady state (Bunge et al., 1995). For finite dose, sampling times should allow determination of the peak flux at the peak time from the flux profile.

8. Skin procurement and preparation

The GN 156 guideline states, "Skin from human or animal sources can be used". The authors of this commentary use skin obtained from rats, backs of stillborn piglets, adult pig ears, and human skin. Pigskin has been suggested as an alternative to human skin due to similar physiological characteristics (Barbero and Frasc, 2009). *In vitro* skin permeation studies of organic substances employing pigskin yielded similar skin permeation curves as published human *in vivo* data (Cilurzo et al., 2007; Schenk et al., 2018). Ethical issues should always be considered when using animal skin, and required permissions should be obtained before starting a study. It is ethically advantageous to use slaughter waste, which is a byproduct from food production, over laboratory animals. Collecting pig ears directly from the slaughterhouse can be an option depending on the procedure at which the ears are treated before being obtained by the researcher (e.g., hair removal using a blowtorch or hot water). We recommend that the OECD guidelines consider including appropriately handled slaughter waste pigskin and adult pig ears for skin experiments in addition to human skin. Criteria for when the slaughter waste pigskin are acceptable alternatives should also be included. In the future, reconstructed human epidermis (RHE) models could be an alternative option once the models are standardized and exhibit a permeability barrier comparable to human skin (Helder

et al., 2020; Abd et al., 2016).

Human skin is generally considered the gold standard because skin permeation data are usually intended for application to the human population. For *in vitro* experiments, human skin is obtained from surgical waste (e.g. from abdominoplasty) and cadavers. Due to inter-donor variability that is inherent in biological membranes, human skin from different donors should be included. Human skin is decontaminated with an antiseptic agent before any trans-cutaneous invasive procedure. How these disinfecting agents affect the skin barrier function in *in vitro* permeation assays is not well known (Karpanen et al., 2008; Nesvadbova et al., 2015). We recommend that when using human skin, a note on which disinfectant that was used be included in the method section, if available (Sidhwa and Itani, 2015).

9. Skin thickness

Skin thickness is specified in GN156 (par. 31) as "when justified, full thickness skin can be used" (up to 1000 μm thick), "split-thickness, typically 200–400/500 μm thick" and "prepared with a dermatome". The dermatome allows setting of a specified thickness, although the skin is not always cut evenly. This introduces variability (Abd et al., 2016), which according to some researchers is reduced if skin is dermatomed to 500–750 μm . The skin thickness should be measured after dermatoming it, but characteristics of an appropriate measuring device are not specified. Several researchers use a micrometer (293-661-10, Mitutoyo) for dermatomed skin and for full-thickness skin (<1 mm thickness), and others use the Oditest tool (H.C. Kroeplin GmbH, Schlüchtern, Germany). The guidelines do not mention how many points to measure to calculate the average thickness nor a "spatial distribution" of measuring points, or if reporting maximum thickness is sufficient.

10. Skin integrity

Skin integrity tests are required to identify samples with barrier function outside "the normal range for that skin type", preferably before permeability measurements are made (GD28). Accepted methods for assessing skin integrity before or after the study (GD28 articles 45 and 47) include electrical resistance to an alternating current (applied at up to 2 V), TEWL, and tritiated water or other reference materials. No further guidelines are provided for performing and interpreting the integrity test or for identifying measurements outside the normal range. As summarized below, skin integrity testing is performed using many different protocols that can produce different results if used without the appropriate considerations. Further guidance on what information about the skin integrity test should be reported and how the rejection (cut-off) criterion is selected would be helpful.

Human skin integrity was first evaluated using tritiated water K_p with a rejection (cut-off) criterion set at $K_p > 2.5 \times 10^{-3}$ cm/h (Franz and Lehman, 1990). Rejection criterion of 1.5×10^{-3} cm/h (Davies et al., 2004) and 4.5×10^{-3} cm/h (Meidan and Roper, 2008) have also been proposed based on subsequent analyses of large numbers of measurements generated in these other laboratories. Faster test methods apply the tritiated water for 1 h (Runciman et al., 2010) or even less. The 5-min exposure assay developed by Franz and Lehman, (1990) and described later by Kasting et al., (1994) avoids fully hydrating the skin prior to applying treatments. The $^3\text{H}_2\text{O}$ cutoff in the latter report was 1.6 $\mu\text{L}/\text{cm}^2$ at 1 h from the dose application, which was subsequently revised upward to 2.0 $\mu\text{L}/\text{cm}^2$ (Morris et al., 2019). Recently, Lehman et al., (2017) used 1.56 $\mu\text{L}/\text{cm}^2$ as the cut-off criteria for tritiated water absorption over 0.5 h after a 5-min application, which was similar to values they derived from three different statistical analyses of more than 400 measurements (0.97, 1.42 and 1.47 $\mu\text{L}/\text{cm}^2$).

