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In vitro Dermal Absorption: Sample Application and Seal Quality in a Franz Diffusion Cell System

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Dermal absorption, in vitro · Cosmetics · Percutaneous absorption

Abstract
One of the known drawbacks of in vitro dermal absorption methods is their high interlaboratory variation. Although often attributed to biological skin differences, it has been shown that validation of other parameters such as temperature and stirring speed can reduce the high variability observed. The Organisation for Economic Co-operation and Development (OECD) and, at the EU level, the Scientific Committee on Consumer Safety (SCCS) have published guidance documents of how to perform these in vitro tests. For the parameter 'sample application' and 'adequate seal', it is indicated to apply the sample homogeneously and provide an adequate seal between the donor chamber and the membrane on which the sample is applied. Here, a simple and visual densitometer-based method is provided, which makes evaluation possible of any application protocol used.

Eric Deconinck and Kristien De Paepe are equally contributing project leaders.

Introduction

Measuring dermal absorption is important to evaluate both the safety and efficacy of a number of consumer products including dermally applied pharmaceutical compounds, pesticides and cosmetics [1–4]. In line with the 'three Rs' principle of Russell and Burch [5], the in vitro measurement of the percutaneous absorption replaces the use of animals in scientific research [6]. For cosmetic products on the EU market, this replacement is mandatory because Regulation 1223/2009 [7] prohibits the sale of cosmetic products that have been tested on animals or contain ingredients for which animal data has been generated, both after certain well-defined dates. Another motivation for the application of in vitro methodology is the ethical aspect and the fact that in vitro methods can be time- and money-saving in comparison with the corresponding in vivo experiments [6].

To limit variations in in vitro methodology, several regulatory organizations such as the Organisation for Economic Co-operation and Development (OECD) and the Scientific Committee on Consumer Safety (SCCS) have provided guidance documents to perform these tests [6, 8–11]. These have improved standardization, but still allow some flexibility which has pro and con arguments [12].

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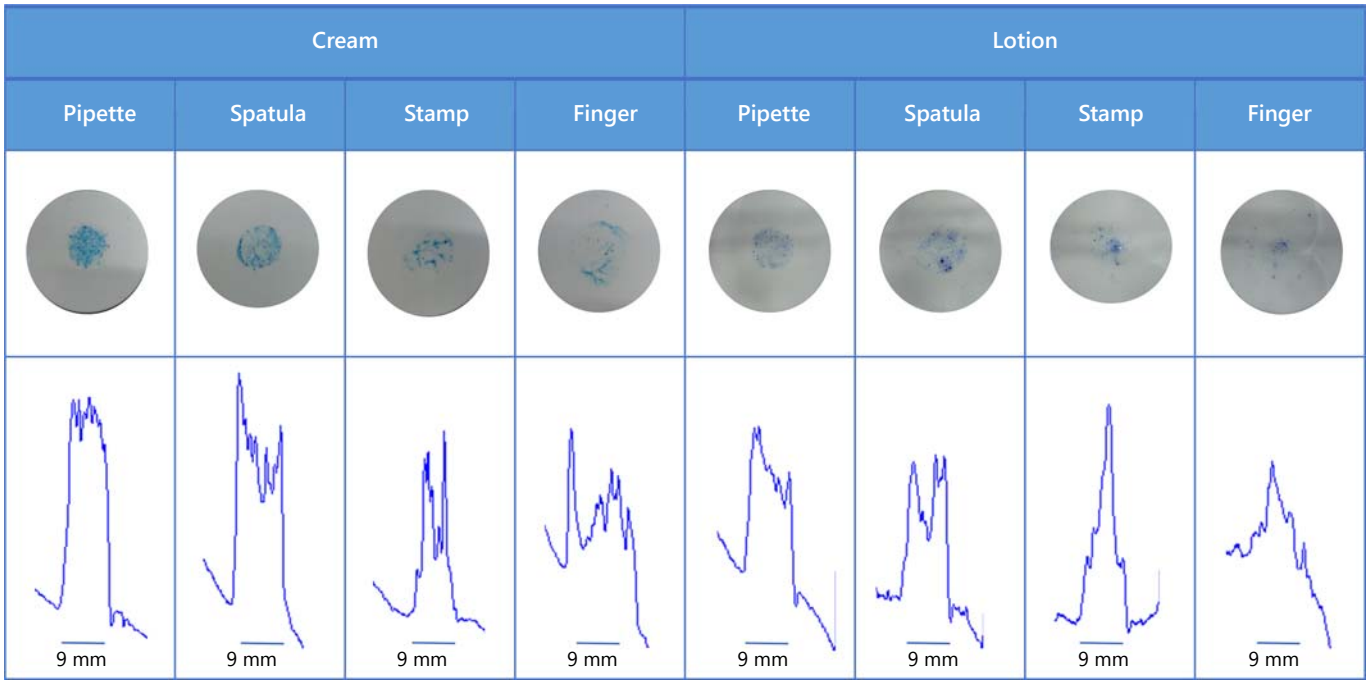

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Fig. 1. Visual result and VIS profile of four different modes of application to assess the uniformity of cream and lotion application in an in vitro dermal absorption experiment using an FDC system.

