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(SCCP)

Opinion on

BASIC CRITERIA FOR THE *IN VITRO* ASSESSMENT OF DERMAL ABSORPTION OF COSMETIC INGREDIENTS - updated March 2006

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1. BACKGROUND

As early as June 1999, before any OECD guideline on percutaneous absorption was available, important scientific articles on the methodology were published [Beck et al. 1995, Diembeck et al. 1999]. The SCCNFP discussed the scientific progress with external experts in the field and subsequently adopted its first "Basic criteria for the *in vitro* assessment of percutaneous absorption of cosmetic ingredients" [SCCNFP/0167/99]. The publication of such a set of criteria was 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 III, IV, VI or VII to Dir. 76/768/EEC. Moreover, the opinion did not only provide 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.

In December 2000, two draft OECD Guidelines on dermal absorption became available, namely 1) OECD Draft Guideline 427 on skin absorption: *in vivo* method [OECD 427], and 2) OECD Draft Guideline 428 on skin absorption: *in vitro* method [OECD 428]. The combination of the OECD-publication with gained practical experience, has led to the incorporation of the *in vitro* dermal absorption study in toxicological dossiers of cosmetic ingredients, plant protection products, biocides, etc [98/8/EC, 2000/6/EC, Sanco/222/2000].

Subsequently, in order to provide an up to date guidance for the application of the *in vitro* methodology for cosmetic ingredients, document SCCNFP/0167/99 was revised and updated in 2003 [SCCNFP/0750/03]. 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dermal absorption rate testing …" [US EPA 1999], the "Guidance document on dermal absorption" [Sanco/222/2000], the "Draft Guidance Document for the conduct of skin absorption studies" [OECD 2000], and the "Technical Guidance Document on Risk Assessment part 1" [ECB 2003] were taken into consideration.

The SCCP has noticed that several dossiers have failed to fulfil the requirements as described in document SCCNFP/0750/03. In particular, a recent revision of all hair dye dossiers revealed a number of problems [Pauwels and Rogiers 2004].

The following actions were undertaken:

- The SCCP established a working group, consisting of SCCP members and external experts in the field of dermal absorption. The present opinion results from the work of the expert group, whose task was to reach a consensus concerning a revision of the "SCCP basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients".

- In order to make the SCCP criteria more widely known, it is intended to expand the chapter on dermal absorption in the SCCP Notes of Guidance, 6th revision.

2. GENERAL PRINCIPLES

Definitions

The definitions with respect to dermal absorption slightly diverge in different official documents. Hereunder, the definitions as proposed by the WHO [WHO 2005] are given here:

The **percutaneous/dermal absorption** process is a global term which describes the passage of compounds across the skin. This process can be divided into three steps:

- **penetration**, which is the entry of a substance into a particular layer or structure such as the entrance of a compound into the stratum corneum;

- **permeation**, which is the penetration through one layer into another, which is both functionally and structurally different from the first layer;

- **resorption** which is the uptake of a substance into the vascular system (lymph and/or blood vessel), which acts as the central compartment.

The purpose of dermal absorption studies of cosmetic ingredients is to obtain qualitative and/or quantitative information on the amounts that may enter, under in-use conditions, into the systemic compartment of the human body. These quantities can then be taken into consideration to calculate the margin of safety using the NOAEL of an appropriate repeated dose toxicity study with 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 penetration and uptake of xenobiotics in the body.

Under *in vivo* conditions, the microcirculatory system (blood and lymph vessels) may carry compounds from the dermis into the central compartment (resorption). *In vitro*, the microcirculation is compromised, thus the potential resorption of a compound cannot be adequately studied in such a setting.

The 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 (so-called split-thickness skin) or possible *in vitro* retention in the dermis and living epidermis (without stratum corneum) must be taken into account when interpreting the *in vitro* results.

The epidermis renews by continuous outward proliferation, differentiation and desquamation. About one layer of corneocytes is shed 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. The WHO recommends human skin as the gold standard.

ii. At the end of the experiment, a full mass balance should be performed.

iii. When considerable cutaneous metabolism of the test compound occurs *in vivo*, further studies may be necessary. It should be noticed that frozen skin preparations may lack the enzyme systems for biotransformation of the test compound and may not provide an accurate picture of the formation of metabolites and their dermal absorption.
Therefore *in vitro* studies using frozen skin may not provide complete information on the dermal absorption of compounds that undergo biotransformation in the skin nor on their potential metabolite(s) formed. The role of cutaneous biotransformation in the absorption process is still a matter of scientific debate.

iv. Sometimes an irreversible binding of an ingredient to the epidermis may occur, followed by elimination through *in vivo* desquamation of the skin surface. When this mechanism is assumed, it must be documented by separate experiments.