Electrical resistance measurements are rapid, robust and reproducible (Franken et al. 2014, 2015a; Larese Filon et al., 2016; Proksch, 2018). The current does not affect the measurement as long as the applied voltage is low enough to prevent skin damage from the

measurement. Increasing area causes a proportional increase in the inverse of the electrical resistance, and therefore, the product of the resistance and area is the correct way to represent the area normalized electrical resistance (White et al., 2011); i.e., 10 k Ω measured across 2.54 cm² = 25.4 k Ω cm², which is equivalent to 39.7 k Ω measured across 0.64 cm²). The large effect of frequency on the measurement has been investigated and correlated to the tritiated water K_p (White et al., 2011). Using the resulting equation, electrical resistance rejection of samples measuring <32 k Ω cm² when determined at 100 Hz and <14 k Ω cm² when determined at 1000 Hz are expected to correspond with a tritiated water cut-off criterion of K_p > approximately 1.5×10^{-3} cm/h (Bunge, 2016; White et al., 2011).

Several researchers use TEWL to assess the water barrier across the skin, with TEWL >10–11 g/m²·h a common cut-off for human skin based on *in vivo* measurements (Pinnagoda et al., 1990). There are important differences in the performance of *in vivo* and *in vitro* TEWL. The donor chamber may prevent contact of the TEWL with the skin surface, necessitating an adaptor or extension to the probe tip that changes the measurement geometry (i.e., the distance of the probe from the skin surface and volume of donor chamber). *In vitro*-to-*in vivo* probe calibration has been recommended (Imhof et al., 2012). The probe type (i.e., open-chamber, closed chamber with a condenser, and an unventilated closed chamber) also affects the results. Generally, the TEWL values determined in human skin are correlated with tritiated water permeation, although different values among the probe types occur (Elkeeb et al., 2010; Elmahjoubi et al., 2009; Farahmand et al., 2009; Imhof et al., 2014), suggesting consideration of probe-dependent cut-off values.

11. Skin viability

Metabolic viability is discussed in GD28 (nos. 32 through 34), which states “the most widely used indicator of skin viability has been glucose utilization since energy metabolism is critical to metabolic integrity”. This test requires glucose to be present in the receptor fluid and a chemical analysis of lactate concentration in the receptor fluid. We believe this method is not widely used, perhaps because it is time consuming and inconvenient to implement. Tissue viability assays (e.g., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (WST-1), and lactate dehydrogenase (LDH) release, resazurin) have historically been used in 2D cell cultures and skin irritation tests in the reconstructed human epidermis (RhE) model (OECD, 2020) but are not yet validated for the study of a complex three-dimensional structure like the skin. Currently, we believe the most appropriate method to evaluate skin viability is the tetrazolium salt assay, which is used to test skin viability prior to skin implantation (Castagnoli et al., 2003).

12. Test substance concentrations and formulations

GN156 (pars. 10 and 49) instructs studies to include several test concentrations that are in the range of realistic human exposures. We believe that the guidelines would be accommodating a broader research field if it could be stated that: if human skin exposures are known, it is advisable to mimic these as far as possible. Naturally, this depends on how sensitive the chemical analysis is, and if the receptor concentrations are above the limit of detection (LOD). LOD and limit of quantification (LOQ) should be determined prior to the relevant exposure conditions and given in the test report. Also, where possible, the formulation containing the test substance should be the same as that to which humans may be exposed.