An important drawback associated with in vitro dermal absorption methods is the high variability of the results, especially when comparing the same methodology between different laboratories, which is often attributed to biological variations of the skin samples used [1]. For this reason, these in vitro methods are often validated using a synthetic membrane avoiding as such naturally occurring skin variation and focusing on variables of the apparatus and human handling practices [1].

In an international multicentre study, participating laboratories determined the permeability coefficient of methylparaben using an artificial membrane. The results were reported with a 35 and 10% inter- and intralaboratory variation, respectively, leading to the conclusion that aside from skin variation other important variables are present [1]. According to Ng et al. [13], control of even small parameters such as stirring speed and correct temperature measurement are important to reduce these variations. In a more recent multicentre comparison study, the variations among different laboratories using human and animal skin were assessed using caffeine, testosterone and benzoic acid as reference compounds. As expected, the intralaboratory variation was higher due to the use of a biological membrane. The authors suggested that besides the biologically introduced variation, the higher variation could also be attributed to the fact that in their study the laboratories worked with finite dose as opposed to infinite dose in the methylparaben study. This means that it is crucial to ensure that the entire skin surface is homogeneously exposed to the test substance, a prerequisite that is mentioned in the SCCS guidance document [8, 12]. When working with infinite doses, this is not a practical problem, but when working with finite doses, with a maximum dose of 10 μl/cm^2 to be applied for liquids, this becomes a technical challenge. To ensure that the applied formulation containing the test substance remains on that part of the skin that is in contact with the receptor fluid, the OECD guideline points out that 'The cell should provide a good seal around the skin' [11]. The latter is often achieved with a seal made of polytetrafluoroethylene or another inert material. Until today no specifications are described on how to verify whether the seal is sufficiently adequate and whether the test substance formulation is homogeneously spread on the skin surface. Therefore in this article, it was investigated how to evaluate both requirements made by the SCCS and the OECD.

Table 2. Overview of the different types of seals and their dimensions

No.	Type	Description [14]	Outer diameter, mm	Inner diameter, mm	Thickness, mm
1	sheet gasket	mechanical seal that fills the space between two	23.0	9.0	0.20
2	sheet gasket	surfaces to prevent leakage	23.0	9.0	0.95
3	gasket	similar to a sheet gasket but with a larger thickness	23.0	9.0	2.00
4	gasket		23.0	9.0	2.50
5	gasket		23.0	9.0	3.00
6	gasket		14.0	9.0	1.35
7	O-ring	mechanical seal that has a torus shape	13.0	9.0	1.90

Table 1. Chemical composition of the formulations used

Ingredients	Cream, w/w %	Lotion, w/w %
Water	78.40	93.40
Cetostearyl alcohol	15.00	0.00
Glycerol	5.00	5.00
Sodium lauryl sulfate	1.50	1.50
Methyl *p*-hydroxybenzoate	0.08	0.08
Propyl *p*-hydroxybenzoate	0.02	0.02

Materials and Methods

The jacketed receptor chamber of the Franz diffusion cell (FDC), purchased from PermeGear Inc. (Hellertown, Pa., USA), has an orifice diameter of 9.34 mm making the application zone 0.69 cm^2 and a volume of 3 ml. In total 9 of these FDCs were placed on a V-series stirrer (PermeGear Inc.). Water was circulated using a separate thermostatically controlled water bath ensuring a membrane surface temperature of 32 ± 1 °C. A synthetic membrane Strat-M™ (Merck Millipore, Darmstadt, Germany) was placed between the donor and the receptor chamber. In compliance with the OECD guideline either 5 mg/cm^2 of a semi-solid cream or 10 µl/cm^2 of a liquid lotion was applied on the donor chamber side [11]. The composition of both formulations is shown in table 1. The receptor chamber mimics the blood compartment and was filled with phosphate-buffered saline solution.

Four different modes of application were assessed. The first procedure uses the pipette tip to disperse the formulation over the application zone. The other methods made either use of a spatula, a round stamp with a diameter of 9 mm, or an index finger covered with a finger cot. For all the applications 4.5 µl of cream or 6.9 µl of lotion was spotted on the centre of the membrane with a positive displacement pipette of 25 µl (Microman®, Gilson, Middleton, Wis., USA). The cream volume complies with the OECD guideline, as 4.5 µl of cream corresponded with 5 mg/cm^2. The quality of product application was visually inspected and the membranes were scanned after 1 h at 664 nm using a thin-layer chromatography densitometer (TLC scanner III; Camag, Muttenz, Switzerland) equipped with a computerized image analyser (Win-Cats® software; Camag) using a slit width and height of 4 × 0.2 mm. To ensure high light absorption and to facilitate visual inspection, the formulations were dyed in a 99/1 (w/w) ratio using a 2% (m/v) methylene blue solution (Fagron, Waregem, Belgium).