3. PRINCIPLE OF THE TEST

At present only skin preparations of natural origin may be used. Although the quality of cultured or reconstituted skin has improved importantly during the last years [Coquette et al. 2000], these cultures do not possess a complete barrier function comparable to that of living skin.

OECD Guideline 428 should be followed as close as possible, taking into account the guidance given here. Any deviation from the OECD or SCCP guidelines should be documented and justified by appropriate scientific argumentation.

The test substance should be applied in an appropriate formulation on the skin sample which is placed in a diffusion cell (cf. **4.1**). The skin is then positioned between the upper and lower chambers of the cell. Diffusion cells may be of static or flow-through design. The integrity of the barrier should be checked by an appropriate method. The test sample should remain in contact with the skin on the donor side for a defined period of time, corresponding to the typical use of the cosmetic end product, such as leave-on or rinse-off conditions. The receptor fluid should be sampled at an early time point (e.g. after 30 minutes), at the end of the experiment and at appropriate time points in between in order to obtain an absorption-time profile. A justification of the procedure used (static or flow-through conditions) should be provided. The skin and/or fluid samples should be analysed by appropriate and validated analytical methods, such as liquid scintillation counting, HPLC, GC or other suitable methods. Information on the sensitivity and repeatability / time-different intermediate precision of the analytical method(s) should be provided.

4. FACTORS AFFECTING DERMAL ABSORPTION AND METHODOLOGY

Dermal absorption can be affected by several factors : e.g. physical and chemical properties of the substance, type and composition of the formulation, occlusion, concentration of the substance in the formulation, exposure pattern, skin site of the body and technical aspects of the respective *in vitro* test [Howes et al. 1996, Schaefer and Redelmeier 1996, ECETOC 1993].

In the following section, an overview is given of factors that may affect dermal absorption in *in vitro* dermal absorption studies.

4.1. Diffusion cell design

The diffusion cell consists of an upper donor and a lower receptor chamber, separated by the skin preparation under investigation. The stratum corneum faces the donor chamber. Diffusion cells should consist of 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 ± 1°C. Additional dermal absorption studies may be required in some specific cases, e.g. when substance exposure at a higher skin temperature may be expected. The receptor fluid in static cells should be well-stirred throughout the study. The advantage of using a flow-through system is continuous sampling; the advantage of using a static system is the increase in sensitivity for test substances only poorly penetrating the skin.

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 must not interfere with membrane integrity. The fluid should not interfere with the analytical procedure.
As a general rule, the receptor fluid should have a physiological pH. Any deviation from this principle should be justified; e.g. in the case of 50/50 ethanol/water (as proposed in the OECD Guideline), evidence should be included in the dossier, showing that this does not significantly affect the integrity of the skin.

In order to avoid serial non-detects at early sample points, the receptor fluid volume should be kept to a minimum.

The receptor fluid, preferably degassed in order to avoid formation of air bubbles during the experiment, should be thoroughly stirred (static cells) or continuously replaced (flow-through cells) during the entire experiment. The choice of static or flow-through conditions in the receptor cell should be made on a compound-by-compound basis, depending on its theoretical absorption properties and the objective of the study. The choice of the test system should be justified in the study report. The amount of penetrated substance in the receptor fluid should not exceed 10% of its saturation level at any time, in order to minimise interference with the free diffusion process that could produce an underestimation of dermal absorption. The substance should be stable in the receptor fluid for the duration of the *in vitro* test and the subsequent analysis.

4.3. Skin preparations

Human skin is obviously the best choice but is not always readily available. Alternatively, pig skin may be used because it shares essential permeation characteristics with human skin. Rat skin is 2 to 10 times more permeable than human skin [Ross et al. 2000] which may result in an overestimation of dermal absorption. Mouse, guinea pig or rabbit skins have no adequate barrier function comparable to that of human skin. The use of cultured or reconstructed skin models is under development and those systems are not yet advised for *in vitro* testing on the basis of their insufficient barrier function [Coquette et al. 2000].

The origin of skin samples should be specified in the respective report. The following information is required:

- Species: by preference human or pig skin should be used. Although rodent skin is not representative for human skin [ECETOC 1993], the results of such studies will not be disregarded, since these models produce an overestimation of the dermal absorption (worst case).
- Skin location: abdomen, leg or breast (human skin); abdomen, breast, back, flanks or ears (pig skin).
- Gender and age: although these factors are not believed to be important variables, they should be stated.
- Fresh/frozen skin: when significant biotransformation of the test compound in the skin is expected, freshly excised, viable skin should be used [Diembeck et al. 1999].
- Details on preservation and storage conditions of the skin should be specified (e.g. skin can be stored in aluminium foil at -20°C or lower) [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, which should be described in the report. 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 technically more difficult to obtain intact split-thickness skin, this could justify the use of full-thickness skin.

