

Guidelines for Percutaneous Absorption/Penetration

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CONTENTS

	Page
1. Background	1
2. Introduction	1
3. Initial considerations	2
4. Principle of the test	2
5. Description of the method	2
5.1 Penetration cell design	
5.2 Receptor fluid	
5.3 Skin disks	
5.4 Membrane integrity	
5.5 Test substance	
5.6 Preparation of the dose	
6. Procedure	4
6.1 Application of test substance	
6.2 Fluid dynamics	
6.3 Temperature	
6.4 Study time	
6.5 Sampling	
6.6 Analysis	
7. Data and Reporting	5
7.1 Data	
7.2 Test report	
7.3 Implications for safety evaluation	
8. References	6
9. Standard Protocols	7
1. Excised human skin	7
2. Pig skin	15
3. Excised pig skin	23
4. Rat skin	32

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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 (≤ 1 mm 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 ($30-32^{\circ}\text{C} \pm 1^{\circ}\text{C}$) 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/cm² 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

Pages

I. Introduction	8
II. Materials	8
III. Methods	8
III.1 Preparation of the formulation	8
III.1.1 Unlabelled test compounds	
III.1.2 Radiolabelled test compounds	
III.2 Preparation of the samples of human skin	9
III.2.1 Origin and storage of biological samples	
III.2.2 Preparation of skin discs	
III.2.3 Control of skin disc thickness	
III.2.4 Specific recommendations for the use of human skin	
III.3. Preparation of the diffusion cells	11
III.3.1 Static mode or dynamic mode	
III.3.2 Receptor fluid	
III.4. Test of skin integrity	11
III.5. Application of formulations	12
III.6. Start of the experimental setup and collection of fractions	12
III.7. Study time	12
III.8. Analysis	12
III.8.1 Unlabelled test compounds	
III.8.2 Radiolabelled test compounds	
III.9. Presentation of the results	13
III.10. Interpretation of the results for safety evaluations	13
III.11. Study report	13
III.12. Archives	13
IV. Literature references	14

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 feasibility 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 immersing 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 ($\mu\text{g}/\text{cm}^2$);
- 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 $2 \text{ mg}/\text{cm}^2$ of a semisolid formulation (cream, ointment, gel, etc.) and about $5 \mu\text{l}/\text{cm}^2$ of liquid preparations (solutions, emulsions, etc.). If semisolid formulations are compared to liquid formulations, identical amounts of $5 \text{ mg}/\text{cm}^2$. When investigating rinse-off preparations (e.g. hair dyes), an infinite dose ($> 10 \text{ mg}/\text{cm}^2$) 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 $\mu\text{g}/\text{cm}^2$ 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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STANDARD PROTOCOL

PERCUTANEOUS ABSORPTION/ PENETRATION *IN VITRO* PIG SKIN

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CONTENTS		Page
I.	General remarks and summary	16
II.	Origin of skin samples	16
III.	Preparation of skin	17
IV.	Preparation, experimental procedure and control of test systems	17
V.	Test of skin integrity	19
VI.	Application of test substances	19
VII.	Rinse-off of test substances	19
VIII.	Time scale of experiments	20
IX.	Scintillation counting	20
X.	HPLC analysis	20
XI.	Analysis of skin samples	21
XII.	Presentation of the results	21
XIII.	Literature references	22

I.I. GENERAL REMARKS

The present Standard Protocol for the in vitro testing of cutaneous absorption/penetration with the pig skin model relies basically on published procedures (ref. 1-6) from our laboratory and has been slightly modified since the first publications.

The test system with pig skin in vitro allows the measurement of cutaneous absorption/penetration of many different components in cosmetics and pharmaceutical products by analysing the receptor fluid and the skin samples with an appropriate methodology (scintillation-counting, HPLC).

I.II. SUMMARY OF THE METHOD

Fresh, unboiled pig skin is provided by a local butcher and is reduced in several steps to 1 mm thick membranes containing stratum corneum, epidermis and some dermis. These membranes can be stored at - 20°C for several weeks and, after thawing at room temperature, can be used for tests of cutaneous absorption/penetration.