The effectiveness of the seal, between the donor chamber and the membrane, was assessed after the dispersion of 6.9 µl of lotion, by using a pipette tip. Seal and donor chamber were placed on top of a membrane, which was in direct contact with the receptor chamber, and the complete set-up was clamped to ensure a tight

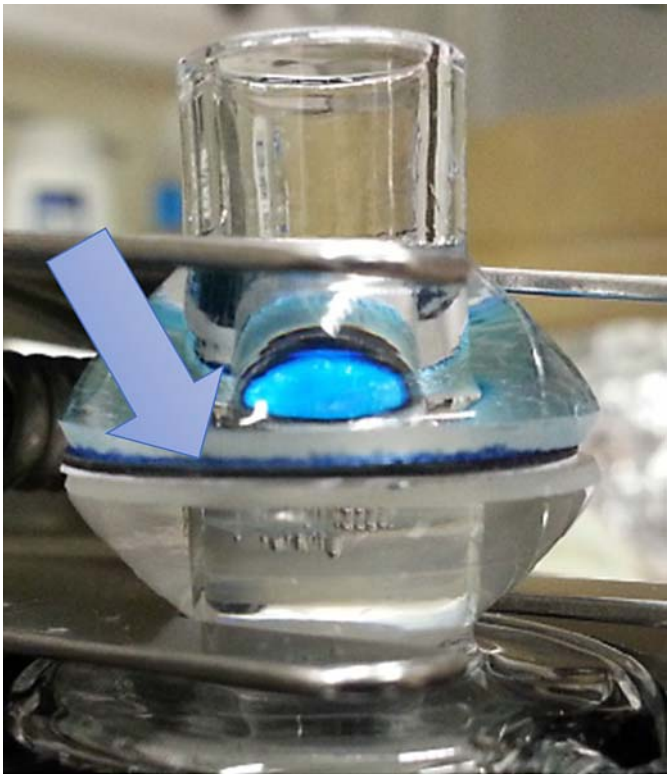

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Fig. 2. Using seal No. 2 (sheet gasket), the lotion formulation dispersed widely outside the application zone (arrow).

