

THE SCIENTIFIC COMMITTEE ON COSMETIC PRODUCTS AND NON-FOOD PRODUCTS
INTENDED FOR CONSUMERS

OPINION

CONCERNING

BASIC CRITERIA FOR THE *IN VITRO* ASSESSMENT OF DERMAL
ABSORPTION OF COSMETIC INGREDIENTS

updated October 2003

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Of 20 October 2003

1. BACKGROUND

During the plenary meeting of the SCCNFP of June 23, 1999, the document "Basic criteria for the *in vitro* assessment of percutaneous absorption of cosmetic ingredients" (doc. n° SCCNFP/0167/99) was accepted. It not only provided the basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients, but also discussed some general principles and practical points of the methodology.

At that time, there was no OECD guideline on percutaneous absorption; the SCCNFP document mentioned above should therefore be seen as a pro-active action of the SCCNFP in order to support and speed up the introduction of *in vitro* dermal absorption studies of cosmetic ingredients in the evaluation of dossiers of cosmetic ingredients belonging to Annexes II, III, IV, VI or VII to Directive 76/768/EEC.

At present, two OECD Guidelines on dermal absorption are available, namely (i) OECD Draft Guideline 427 on skin absorption : *in vivo* method [OECD 427], and (ii) OECD Draft Guideline 428 on skin absorption : *in vitro* method [OECD 428].

Over the years, practical experience has been gained in using the *in vitro* methodology : *in vitro* dermal absorption studies are now currently used and results are incorporated in toxicological dossiers of cosmetic ingredients, plant protection products, biocides, etc [98/8/EC, 2000/6/EC, Sanco/222/2000].

In order to provide up to date guidance for the application of this *in vitro* methodology for cosmetic ingredients, document SCCNFP/0167/99 has been revised and updated. Existing documents such as the "Proposed rule for *in vitro* dermal absorption rate testing ..." [US EPA 1999], the "Guidance document on dermal absorption" [Sanco/222/2000], the "Draft Guidance Document for the conduct of skin absorption studies" [OECD 2000], and the "Technical Guidance Document on Risk Assessment part 1" [ECB 2003] have been taken into consideration when updating the opinion SCCNFP/0167/99.

2. GENERAL PRINCIPLES

The purpose of dermal absorption studies of cosmetic ingredients is to obtain quantitative information on the amounts that can enter, under in-use conditions, into the systemic compartment. These quantities can then be taken into consideration to calculate a safety factor using the NOAEL of the respective substance.

Justification for the use of *in vitro* dermal absorption studies on isolated skin is based on the fact that the epidermis, in particular the stratum corneum, forms the principal *in vivo* barrier of the skin against the penetration and uptake of xenobiotics in the body.

Under *in vivo* conditions, the microcirculatory system (blood and lymph vessels) carries compounds from the dermis into the central compartment. *In vitro*, the microcirculation is obliterated. Consequently, under *in vitro* conditions, dermal tissue may retain penetrating compounds that, *in vivo*, would have been removed into the systemic compartment. Thus, either the dermis must be removed prior to *in vitro* investigations (epidermal membrane) or such possible *in vitro* retention in the dermis must be taken into account when interpreting the *in vitro* results.

The epidermis renews itself by continuous outward proliferation, differentiation and desquamation. About one layer of corneocytes is shedded off per day. After topical application, xenobiotics detected *in vitro* in the skin, particularly in the stratum corneum and the pilosebaceous units, might *in vivo* have been lost from the skin via desquamation or sebum secretion, respectively. Because these processes are not present *in vitro*, the final epidermal (stratum corneum) levels *in vitro* could be elevated compared with the corresponding *in vivo* levels.

According to these principles, the following should be included in the protocol for *in vitro* dermal absorption studies:

- i. Studies should be performed on appropriate standardised skin preparations. The respective choice should be justified in the protocol.
- ii. At the end of the experiment, a full mass balance should be performed.
- iii. When considerable cutaneous metabolism of the ingredients to be tested occurs *in vivo*, further studies are necessary. It should be noticed that frozen skin preparations lack the enzyme systems to biotransform the test compound and do not provide an appropriate picture of the potential metabolites and their dermal absorption. Therefore *in vitro* studies using frozen skin, do not provide information on the dermal absorption of a substance, neither do they give information on the metabolite(s) of the test material that undergoes extensive biotransformation.
- iv. Whenever irreversible binding of an ingredient to the epidermis, followed by elimination through *in vivo* desquamation of the skin surface, is assumed, this must be documented by separate experiments.

3. PRINCIPLE OF THE TEST

At present, it is only possible to use skin preparations of natural origin. The quality of cultured or reconstituted skin has improved importantly during the last years [Coquette et al. 2000], but does not yet represent the full barrier function as known *in vivo*.

OECD Guideline 428 should be followed as close as possible, taking into account the guidance given here. Any deviation from these should be accompanied by a specific justification of the particular changes made and appropriate scientific references should be mentioned.

