COLIPA GUIDELINES

Guidelines for Percutaneous Absorption/Penetration

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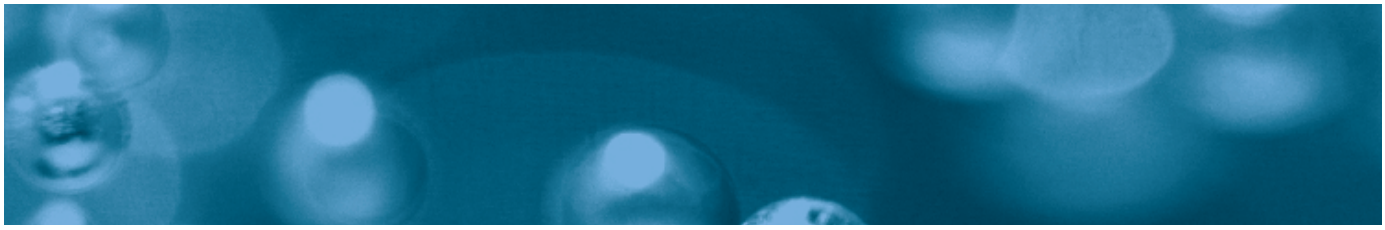

CONTENTS

1. BACKGROUND

The 6th Amendment to the Cosmetics Directive (93/35/EEC) includes a potential ban on the use of animals in the testing of cosmetic products and their ingredients, from January 1, 1998.

With regard to the assessment of cutaneous absorption/penetration properties of substances, in vitro methodologies are recommended for ethical reasons and feasibility. Excised and reasonably standardised skin is easily obtainable from various species whereas human skin from individuals is available only under favourable circumstances and in limited quantities from surgical or post-mortem sources.

The value of organotypic skin models for penetration studies has to be demonstrated by further research. The viability of the skin is not a pre-requisite for penetration testing, since the process depends on passive diffusion and not on active transport. Furthermore, biotransformation is of minor interest and importance in the majority of cases. In the exceptional case of relevance, biotransformation can be monitored.

2. INTRODUCTION

Percutaneous penetration is the passage of material from the stratum corneum surface of the skin to the systemic circulation. Percutaneous absorption is the passage of topically applied materials into the skin.

In vitro methods used now in many laboratories, utilise transport across full- or split-thickness animal or human skin to a receptor fluid reservoir. It is possible to estimate in vivo absorption by extrapolating from suitable in vitro data.

This guideline for the testing of percutaneous penetration in vitro describes a general procedure for measuring the penetration of test substances through excised mammalian skin. The barrier properties of skin are usually maintained after excision from the body and appropriate storage in a freezer for up to 3 months, since penetration is driven by passive diffusion and there is no evidence for active transport (ref. 5). Therefore, skin viability is not a prerequisite for these studies. The principal diffusion barrier has been identified as the stratum corneum, the integrity of which has to be controlled.

Details of the procedures are presented in the form of examples, in the appendix (section 9). The appendix contains examples of the following standard protocols, according to a ranking in terms of relevance to mimic human conditions (ref. 6):
- human skin
- pig skin
- rat skin

3. INITIAL CONSIDERATIONS

The in vitro methods described in these guidelines provide information in all cases where the stratum corneum is the rate limiting barrier to percutaneous absorption and are, therefore, applicable to those compounds which are sufficiently soluble in the intended receptor fluid. There is considerable experience to allow these in vitro methods to be used for the assessment of percutaneous absorption/penetration and for the prediction of the in vivo situation (ref. 1). The use of appropriately prepared skin membranes (full thickness, split-thickness and isolated epidermis) may allow the measurement of the amount of substance delivered to the systemic circulation and the amount delivered to the epidermis or dermis (ref. 6).

The advantages of the in vitro method are that it can be used with skin from human and other species, it does not need live animals and it is suitable for compounds extensively metabolised in the body. The limited supply of human skin means that the routine use of this material is unlikely and hence animal skin will normally be used. There are now many comparisons in the literature for predictions of in vivo absorption/penetration from in vitro data to be made with some confidence for the majority of compounds (ref. 1 to 4).

4. PRINCIPLE OF THE TEST

The test substance, either as such or dissolved in an appropriate solvent or galenic formulation, thereby yielding the test sample, is applied to the intact surface of the skin disk separating the upper and lower chambers of a diffusion cell, which can be run in static or flow-through mode.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 is sampled once at the end of the experiment or at various time points in between. The skin and/or fluid samples are analysed by an appropriate method (e.g. scintillation counting, HPLC, GC). The integrity of the barrier should be checked by an appropriate method (see paragraph 5.4).