Workers are often exposed to organic solvents as such. In general, permeability of a compound applied to the skin neat cannot be used to estimate K_p for that compound in water or another vehicle. However, a K_p calculated by dividing the permeation rate with the density of the test

substance may provide a reasonable estimate for that solvent in a vehicle with which it is fully miscible if neither the solvent or the vehicle alters the skin barrier significantly. Alternatively, the steady state flux of a neat organic solvent through the skin (a measure of maximum flux, J_{max}) can be divided by its water solubility, S_w , to provide a first estimate of K_p from an aqueous solution. But this relationship ignores hydration effects or co-swelling by water miscible solvent systems that can increase the latter value by up to an order of magnitude. Neat polar solvents can dehydrate the skin (Bunge et al., 2012; Frasch et al., 2014a) with a resulting increase in the barrier function (i.e. a decrease in K_p relative to the same solvent dissolved in water). For these compounds, K_p determined for the neat compound should not be used to predict flux for dilutions of it in water. To identify compounds with this behavior requires measurements of aqueous solutions of the compound.

As discussed above, permeation experiments conducted using different vehicles will yield different K_p values, even if the vehicle does not alter the skin, because K_p depends on partitioning between the stratum corneum and the vehicle (Bronaugh et al., 1986; Dugard and Scott, 1986). Because of this, and also vehicle changes to the SC, the flux values are different for the same concentration of a test substance in different vehicles; e.g., Abd et al., (2019); Barry et al., (1985); Champmartin et al., (2020). For the same reasons, studies of pesticides have observed enormous differences in permeation rates between active ingredients and formulations of the same active ingredient as well as between formulations containing the same amount of the active ingredient (Berthet et al., 2014).

The broad recommendation (GD28, par. 52) that “solids, such as powders be moistened with water to assist skin contact and quantitative and homogeneous application of the dose” is ill-considered, except perhaps when used as delivery agent that is known to evaporate soon after application. Moisture in surface solids will change during an experiment, by evaporation, absorption into dry skin, and transfer from the receptor fluid, even if humidity is controlled. Moreover, it is difficult to identify an appropriate standard amount to apply that would mimic the “presence of perspiration” for “normal ‘in-use’ conditions” (GD28, par. 52) given the wide variation in perspiration rates by body region and between individuals, and the rapid evaporation that occurs, except in high humidity environments or under occlusion. In addition, the perception that moisture (including artificial sweat) increases skin absorption from solid powders and films is not always valid. Chemicals that are solids or sorbed to solids do absorb into skin or membrane surrogates for skin without being moistened (e.g., McCarley and Bunge, 2003; Peckham et al., 2017), and a film of water or sweat can potentially act as a barrier for compounds with very low water solubility.

Artificial sweat has been used as vehicle for testing skin permeation of metals (Larese Filon et al., 2013; Midander et al., 2016) based on the premise that workers are in contact with most metals as solids in powders or deposited particles trapped in sweat. Many different compositions are described in literature for sebum and artificial sweat, and some formulations have been proposed (CEN, 2011; Stefaniak and Harvey, 2006). We would like to see the OECD guidelines give more details here, including a preferred formulation for artificial sweat and sebum as well as their pH. The test report of publication should include a description of the formulation used in the method section.

13. Application to the skin

GD28 (par. 57 and p. 30) and GN156 (2011 p. 63 and 2019 p. 56) define “infinite dose” as the amount of test preparation applied to the skin where a maximum absorption rate of the test substance is achieved and maintained, and “finite dose” as the amount of test preparation applied to the skin where a maximum absorption rate of the test substance may be achieved but is not maintained. These definitions clarify correctly that infinite and finite dosing are determined by permeation behavior and not the applied dose size (Hewitt et al., 2020). Nevertheless, GD28 specifies amounts for finite and infinite dose experiments. For

a finite dose experiment, GD28 (par. 56) recommends an applied dose of “up to 10 mg/cm² or 10 µL/cm²” because “larger amounts tend to run-off in actual exposure conditions”. For infinite dose experiments, GD28 (par. 58) explains that “typical doses of 100 µL/cm² (or >10 mg/cm²) or more, may be appropriate to obtain steady state conditions from which the steady state flux or absorption rate and K_p value (cm/hour) can be calculated. Such a volume ensures continuous excess of test preparation in the donor compartment.”

We support the definitions in GD28 that separate finite and infinite doses from the volume of the deposit. They correctly represent considerations necessary for comparison with actual exposure conditions (finite dose) or for obtaining consistent steady state flux and K_p values (infinite dose). In the latter case, a sufficient dose volume must be applied to ensure that the concentration of the dose solution is maintained nearly constant, e.g. $\pm 10\%$, during the duration of the test. Concentration can change either up or down depending on the relative evaporation and permeation rates of solvent and test solute, or to adsorption of the test solute on the donor compartment wall. We re-emphasize that K_p values obtained using nonaqueous donor solutions must not be directly compared to those from aqueous solutions, nor should they be used to predict delivery from compositions other than the one tested or very closely related chemistries.