For the test procedure, the circular skin membranes are fixed in appropriate chambers where the bottom-sided dermis is in contact with receptor fluid, and the horny layer is exposed to air. The actual absorption/penetration experiment is preceded by a skin integrity test performed with tritiated water. Applications of test substances are mainly done in gel-like formulations, occasionally also as liquids, over a time span of 30 minutes, followed by rinsing of the skin surface with water and shampoo. In general, experiments are stopped after 72 hours, but depending on the substance and the available information, experiments can be stopped after 24 hours. The measurements of the test substance(s), including all steps from the starting material to the receptor fluid, the rinsing solution and the remainder in the skin, produce a final mass balance, which is useful for the internal control of the experimental procedure.

II. ORIGIN OF SKIN SAMPLES

II.I. Skin from the back and the flanks

The entire skin of the back and flank of male, castrated pigs weighing 100 kg (average), is obtained from a local butcher. The unboiled skin of one animal is obtained within 1-2 hours after slaughter and is refrigerated during this period.

II.II. Skin from pig ears

Skin from the ear causes some problems for the dissection of the membranes, but it can be easily obtained from any butcher or slaughterhouse.

III. PREPARATION OF SKIN

III.I. Skin from back and flank

The skin is marked by the butcher in such a way that the four pieces delivered can be easily assigned to their original location. The skin is cleaned with tap water under a shower. The bristles are cut with an electric cutter (Elektra II, Aesculap-Werke AG, Tuttlingen, Germany). The skin surface is briefly washed with 70% ethanol and blotted with soft household paper and then stored for approximately 16 hours at 4°C in a refrigerated room. Strips of 7.5 x 40 cm are thereafter cut with a scalpel and marked for identification of the initial position. Subcutaneous fat is removed with an electrical knife (Moulinex SA, Zürich, Switzerland) using a guide of 4 mm thickness. A 1 mm thick layer is cut off using an air-driven high speed dermatome (Stryker, Hausmann SA, St.Gallen, Switzerland). It is important that during this cutting procedure the dermatome be fixed and guided by a holding device (ref. 1,2) ensuring a constant angle of inclination of the oscillating blade towards the skin, thus providing a constant thickness of the resulting skin strips which comprise the horny layer, the epidermis and part of the dermis, but no subcutaneous fat. The skin membranes are either used immediately for experimentation or sealed in plastic bags and stored frozen in a flat, horizontal position at -20°C for up to eight weeks.

III.II. Skin of the ear

After cleansing and removal of bristles (see III.1.) the ear is fixed with needles on a cork board or similar device so that the favored flat regions of the outer surface of the ear can be isolated with a scalpel. Subcutaneous fat is scarce. If necessary, it is removed with scalpel and forceps. The thickness of such a preparation is definitely greater than 1mm. Therefore, the PTFE chambers described under IV.1. are not suitable, but chambers with a different clamp system (e.g. Laboratory Glass Apparatus, Berkeley, U.S.A.) can easily be used. Skin samples from the ear are immediately mounted in the chambers and used mainly for absorption/penetration experiments in connection with studies of metabolism in the skin.

IV. PREPARATION, EXPERIMENTAL PROCEDURE AND CONTROL OF TEST SYSTEMS

IV.I Absorption/penetration system

The membranes needed for a given flow-through chamber system are cut from the thawed skin strip with a steel punch of appropriate size and immediately mounted in the chambers. In addition to the large chambers made of PTFE (ref. 1,2), with a surface of 27.3 cm², smaller chambers of identical design with a surface of 9.6 cm² or 1.8 cm² are available. In addition, the earlier mentioned all-glass absorption/penetration system (Laboratory Glass Apparatus, Berkeley, U.S.A.), with a surface of

0.785 cm² is used. Immediately after mounting of the skin membranes (usually 6 membranes/experiment) the dermis side directed to the receptor chamber is brought in contact with the receptor fluid (0.14 M NaCl + 2 mM K₂HPO₄ + 0.4 mM KH₂PO₄ with 100 IU Penicillin and 100 mg Streptomycin per ml) by connecting the tubings, which are already connected to a peristaltic multichannel pump (Type IPN 16, IsmaTec SA, Zürich, Switzerland) with the individual chambers. To prevent air bubbles, each chamber is initially filled with receptor fluid by a syringe. The very thin bottom of the PTFE chamber allows the control of eventual air bubbles. With glass chambers, the connecting tubings are simply slipped over the glass-made in- and outlets and the skin is clamped on the previously filled receptor chamber.