5. DESCRIPTION OF THE METHOD

5.1 Penetration cell design

The penetration cell consists of the upper donor and the lower receptor chamber, separated by a skin membrane. The epidermis faces the donor chamber and the lower surface the receptor chamber. The cells must be made from an inert and non-absorbing material (e.g. glass or PTFE). Temperature control is crucial throughout the experiment and it should be maintained at in vivo skin conditions (see paragraph 6.3). The receptor fluid must be well-mixed throughout the experiment. Sampling should be feasible without interrupting the experiment, by appropriate cell design.

5.2 Receptor fluid

The composition of the receptor fluid should not limit the extent of penetration of the test substance, i.e. the theoretical total solubility should be guaranteed. The receptor fluid must not affect the integrity or alter the permeability properties of the skin. A saline or buffered saline solution is recommended for hydrophilic compounds. For lipophilic molecules, addition of serum albumin or other appropriate solutors, such as non-ionic surfactants, is recommended. The appropriate receptor fluid volume is determined by the solubility and analytical detectability of the test substance. It can be adapted by choosing a receptor chamber of adequate volume (static) or by varying the setting of the pump (flow-through).

5.3 Skin disks

The test should be carried out with an appropriate number (i.e. minimum six) of skin disks of similar integrity. Split-thickness skin (epidermis and upper dermis obtained by dermatomisation or epidermis only obtained by pre-treatment with heat, enzymes or chemicals) may be used. Full thickness skin may be used provided that the skin compartments are analysed in detail. Details on preparation are found in the standard protocols. In case of lipophilic compounds split-thickness skin (≤ 1mm thick layer) or epidermal preparations are to be preferred to limit the dermal retention in vitro. The skin should be clipped before preparing membranes. Skin thickness should be measured by an appropriate method.

5.4 Membrane integrity

Since barrier integrity is crucial for the experiment, it should be checked by measuring the skin penetration of a marker molecule, e.g. tritiated water, for which suitable historical control data are available. Alternatively, the integrity can also be tested with a non-radioactive probe, e.g. caffeine, or with physical methods like TEWL or TER (Transepidermal Water Loss or Transdermal Electrical Resistance respectively). Details of such procedures are given in the appendix.

5.5 Test substance

Toxicological and physico-chemical data, purity, solubility, stability, octanol-water partition coefficient and analytical methods and their detection limits should be known for the test substance. Relevant information on the formulation in which the test substance is used should be available.

5.6 Preparation of the dose

Depending upon the purpose of the experiment, the test substance may be used as such, in solution or in formulation, where appropriate, to allow good contact with the skin or to simulate the intended use conditions. The stability of the test sample and substance under the proposed conditions of administration and usage should be known.

6. PROCEDURE

6.1 Application of test substance

The dose as well as the contact time with the skin should mimic the intended use conditions as closely as possible or be higher to obtain relevant safety data under exaggerated use conditions (for assessment of foreseeable misuse). The amount of the formulation to be applied should be adapted whenever possible to the consumer use values published by Colipa (doc. 93/067). If permeability constants are being determined then infinite dose conditions are required.

6.2 Fluid dynamics

The receptor fluid, preferably degassed (e.g. by sonication), must be thoroughly stirred at all times or be continuously replaced in flow through chambers. The choice of static or flow-through conditions in the receptor cell should be made on a compound-by-compound basis, depending on its absorption properties and the goal of the study. It must be ensured that the amount of penetrant in the receptor fluid is less than 10% of its saturation level, in order to prevent significant back penetration and hence underestimation of absorption.

6.3 Temperature

Because the rate and extent of skin absorption is temperature dependent, the skin temperature should be maintained constant (30-32°C ± 1°C) either by use of penetration chambers with water jackets or temperature-controlled incubators.

6.4 Study time

The study time is determined by the subsequent reasonable characterisation of the absorption profiles and the intended use conditions. The exposure time and sampling period should be defined in the protocol. A normal study time of 24 hours is recommended.

6.5 Sampling

The frequency of sampling will depend on the rate and extent of dermal penetration, but should be sufficient to allow the extent or rate of penetration and/or the profile to be determined.

Where appropriate, the surplus of non-penetrated test substance should be determined in skin samples, rinsings and cell washings. The overall recovery of test substance should be at least 85%. If low recoveries of the test substance are obtained with all cells, a search should be conducted to determine the cause(s) which may include binding to proteins, to penetration cell surface and tubings, as well as possible evaporation or loss by chemical reaction.