Finite dose delivery requires thoughtful choices of dose vehicle and volume. Anticipated human *in vivo* exposure conditions should always be the first and foremost consideration. They should be matched as closely as possible commensurate with analytical sensitivity and satisfactory dose distribution and recovery. Exaggerating a finite dose leads to inaccuracies in estimating delivery in a typical exposure situation, however. For example, facial moisturizers are usually applied at a level of 1–2 mg/cm² and exposures from aerosolized pesticides or volatile organic solvents may be much smaller. When such formulations are applied to skin in much larger amounts, the potential for full hydration of the skin or disruption of the skin lipid barrier increases. Furthermore, if the solvent is volatile, increasing the dose increases the deposited load of test compound on the skin, which reduces its percentage absorption if a solubility limit is approached (Frasch et al., 2014b). Hence, exaggerated doses may either increase or decrease permeation, depending on the circumstances and on how the data are represented. Strict adherence to the OECD 10 µL/cm² finite dosing guideline may thus lead researchers to obtain “precisely the incorrect answer”. We encourage regulatory bodies to consider these factors when designing guidelines and guidance documents.

Application volumes less than 10 µL/cm² are difficult to distribute evenly onto the skin. This can increase variability in measurements of skin permeation and skin recovery. Surface tension of the test formulation and its ability or lack thereof to wet the skin or the walls of the donor compartment are major considerations in dose distribution. For example, very small doses of purely aqueous solutions, e.g. < 10 µL/cm², may bead up on the (low surface energy) skin surface, whereas larger doses that reach the walls of a (high surface energy) glass donor cell may climb the wall and deposit much of the dose on the wall and an outer annular ring of skin as they evaporate. The authors handle this difficulty differently and according to their research area and the characteristics of the test formulations they study.

Public health researchers recommend applying volumes that reliably cover the skin surface completely. Thus, as a first approach, for preliminary experiments, they encourage applying 30–100 µL/cm² for “finite dose” experiments. This should ensure an even distribution even if the skin becomes concave once mounted on the system. Public health researchers do not recommend adding other solvents not present at the workplace (e.g., ethanol) as their primary goal is to mimic a particular exposure scenario. Researchers in the pharmaceutical and cosmetics area suggest altering the formulation to facilitate its spread, for example, by lowering the surface tension of the test formulation or by thickening it. For aqueous formulations applied in the dose range of ≤ 10 µL/cm², these researchers recommend adding up to 50% v:v ethanol or 1–2% of a

nonionic surfactant such as polysorbates 20 or 80. Thickening the formulation with 1–3% carboxymethylcellulose or a related cellulosic thickener may be effective. For poorly water soluble solutes, the 4:1 v:v acetone:olive oil (AOO) could be useful. Some authors from both groups suggest that the guidelines let the researcher determine (with suitable explanations) the appropriate volume to deposit on the skin. These different approaches for applying finite doses and small volumes to the skin need further discussions to reach consensus.

We recommend that the discussion of finite and infinite dose should be kept separate from the volume of the deposit, as these are not always related. Further, we recommend that the guidelines let researchers determine (with suitable explanation) the appropriate volume to deposit on the skin. Substances that permeate the skin rapidly may require more than 100 µL/cm² to maintain a constant vehicle concentration long enough to reach steady state, while 10 µL/cm² might represent an infinite dose in some situations (e.g., a slowly permeating chemical in vehicle that spreads well across the skin surface). The choice of small applied volumes should consider surface wetting, viscosity, and density of the vehicle, which affect whether this amount of vehicle can cover the skin surface completely and homogeneously. For the application of liquids, we suggest using precision pipettes or syringes or positive displacement pipettes with weighing before and after depositing the substance.