When epidermal membranes are used for the *in vitro* dermal absorption study, the reason for this should be justified. Epidermal membranes are sometimes quite fragile and some mass balance techniques (e.g. tape stripping) cannot be applied to this model. It must also be mentioned that epidermal membranes may overestimate human *in vivo* skin absorption [Van de Sandt et al. 2000].

The minimum skin area to be covered is considered to be 0.64 cm^2.

4.4. Skin integrity

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

4.5. Skin temperature

Because the rate and extent of skin absorption is temperature-dependent, the skin disc temperature should be maintained constant (32 ± 1°C, corresponding to the normal human skin surface temperature). The method of temperature maintenance 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) should be given.

The purity of the test substance should be described and should be comparable to that of the substance in marketed products (see 4.10). It is recognised that radio-synthesis of [^{14}C]- or [^{3}H]-labelled substances may result in a somewhat different purity and/or impurity profile than that of substances produced by large-scale chemical production.

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

A separate set of criteria dealing with dermal absorption issues related to nanoparticles/nanotubes will, if necessary, become available as an annex to this Opinion.

4.7. Preparation of the dose and vehicle / formulation

The dose and vehicle / formulation should be representative for the in use condition(s) of the finished cosmetic product. The quantitative composition of every formulation used during the experiment, should be given.

More than one concentration of the test substance, including the highest requested one, is used in typical formulations spanning the range of human exposure. These concentrations should be selected in such a way that the range of the linear curve of concentration versus dermal absorption is demonstrated.

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 of the test formulation as well as its contact time (exposure) with the skin should resemble 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 is between 2-5 mg/cm^2 for solids and semi-solid preparations, and up to 10 µl/cm^2 for liquids. For oxidative hair dye formulations, 20 mg/cm^2 is applied. Deviations should be explained. The volume of formulation used should be appropriate to spread the sample homogeneously over the skin surface. This depends on the viscosity and lipophilicity of the formulation. Both mass and volume applied should be stated in the test report.

4.9. Study period and sampling

The exposure time and sampling period(s) should be defined in the protocol. The normal exposure time is 24 hours with regular sampling intervals. Longer duration of the study may result in membrane deterioration and requires careful control of membrane integrity. The exposure time should be consistent with the intended use of the cosmetic formulation. E.g. for oxidative hair dye formulations, the time of contact could vary from 15 to 45 minutes or even more according to the in-market use. The skin surface will also be rinsed using a procedure mimicking the consumer situation. Sampling of the receptor fluid is continued until e.g. 24 hours.

The frequency of sampling should be chosen adequately to allow the determination of the extent/rate of absorption and the absorption profile. Kinetic measurements have to be obtained for at least 6 post-application time points, including one early time point (30 minutes), in order to be able to estimate the absorption kinetics. For rinse-off products, measurements after rinsing have to be taken. The full sampling procedure must be described in the report.

4.10. 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 micro-autoradiography, may be useful tools for skin distribution assessments.

4.11. Data collection

The test compound must be determined in the following compartments:

- Product excess on the skin (dislodgeable dose)
- Stratum corneum (e.g. adhesive tape strips)
- Living epidermis (without stratum corneum)
- Dermis
- Receptor fluid

For the reason of an appropriate mass balance, it is necessary to check for substance adsorbed to the equipment (included in rinsing solutions and/or compartments).

4.12. Mass balance analysis / recovery

The mass balance of the applied dose must be determined.

The overall recovery of test substance (including metabolites) should be within the range of 85-115%. Lower or higher recovery rates should be investigated and/or explained.

4.13. 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.
  Experience has shown that the high variation in dermal absorption may partly be explained by differences in skin samples. Therefore skin samples should be taken from a suitable anatomical site and the study should use a sufficient number of samples and replicates.
- The technical ability of the performing laboratory and the validity of the method used should be assessed at regular intervals, at least twice per year, by using reference compounds like caffeine or benzoic acid. These data should be included in the study report [OECD 2004, Van de Sandt et al. 2004].
- The results of the dermal absorption studies should be reproducible. A minimum of **six** evaluable samples (human or pig skin), from each of at least **three** donors, should be used per dose tested. 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.
- For oxidative hair dyes, the relevant combination(s) of hair dye, coupler(s) and developer(s) should be tested.

5. RESULTS

Dermal absorption should be expressed as an absolute amount [µg/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 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 are not expected to contribute to the systemic dose.

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

All measurements, statistical processing and obtained kinetic curves have to be provided.

In case the results are derived from an inadequate *in vitro* study, the default value of 100% absorption is applied.

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Dr. S.C. Rastogi	Dr. I.R. White	