6.6 Analysis

The receptor fluid must be analysed and, if possible, an analysis should be made of the amounts found in individual skin layers and on the skin surface. Suitable analytical procedures must be used, e.g. scintillation counting, HPLC or GC.

7. DATA REPORTING

7.1 Data

The absorption profile normally is determined up to 24 hours post application with usually six cells of similar barrier integrity. When adequate data are available, the lag time and the absorption rates may be calculated. Permeability constants can only be calculated from infinite dose experiments.

7.2 Test report

The test report should include the following information:

- Test substance:
  - chemical structure, physico-chemical properties and purity;
  - identification data.

- Vehicle (if appropriate):
  - type of formulation;
  - justification for choice of formulation.

- Test conditions:
  - source and site of skin sample, method of preparation, integrity;
  - details of test sample preparation, final concentration, stability and homogeneity of the preparation, especially when blended with radiolabelled test substance;
  - details of the administration of the test substance (exposure time and conditions, e.g. occlusion or not, rinse-off, leave-on);
  - justification for choice of skin source and skin preparation;
  - analytical method, sample clean-up, recovery and detection;
  - overall recovery of material, if possible.

- Reporting of results:
  - tabulation of individual results at each time point;
  - lag times, absorption rates, absorption profiles, if appropriate.

Data can be presented in various ways. Depending on the experimental set-up, percent dose, micrograms, micromoles or a penetration constant can be displayed. The presentation of cumulated percent dose or micrograms/cm^2 in tables and graphs is recommended. Graphs are prepared with appropriate computer programs which may be equipped with functions for statistical treatment of data.

- Statistical methods

- Discussion of results and conclusions:
  It should be remembered that percentage absorbed values are dependent on specific exposure and formulation conditions. In some cases, a permeability constant may provide useful interpretation of the results.

7.3 Implications for safety evaluation

Percutaneous absorption/penetration is a key parameter for the safety evaluation of cosmetic ingredients, as well as of the finished products containing them. Determination of absorption/penetration allows the interpretation of systemic toxicity data in terms of exposure via topical application. Furthermore, the utilisation of the procedures described in this guideline will provide a suitable substitute for in vivo experiments to measure percutaneous absorption/penetration. Depending on the outcome of such studies, with the context of the overall safety evaluation, further animal toxicity studies would be minimised. The results of such studies may also provide guidance on the optimisation of the design of further required tests.

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STANDARD PROTOCOL

PERCUTANEOUS ABSORPTION/ PENETRATION *IN VITRO* EXCISED HUMAN SKIN

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CONTENTS

I. INTRODUCTION

This standard protocol describes in vitro percutaneous absorption/penetration studies of cosmetic ingredients through human skin. Such studies are performed to measure the amount of test compound penetrating through the skin barrier for safety evaluation. Recommendations published by an AAPS-FDA panel on percutaneous absorption studies of drugs have been taken into account for this protocol (ref. 1).

The in vitro percutaneous absorption/penetration of the test compound is assessed using samples of isolated human skin placed in static or dynamic diffusion cells (ref. 2,3,4).

All the mentioned SOP (Standard Operating Procedure) have been specifically written up for the respective laboratory.

II. MATERIALS

- Dynamic mode: flow-through diffusion cell (ref. 3)
- Static mode: static Franz diffusion cell (ref. 2)

Usual laboratory equipment as indicated in a specific SOP.

III. METHODS

III.1. Preparation of the formulation

III.1.1. Unlabelled test compounds

The specifications of each test compound must be defined prior to the formulation: a recent analysis of the respective batch by the analytical department must be included in the final study report.

The qualitative controls of the formulation are performed by the formulator.

The quantitative controls are performed according to the SOP "*Control of an unlabelled formulation*". This control is based on HPLC or another suitable method in order to ascertain the concentration of the test compound in the formulation.

III.1.2. Radiolabelled test compounds

The degree of radiolabelling is specific for each formulation: the radiolabelled test compound is diluted according to its initial specific activity.

The supplier of the radiolabelled compound must provide a complete analysis bulletin including the radiochemical purity and date of the analysis; radiochemical purity must be reassessed before beginning the formulation and in the final formulation by the technical investigator; analytical bulletins should be included in the final study report.

Since only small quantities of radiolabelled formulations can be prepared each time, unlabelled pilot formulation assays on the respective scale should be performed and analytical results should be provided by the study promotor/department requesting the study. The latter ascertains the feasability of the formulation and its stability. The formulation should remain stable for the duration of the study.