Application of dry particles present some potential problems, such as electrostatic charge and uneven distribution. Also, one has to consider whether to load particles onto the skin before or after mounting the skin in the diffusion cell, recognizing the risks that some of the dose could be lost. A spatula can be used to drop the powder onto the skin, which basically mimics the workplace scenarios. For example, in the Lewinski et al., (2017), the amount of iron oxide nanopowder applied was determined from the change in the before and after spatula weight (using a static neutralizer to reduce loss due to electrostatic charge). A similar technique has been used to apply other non-liquid or viscous substances (e.g., biocidal creams containing either methylchloroisothiazolinone (MCI) or methylisothiazolinone (MI) (Berthet et al., 2017)). Solids might also be applied to the skin using discs uniformly covered with the test substance, which are placed on top of the skin mounted in the diffusion cell (Belsey et al., 2011). Particulate or dissolved solids can be applied to skin in acetone or other volatile solvent that subsequently evaporates. Whether the solvent contains dissolved solids or suspended particles, the residue of precipitated solid or dispersed particles left on the skin surface after the solvent evaporates will not be uniform in many cases. Variations in surface tension and preferential wetting of the skin surface or the donor chamber material are all known to cause uneven distribution of solids; e.g., Deegan et al., (1997). Before using solvents for this purpose, it is important to verify that their presence during the (usually short) time required to evaporate have no, or only minimal, effects on skin viability and integrity. We recommend including discussions of these approaches in the guidelines.

14. Temperature

According to the TGD428, it is preferable that the skin and diffusion chamber are kept constant at $32^\circ \pm 1^\circ \text{C}$ with a humidity of 30–70% throughout the experiment. However, the blood temperature in the skin is in the range of 33 – 35 °C (Savage and Brengelmann, 1996) under natural conditions, in addition, the skin exposures might occur in conditions with elevated temperatures and thus skin temperatures would be higher than 32 °C (Duracher et al., 2009). Gradual increase in temperature in the physiological range (25, 32, 39 °C) has been shown to facilitate skin permeation of both lipophilic (anisolet) and hydrophilic substances (1,4-dioxane, ethanol). After 3 h of exposure, absorbed amounts doubled or nearly tripled with the rise in temperature (25–39 °C) (Kilo et al., 2020). Some researchers have opted to conduct their *in vitro* experiments with the receptor fluid at 37 °C, and the reason was to ensure that the physiological skin temperature will not fall below, but be

maintained at 32 °C (Franken et al. 2014, 2015a, 2015b; Franz, 1975; Jansen Van Rensburg et al., 2017). We recommend that the OECD guidelines keep the temperature guideline at 32° ± 1 °C for skin and diffusion chamber, and that researchers make sure this temperature is maintained throughout the experiment.

15. Recovery procedures

Procedures for removing the test chemical from the skin are not provided in any of the OECD guidelines. We recommend that the revised guidance discuss the mass balance and the elements of an acceptable recovery procedure, which by its design should be able to demonstrate that test substance recovery is reliable and complete. The skin should be cleaned, and the donor and receptor chambers rinsed two or three times with solutions that have adequate solubility for the test substance (and metabolites if present). The collected samples should be analyzed separately. The amount of test substance/metabolites in the final rinse should be small indicating that the rinses have effectively recovered the material. Procedures for extracting or otherwise recovering the test substance from the skin, tape strips of the skin, and other materials used in the cleaning process (e.g., swabs) should be appropriate for the test substance, robust, complete, validated and discussed in the paper or report. Skin digestion or freeze-drying may be good choices for metals, but not for organic substances that might be destroyed in skin digestion or lost by evaporation in freeze-drying. Skin digestion with nitric acid will also dissolve metal particles, making separate identification of particulate and dissolved metal impossible. A recent study reported that freeze-drying the skin followed by shaking with a stainless steel bullet did not produce a powder from the skin (Midander et al., 2020). Agitation or sonication can increase extraction efficiencies. When available, radiolabeled molecules make the recovery procedure easier, although metabolites and parent compound cannot be distinguished unless another analytical method (e.g., HPLC) is also used.

Tape stripping for *in vitro* assays can be difficult to perform, as the skin is very fragile at the end of the experiment. The number of tape strips needed is described in GN156 as a “few” tape strips to remove the test substance retained in the top layers of the stratum corneum (par. 73), and two tape strips for plant protection products (par. 74). Extraction and analysis of non-radiolabeled chemicals from the tape strips can be challenging. The guidelines should recommend that the study report includes the number of tape strips used, the type of tape strips, the tape stripping procedure (e.g., pressure applied to the tape strip before removal), and the extraction method for non-radiolabeled chemicals (Dennerlein et al., 2017). The amount of stratum corneum collected on each tape strip is known to be variable and generally decreases with depth into the stratum corneum. Different types of tape remove different amounts of stratum corneum. Although time consuming, an analysis of the amount of stratum corneum collected on the tapes (e.g., by protein analysis or by spectroscopic or gravimetric determinations) provides useful information about the fraction of the stratum corneum that is removed. If the analytical method is sensitive enough to quantify the amount of test substance on individual tapes or small groups of tapes, then depth profiles of the test substance amount per area or concentration (i.e., test substance amount divided by the mass of stratum corneum) can be used to evaluate if most of the test substance in the stratum corneum is or is not collected. We support harmonizing the different OECD guidelines concerning tape stripping and adding practical and technical information on using the tape stripping method.