The multichannel pump is placed outside the incubator at room temperature, while the receptor reservoir fluid is placed in the incubator to provide the appropriate temperature.

IV.II. Incubators

To maintain a temperature of 32-33°C (heating/thermostat) and 50-60% relative humidity (largely provided by the liquids in the system), unsophisticated incubators (Types 2736 and 2737, Köttermann, Hänigsen, Germany) are used. Temperature and humidity are continuously recorded with a combined measuring device (Krüger & Co., Messgeräte, Degersheim, Switzerland). The all-glass system with double walls can be used without an incubator, if a thermostated waterbath is available.

IV.III. Circulation system and fractionation

The multichannel pump outside the incubator aspirates receptor fluid from a container inside the incubator and distributes it to the receptor chambers (maximum 6) in the incubator and from there to the collection vessels or to fraction collectors placed outside the incubator at room temperature. The flow rate is regulated to deliver 10ml per hour to the large chamber system. For smaller chambers, the flow is reduced proportionally to the available surface. The collection vessels are changed at defined time points. Fraction collectors are used if collection of fluid at short intervals is desired.

IV.IV. Control of test systems

The cleaning of the test systems after each experiment is of critical importance in case of radiolabelled test substances. With low absorption/penetration values and concomitant low radioactivity input, the carry-over contamination of tubing and chamber from a preceding experiment with high radioactivity can be devastating for the interpretation of data. To avoid such problems, the chambers are thoroughly washed after each experiment with a detergent, rinsed for several hours under tap water, then rinsed with distilled water and finally with 70% ethanol. The tubing is rinsed with 10% NaOH at elevated pump speed for several hours, then rinsed overnight with deionised water. In case of an obstinate contamination, the use of a special cleanser (e.g. Pico-Kleen from Packard) can help or eventually the whole tubing is renewed. A ¹⁴C-contamination can eventually be seen during the measurement of tritiated water from the skin integrity test by increased values in the ¹⁴C-channel of the scintillation counter.

V. TEST OF SKIN INTEGRITY

The integrity of the skin barrier is tested prior to each experiment with tritiated water. Steady state is reached 2-3 hours after application of 10 ml water containing 3.7 μCi $^3\text{H}_2\text{O}$ to the largest chambers having a surface of 27.3 cm^2 . For smaller chambers, the volume is reduced proportionally to the surface. Evaporation is prevented by covering the surface with parafilm. The collection vessels are changed every hour. The radioactivity is measured after mixing 0.5 ml of each fraction with 2 ml scintillation cocktail (UltimaGold, Packard, Zürich, Switzerland) in a TriCarb-2000-CA scintillation counter (Packard). The mean value measured for 3 blanks containing 0.5 ml fresh receptor fluid and 2 ml scintillation cocktail is subtracted from the measured values. After 5 hours the $^3\text{H}_2\text{O}$ -liquid is aspirated and the skin is blotted dry with Kleenex tissue. The values obtained between 2 and 5 hours are checked for linearity and the cumulative absorption/penetration over 5 hours is calculated. From a large number of measurements made in the past, it is concluded that membranes with a 5 hours absorption/penetration up to 1% of the dose can be used with confidence. Our historical control over a period of 6 months is 0.58 +/- 0.17% (n = 60, membranes with $^3\text{H}_2\text{O}$ values > 1% excluded).

VI. APPLICATION OF TEST SUBSTANCES

Test substances are applied 1 hour after removal of the tritiated water. Usually the compound to be tested is applied to a surface of 9 cm^2 of the large chambers, to 4 cm^2 of the medium sized, and to 1 cm^2 of the small chambers. The surface to be covered is marked with a soft marker (Stabilo OH-Pen). With the all-glass system the whole surface of 0.785 cm^2 is covered. The delimitation of the marked area is readily maintained when gel or cream formulations are applied with a spatula. Liquids are more difficult to apply to a defined area and not more than 25 microliters/ cm^2 should be applied with a micropipette. Using higher amounts of liquid usually results in a treated surface of undefined size. Of course, it is also possible to work with an infinite dose, as in the case of the integrity test with tritiated water.