The qualitative controls of the formulation are performed by the formulator.

The quantitative controls are performed according to a specific SOP. This control is based on HPLC or another method suited to ascertaining the concentration of the test compound (radiolabelled and unlabelled) in the formulation.

Specific radioactivity of the formulation is determined by scintillation counting using an external standard; the quench curves are established according to a specific SOP.

III.2. Preparation of the samples of human skin

III.2.1. Origin and storage of biological samples

Human skin used for these experiments is obtained from surgery. The skin samples can be obtained from a variety of anatomical sites including the breast (mastectomy and reduction mammoplasty) and abdomen (cosmetic reductive surgery). Skin must be collected as soon as possible after surgery. All skin samples are checked visually to ensure they are healthy and unaltered by clinical removal conditions. For transportation from the hospital/clinic to the laboratory, the skin should be kept in an isotherm container at 4°C. In the laboratory, each skin sample should be identified (identification number, type, age, gender, date of operation) and have the subcutaneous fat removed. The whole process of transporting and preparing the skin samples must be achieved as quickly as possible.

The skin samples are put into plastic bags, sealed and stored at - 20°C until use. Skin samples can be kept in a freezer for up to 3 months. For in vitro percutaneous absorption/penetration experiments, it has been shown that human skin permeability is unaffected by freezing (ref. 5, 6). These different stages should be performed according to a specific SOP *"including security measures for handling of biological materials"*.

III.2.2. Preparation of skin discs

An in vitro study using isolated skin should simulate as closely as possible the in vivo conditions. In vivo, a compound must diffuse up to the upper papillary dermis before being taken up by blood vessels and then entering the systemic circulation. Thus, depending on the solubility of the test

compound, to limit the effect of the dermal retention in vitro, especially with hydrophobic compounds, split-thickness skin (dermatomed skin or isolated epidermis) should be used (ref. 7).

Each skin sample is thawed at room temperature and prepared the day of the experiment according to a specific SOP:

• ***Dermatomed skin*:**

An electric dermatome (Davies simplex, THACKRAY SURGERY) is used to cut horizontal slices of skin. The thickness of the cut is controlled by a lever on the side of the dermatome head with the indicated calibrations. Full-thickness skin is fixed on a dissection board, epidermal side up, and sections are cut at 300 to 500 μm. The thickness of the membrane obtained is the result of the pressure applied and the angle of the dermatome as it is pushed across the skin. During this procedure, a constant angle of inclination of the oscillating blade relative to the skin must be maintained. The dermatomed layer thus obtained includes the epidermis and some dermal tissue.

• ***Isolated epidermis*:**

Epidermal membranes are prepared from full-thickness skin by a heat separation method (ref. 8). Water is heated in a beaker to 58-60°C. Full-thickness skin is suspended in the water with forceps for 60 seconds. Then the epidermis is gently peeled off from the dermis using forceps. The remaining epidermal membrane is cut to fit the diffusion cell area. Each epidermal membrane should be checked for integrity with the aid of a stereomicroscope.

III.2.3. Control of skin disc thickness

Skin thickness should be measured with a Digitaler Meßtaster (MT12, HEIDENHAIN) and a bidirectional counter (VRZ 401, HEIDENHAIN) or with equivalent equipment. The measurements should be performed according to the directions for use. Ten measurements per skin disc should be made. This process should be performed according to a specific SOP.

III.2.4. Specific recommendations for the use of human skin

As there is wide variability in human skin permeability (ref. 9), not less than 3 skin donors should be used to take account of interindividual variability besides the intraindividual variability; a proper statistical design should be used.

Age and anatomic site influence percutaneous absorption (ref. 9,10). Thus, skin samples from the same anatomical region and in the same age range (20-60 years) should be used to limit these variations.

To preserve the integrity of human skin membranes mounted in diffusion cells, the study time should never exceed 24 hours.

III.3. Preparation of the diffusion cells

The number of diffusion cells per experiment and the number of runs per study, must be specified in the protocol. The study should involve a minimum of six samples of similar integrity. This process should be performed according to a specific SOP.

III.3.1. Static mode or dynamic mode

The choice of static mode or dynamic mode depends on the absorption/penetration properties of the test compound and depends on the aim of the study.

The skin samples are placed as a barrier between the two halves of the diffusion cell, the stratum corneum facing the donor chamber; the donor side of the cell is open to the air (non occlusive conditions). The skin sample is kept at (32 +/- 1)°C by circulation of temperature controlled receptor fluid through the cell (dynamic diffusion cell) or by emersing the cell (static diffusion cell) in a temperature-controlled water bath.