The test substance is applied in an appropriate formulation on the skin sample which usually is placed in a diffusion cell (cf. 4.1). The skin is positioned between the upper and lower chambers of the cell. The latter may be either of static or flow-through design. The integrity of the barrier should be checked by an appropriate method. The test sample remains in contact with the skin on the donor side for a defined period of time (leave-on or rinse-off respectively, depending on the intended use conditions). The receptor fluid may be sampled once at the end of the experiment or preferably at various time points before the end so that an absorption profile may be constructed. A justification of the procedure used (static or flow-through conditions) should be provided. The skin and/or fluid samples are analysed by appropriate and validated analytical methods (scintillation counting, HPLC, GC, etc.) of which the sensitivity for the particular ingredient under investigation, should be mentioned.

4. FACTORS AFFECTING DERMAL ABSORPTION AND METHODOLOGY

Dermal absorption is affected by several factors : e.g. physical and chemical properties of the substance, type and composition of the formulation, occlusion, concentration within the formulation, exposure pattern, skin site of the body [Howes et al. 1996, Schaefer and Redelmeier 1996, ECETOC 1993].

In the next section, a brief overview is given of factors that may affect dermal absorption using common *in vitro* methodology.

4.1. Diffusion cell design

The diffusion cell consists of an upper donor and a lower receptor chamber, separated by a skin preparation. The stratum corneum faces the donor chamber. The cells are made preferably from an inert non-adsorbing material. Temperature control of the receptor fluid is crucial throughout the experiment. The skin surface temperature in the diffusion cell should be kept at the *in vivo* skin temperature of 32°C. Additional dermal absorption studies may be required in some specific cases (e.g. exposure to a higher skin temperature). The receptor fluid is well-mixed throughout the experiment. The cell design (flow through) allows multiple sampling without interrupting the experiment.

4.2. Receptor fluid

The composition of the receptor fluid is chosen so that it does not limit the extent of diffusion of the test substance, i.e. the solubility and the stability in the receptor fluid of the chemical under investigation have to be guaranteed. Saline or buffered saline solutions are commonly used for hydrophilic compounds. For lipophilic molecules, serum albumin or appropriate solubilisers/emulsifiers are added in amounts which do not interfere with membrane integrity. The properties of the receptor fluid should be such that there is no interference with the analytical procedure. Ethanol/distilled water (1/1) as proposed in the OECD Guideline for lipophilic compounds is considered to be non-appropriate.

The receptor fluid, preferably degassed, is thoroughly stirred or continuously replaced in flow-through cells during the whole experiment. The choice of static or flow-through conditions in the receptor cell is made on a compound-by-compound basis, depending on its absorption properties and on the goal of the study. It must be specified in the test report. It has to be ensured that the amount of penetrant in the receptor fluid is less than 10% of its saturation level at any time. This minimises any interference of the free diffusion process that could lead to underestimation of dermal absorption. The substance must remain stable in the receptor fluid for the duration of the *in vitro* test and the subsequent analysis.

4.3. Skin preparations

Human skin would be the obvious choice but is not always readily available. Pig skin is used because it shares essential permeation characteristics with human skin. Rat skin is grossly 2 to 10 times more permeable than human skin [Ross et al. 2000] leading to overestimation of human dermal absorption, whilst mice, guinea pigs and rabbits have no comparable barrier function. The use of artificial skin is still under development and not yet advised for *in vitro* testing because of differences in barrier function [Coquette et al. 2000].

The origin of skin samples must be specified in the respective report in terms of :

- species : only human or pig skin should be used, skin of rodents is not representative for human skin [ECETOC 1993];
- location on the body : for human skin : abdomen or breast; for pig skin : abdomen, breast, back, flanks and ears;
- gender and age : these factors are not considered to be important variables but should be stated;
- fresh/frozen skin : when a significant biotransformation of the test compound in the skin is expected, freshly excised skin must be used under viable conditions [Diembeck et al. 1999];
- details on preservation and storage of skin : skin can be stored in aluminium foil at 20 °C or lower (conditions should be specified) [Howes et al. 1996, Bronaugh et al. 1986]. During transport skin samples should be kept at or below 4°C.

Skin samples that may be used are split-thickness (200-500 µm) or full-thickness (500-1000 µm) skin preparations [Sanco/222/2000]. Dermatomed skin is often used. Skin thickness should be measured by an appropriate method and the latter should be stated. The skin samples should be prepared to fit the experimental cell.

- For human skin : split-thickness skin should be the general rule. If for a specific reason, full-thickness is required, this should be justified.
- For pig skin : since it is more difficult to obtain intact split-thickness skin, this could justify the use of full-thickness skin.

When epidermal membrane is used for the *in vitro* dermal absorption study, the reason for this should be justified. Epidermal membranes are quite fragile and mass balance techniques often cannot be applied in this case. Moreover, epidermal membranes sometimes overestimate human *in vivo* skin absorption [Van de Sandt et al. 2000].