16. Chemical analysis

Analytical methods have advanced since the OECD guidelines were written, allowing for lower detection limits for many chemicals. However, there may still be difficulties in achieving sufficiently low detection limits for some test substances. Methods for lowering the detection limit

include reduction of the volume of the receptor fluid and avoidance of additives that might interfere with the analysis. The latter approach might be especially challenging when working with viable skin, which needs sugar to ensure metabolism and antibacterial substances to prevent the proliferation of bacteria that could degrade skin samples. We recommend that the limit of detection in the receptor fluid be tested and that sufficient test substance be applied to obtain a skin permeation curve even though this might deviate from the exposure scenario approach. This will avoid a false conclusion that the test substance does not cross the skin barrier within 24 h. We recommend using radiolabeled test substance whenever possible.

One example from metal analysis using ICP-MS is to use matrix matched standard curves and internal standard addition in all samples to keep track of recovery and allow for correction due to matrix effects. Standard solutions are usually prepared in diluted nitric acid for calibration, while samples can be diluted in phosphate buffer solution (PBS), artificial sweat, metal working fluid, and particle suspensions. In the experiments with continuous measurement (Midander et al., 2020), a matrix matched calibration curve is required since PBS from the receptor is continuously circulated through the ICP “as is” (pH 7.4). GD28 does not cover the chemical analytical aspects of the skin permeation experiments. It indicates that unlabeled test compounds should only be used in studies with lipophilic compounds if analytical sensitivity permits skin levels of the test compound to be measured at the end of the study. We recommend to use matrix matched standard curves and internal standard addition in all samples to keep track of recovery and allow for correction due to matrix effects.

17. Replicates

Recommendations on the number of replicates and also the number of different donors should consider the purpose of the measurements; e.g., more donors and replication might be required for bioequivalence assessment of topical dermatological products than for risk assessment. Furthermore, the number of blank cells should be specified to confirm the absence of contamination.

The guidelines should give instructions on how to calculate the permeation parameters for replicated measurements: how to calculate a mean and confidence interval over all skin samples from one or more donors (as opposed to averaging all the time-points and calculating a permeation rate from those averages). Provision of a spreadsheet performing calculations of example test substance data would promote harmonization among *in vitro* skin absorption experiments.

18. Test report

TGD428 (par. 22) outlines the contents of the test report, encompassing several of the issues discussed herein. Thus, the test report should include, for example, descriptions of the test substance, test preparation, test conditions and recoveries, and justification of choices made. In addition to these requirements, we recommend a prescription that all ingredients in the test preparation must be listed, including those classified as “inert”, in order to understand what might affect the permeation (Qiu et al., 1997; Ross and Shah, 2000). As described above, dermal absorption of the same test substance from different vehicles is different.

19. Conclusion

We, the authors, discussed how to harmonize future skin absorption experiments. Our discussions pertained to all three guidance documents and one overarching recommendation is that the revision of GN156 should be followed by revisions of the GD28 and TGD428; as all three documents need to be updated and harmonized. We have reviewed the OECD Guidelines TGD428, GD28, and GN156. Here, we have summarized some discrepancies and lack of detailed guidance on important

topics. We recommend that the OECD guidelines use the same terminology. GN156 should be revised to clearly address all types of chemicals tested for skin permeation. The guidelines should include a section on theoretical basis of the skin permeation parameters and possible limitations in using these in risk assessment. Details are needed in several sections such as examples of appropriate vehicles and receptor fluid for poorly soluble analytes. Reliable methods for assessing viability and integrity, including criteria for acceptable integrity, should be described. The main criterion for the volume of vehicle applied to the skin should be a uniform and homogeneous deposit of the vehicle over the entire surface of the skin covering the diffusion cell. Finally, the guidelines should include a protocol describing how to conduct mass-balance, tape strip methods, and skin extractions. These changes would contribute in harmonizing future *in vitro* skin permeation experiments.

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