The receptor chamber is filled with receptor fluid, capped and allowed to attain the correct temperature 1 hour before the beginning of the experiment. In dynamic mode, care is taken to ensure that no air bubbles form on the underside of the skin throughout the experiment.

III.3.2. Receptor fluid

To maintain the integrity of the skin samples and to collect the test compound, the receptor fluid is saline or buffered saline solution; a non-ionic surfactant can be added to solubilise the test compound especially for lipophilic compounds (ref. 11).

Before the experiments, it should be checked that the test compound is stable in the receptor fluid (in experimental conditions) for a period corresponding to the duration of the experiment.

III.4. Test of skin integrity

Each sample of human skin must be checked for integrity before the application of the test compound. This test must not affect the quality of the skin samples and must not influence the penetration of the test compound.

The integrity of the skin samples is checked with the the aid of a stereomicroscope and/or by measuring TEWL (Trans Epidermal Water Loss) (ref. 12). The procedure is performed according to a specific SOP.

III.5. Application of formulations

The quantities to be applied are expressed as follows:

- amount of formulation applied (mg/cm^2);
- amount of test compound applied (μg/cm^2);
- in case of radiolabelled compounds: amount of radioactivity applied (Bq/cm^2).

A finite dose of the test compound is applied to the skin surface (ref. 13) i.e. about 2 mg/cm^2 of a semisolid formulation (cream, ointment, gel, etc.) and about 5 μl/cm^2 of liquid preparations (solutions, emulsions, etc.). If semisolid formulations are compared to liquid formulations, identical amounts of 5 mg/cm^2. When investigating rinse-off preparations (e.g. hair dyes), an infinite dose (> 10 mg/cm^2) of the test compound is applied to the skin surface to mimic the use conditions. The rinsing procedure must be specified in the protocol.

The application is performed according to a specific SOP.

III.6. Start of the experimental setup and collection of fractions

The experiments are started immediately after the application of the formulation, according to a specific SOP.

In the static mode, aliquots of the receptor fluid are taken either throughout the experiment or at the end of the experiment. In the dynamic mode, the receptor fluid is pumped from a reservoir into and through the cell by a peristaltic pump. After exiting the cell, the receptor fluid is collected in an automatic fraction collector.

III.7. Study time

The study time is chosen according to the nature and intended use of the test compound. Rinse-off preparations are left on the skin for a restricted time (e.g. 30 minutes in the case of hair dyes). Then the skin and the upper part of the diffusion cell are rinsed and the experiment is continued for a maximum of 24 hours. Leave-on preparations are left in place for the entire study time (maximum 24 hours).

The diffusion cells are then taken apart according to a specific SOP.

III.8. Analysis

In all cases, the receptor fluid samples are analysed. In case of dermatomed skin, the epidermis is separated from the dermis and the remaining dermis sample is analysed.

III.8.1. UNLABELLED TEST COMPOUNDS

If relevant and if a suitable analytical method exists (in terms of detection limit, sensitivity, etc) an analysis will be made of the amounts of the test compound on the skin surface, in rinsings, cell washings and skin layers. The preparation of samples, extraction steps and analysis by HPLC or another suitable method depend on the test compound and should be described in the appendix of the study report.

This procedure should be performed according to a specific SOP.

III.8.2.RADIOLABELLED TEST COMPOUNDS

An analysis of the test compound on the skin surface, in rinsings, cell washings and skin layers should be made. This procedure should be performed according to a specific SOP. The analyses are carried out by means of scintillation counting according to a specific SOP. Scintillation counting is performed using an external standard; the quench curves are established according to a specific SOP.

III.9. Presentation of the results

The results are expressed in $\mu g/cm^2$ and % of the applied dose in the form of tables and graphs.

III.10. Interpretation of the results for safety evaluations

Classical in vitro percutaneous absorption studies only consider as penetrated the amount of compound entering into the receptor fluid. This interpretation is only correct when the skin samples are completely devoid of dermal tissue, i.e. when the epidermis has been removed from the dermis by heat (in vivo the dermis is vascularised, i.e. is part of the central compartment).

The dermatome-based separation technique leaves some upper dermal tissue underneath the epidermis. Hence, the epidermal and the dermal compartments have to be considered separately. The amount of test compound found after the study time in the receptor fluid, plus that found in the dermis, must be considered to have crossed the skin barrier i.e. as penetrated.

III.11. Study report

The study report has to be finalized according to a specific SOP.

III.12. Archives

The protocol, raw data and study report are kept on file by the study director's office for at least 5 years.

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