4.4. Skin integrity

Barrier integrity is crucial for the experiment, and must therefore be checked. This is achieved by either measuring the penetration of a marker molecule, e.g. tritiated water, caffeine or sucrose, or by physical methods like TEWL (Transepidermal Water Loss) or TER (Transcutaneous Electrical Resistance) measurements. Data obtained should be reported.

4.5. Skin temperature

Because the rate and extent of skin absorption is temperature dependent, the skin disc temperature is maintained constant ($32 \pm 1^\circ\text{C}$ = skin surface temperature *in vivo*), using an appropriate device, which should be described in the report.

4.6. Test substance

The relevant physical and chemical data (e.g. MW, log P_{ow}, solubility, stability, and pK_a of the test substance) must be given.

Also the purity of the test substance has to be given and it should be similar as that of the marketed compound (see 4.11).

As already mentioned under **4.2**, the solubility and stability of the test substance in the receptor fluid for the whole test duration should be documented.

4.7. Preparation of the dose and vehicle / formulation

The dose and vehicle/formulation must be representative for the in use condition(s) of the finished cosmetic product.

Normally, more than one concentration of the test substance, including the highest requested one, is used in typical formulations spanning the range of human exposure. The quantitative composition of the vehicle/formulation should be specified. The stability of the test substance under the foreseeable conditions of application and usage must be ascertained.

4.8. Dose and volume of test substance

The dose as well as the contact time (exposure) with the skin are chosen to mimic intended use conditions. The amount of the formulation to be applied is adapted to the consumer use values described in the Notes of Guidance [SCCNFP/0690/03], and usually lies between 2-5 mg/cm² for solids and semi-solid preparations, and up to 10 µl/cm² for liquids. Deviations should be explained. The volume of formulation used should be appropriate to spread the sample homogeneously on the skin surface. This strongly depends on the viscosity of the formulation.

4.9. Study period

The exposure time and sampling period(s) should be defined in the protocol. The normal exposure time is 24 hours. Longer duration may result in membrane deterioration and requires membrane integrity to be carefully checked. Depending on the intended use, a shorter exposure time for certain preparations may be considered, e.g. oxidative hair dyes usually have a contact time of 30 minutes to mimic in-use conditions.

4.10. Sampling

The frequency of sampling depends on the rate/extent of dermal absorption. It should be chosen adequately to allow the determination of the extent/rate of absorption and the absorption profile. It must be described in the report. The absorption profile is determined up to 24 hours.

4.11. Analytical methods

Appropriate analytical techniques, e.g. scintillation counting, HPLC or GC, should be used. Their validity, sensitivity and detection limits should be documented in the report. When an increase of sensitivity is needed, the test substance should, whenever possible, be radio-labelled.

Qualitative or semi-quantitative methods such as microautoradiography can be useful tools for skin distribution assessments.

4.12. Data collection

Amounts of the test compound must be determined in :

- the surplus on the skin

- the stratum corneum (e.g. adhesive tape strips)
- the epidermis without stratum corneum
- the dermis
- the receptor fluid.

It is necessary to check for substance adsorbed in the equipment.

4.13. Mass balance analysis / recovery

The mass balance of the applied dose must be determined with the data mentioned above. The overall recovery of test substance (including its metabolites) should be within the range of 85-115%. If lower recoveries of the test substance are obtained, the reasons need to be investigated and explained.

4.14. Variability / validity / reproducibility

- The variability of dermal absorption studies depends on the penetration rate of a particular ingredient; the lower the penetration rate, the higher the variability. This high variability is due to known intra-individual and inter-individual characteristics of the stratum corneum barrier. The relative variability of the method should be documented.
- The validity of the method used should be assessed at regular intervals by including penetrating compounds like caffeine or benzoic acid as reference substances. These data should be included in the study report.
- The reproducibility of the method should be shown by the use of minimum six evaluable samples (human or pig skin), from each of at least 3 donors per dose. The coefficient of variation should be less than 30 %. If statistical evaluation is not possible, the highest observed penetration value will be used in the systemic exposure dosage (SED) calculation.

5. RESULTS

Dermal absorption should be expressed as an absolute amount [$\mu\text{g}/\text{cm}^2$ of skin surface] and as a percentage of the amount of test substance contained in the intended dose applied per square centimetre of skin surface.

The amounts of penetrated substance(s) found in the receptor fluid are considered to be systemically available. The epidermis (except for the stratum corneum) and dermis are considered as a sink, therefore the amounts found in these tissues are equally considered as absorbed and are added to those found in the receptor fluid. The amounts that are retained by the stratum corneum at the time of sampling are not considered to be dermally absorbed, and thus they do not contribute to the systemic dose.

The absorption rate and mass balance should be calculated separately for each diffusion cell. Only then, the mean \pm S.D. and median with 10% and 90% percentiles can be calculated.

6. REFERENCES

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