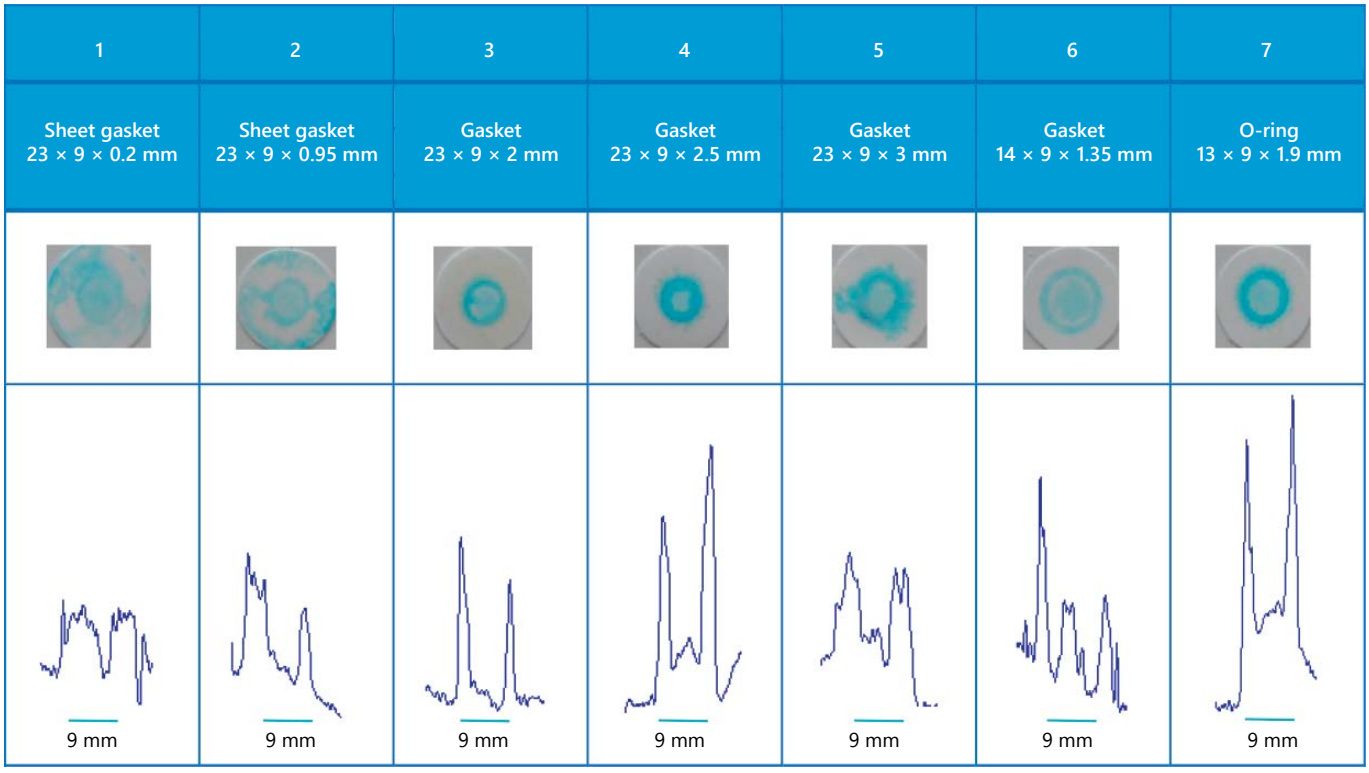

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Fig. 3. Visual result and VIS profile of seven different seals used in an FDC system for measuring the in vitro dermal absorption.

fit. In total 7 different seals (table 2) were tested and the inner and outer diameters were measured with vernier callipers (Mitutoyo, Kruibeke, Belgium) and an Absolute digimatic micrometer (Mitutoyo, Kruibeke, Belgium) was used to measure the seal thickness. After 1 h the membranes were visually evaluated and scanned as described above.

Results and Discussion

Uniformity of Sample Application

Applying the formulation homogeneously on the skin or artificial membrane used in an FDC is an important parameter for in vitro dermal absorption methodology [6, 12, 15]. In practice this is often done with a spatula or manually with a finger cot. Especially the latter is difficult when the FDC system has a small orifice diameter. For liquid formulations, the sample is mostly applied with a micropipette and then spread evenly using a pipette tip. For 'even' or 'uniform' spreading, no practically oriented literature is available. Therefore in this article an easy-to-apply technique is proposed, that allows a qualitative analysis of the dispersion mode. After sample application, the membrane is scanned with the TLC scanner. The visible (VIS) absorption of the membrane is considered as background signal and only when the scanner crosses the application site the VIS intensity increases. In a perfect situation this would lead to a block profile of which the base equals the background signal of the membrane and the top corresponds to the absorption of the applied sample. The length of this block signal can be measured and should ideally not deviate from the orifice diameter of the experimental set-up, being here 9 mm. When the sample is not homogeneously applied, the VIS profile will differ from this block profile. This was tested for the four modes of application, and repeated 3 times for each formulation. A representative result for each application mode is shown in figure 1.

Applying cream or lotion with a pipette tip results in the overall profile that best approaches the ideal block profile. The application with a spatula shows the tendency for higher formulation concentrations at the outside of the application zone and less at the inside. For the stamp and the finger cot, the opposite observation was made.

This does not mean that the other application modes are insufficient. It only shows that in our current conditions of the FDC system and the type of formulations used, the micropipette provides the most homogeneous dispersion.

Providing an Adequate Seal

For liquid formulations an adequate seal between the donor chamber and the membrane is required [11]. Already during the first tests, it was observed that a standard sheet gasket (No. 2) provided for this apparatus was not sufficient. The liquid formulation did not remain on the membrane that was in contact with the receptor fluid but spread out over the entire surface of the membrane. Due to capillary forces the lotion was also detected on the upper part of the seal and the donor chamber. This was easily visualized by adding methylene blue to the lotion as shown in figure 2. To solve this problem several types and dimensions of seals were tested. The results are shown in figure 3.

The visual images and VIS profiles show that seals 1, 2 and 5 were insufficient and did not keep the formulation within the application zone. Seals 3, 4, 6 and 7 resulted in a blue spot that was paler in the centre and more concentrated to the outside; this means that the type of seal has an effect on the dispersion of the applied sample. The best result was seen with the O-ring (seal 7). Although the latter seal concentrated the lotion towards the outside of the application zone, the overall absorption profile was superior compared to the other seals tested.

Conclusion

Even when fully complying with the OECD and SCCS guidance documents for in vitro dermal absorption, the study results presented here clearly show that applying the sample in a homogeneous way and providing an adequate seal are not as straightforward as expected. Both parameters might be important variation factors in this in vitro technique. Using an easy-to-handle densitometer, sample application can be visualized for almost every formulation without altering its composition; it also allows laboratories to check and validate their application protocols for various formulations. No overall guidance exists for this type of variables. Depending on the FDC dimensions, the results might be different, but by using this easily applicable technique, both parameters can be qualitatively assessed.

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