VII. RINSE-OFF OF TEST SUBSTANCES

When testing hair dyes in formulations, the bulk of the formulation is removed 30 minutes after application with a PTFE spatula followed by rinsing with a mild shampoo and lukewarm water in several washing steps. The washing procedure imitates as close as possible the intended use conditions. Of course, it can be modified for other applications. As a rule of thumb it is proposed to remove as much as possible of the test substance from the skin surface without damaging the skin by mechanical or chemical influence. In experiments performed without a washing step after 30 minutes, the washing procedure is carried out at the end of the experiment, if there is any interest in the analysis of the skin sample.

VIII. TIME SCALE OF EXPERIMENTS

Experiments for the determination of the cutaneous absorption/penetration are principally continued over a period of 72 hours. However, if the absorption/penetration kinetics for a given substance are already known, further experiments, for example comparisons of different formulations, can be performed as short term tests over a period of 16-24 hours.

IX. SCINTILLATION COUNTING

The measurements of radioactivity by scintillation counting of ^{14}C or ^3H are performed in a Packard Tricarb-2000-CA scintillation counter, which is equipped with a considerable number of computer guided control functions. The samples to be measured are dispensed in appropriately sized scintillation vials and mixed with the appropriate scintillation cocktail, e.g. Ultima Gold from Packard, in ratios specified by the producer for various contents of salt or solvent.

Before starting the measurements the vials are conditioned for at least 1 hour in the counter. Measurements are performed with two specific programs for high and low counts and separate determination of ^{14}C and ^3H . The following aliquots, providing the results and mass balance of the experiment, are measured :

- a) ^3H Application solution of tritiated water, 0.1 and 0.5 ml.
- b) ^3H Elution solution from the receptor chamber, 0.5 ml of each fraction during 5 hours integrity test.
- c) ^{14}C Dilutions of the labeled application formulation (4 separate weighings).
- d) ^{14}C Elution fluid from the receptor chamber, 0.5 - 1 ml of each fraction during 24 - 72 hours after application of test substance.
- e) ^{14}C Diluted solutions, containing dissolved substance from devices used for application and removal of formulation and washing solutions.
- f) ^{14}C Neutralised aliquots of dissolved skin.
- g) $^3\text{H}/^{14}\text{C}$ Blanks, containing mixtures with appropriate content of salt or solvent, without radioactivity.

X. HPLC ANALYSIS

HPLC analysis is carried out according to established laboratory methods for the compounds of interest. Good results are usually obtained using solid phase extraction with Sep-Pak C18 cartridges from Waters (Millipore Corp). In many cases, these cartridges can be placed in front of the collection vials and retain the analyte completely. This eliminates a number of steps of the sample preparation. The suitability of this method has to be rigorously tested for each analysis.

XI. ANALYSIS OF SKIN SAMPLES

11.1. Analysis of the skin by scintillation counting

At the end of the experiment, the application area is cut out and placed into a glass container with 10 N KOH for 4 hours at 55-60°C to dissolve the skin sample. The amount of KOH is at least 0.5 ml/cm² of skin. The yellowish-brown liquid is subsequently neutralised with HCl, the total volume determined and aliquots (0.05-0.5 ml) prepared for scintillation counting.

11.2. Analysis of the skin by HPLC

The test substance is extracted from the exposed skin with an appropriate organic solvent (mainly n-butanol for hair dyes) and the recovered solvent evaporated in vacuo. The residue is dissolved in mobile phase and filtered before injection. Obviously the method has to be optimised for each substance with respect to recovery and matrix components interfering with the analysis. Controls are prepared by applying defined amounts of test substance in ethanol or methanol to a defined skin surface for at least 60 minutes under experimental conditions and are then extracted in the same way as the experimental samples.

11.3. Separate analysis of horny layer and residual skin

To separate the horny layer from the rest of the membrane used for the experiment, the isolated skin piece is wrapped in aluminium foil and placed on a heating plate (90°C) for 30-60 seconds. Thereafter, the horny layer can be isolated in large patches with forceps. The separation is not to be considered as complete, especially in the presence of hair or bristles, but the procedure often gives valuable information on the localisation of the substance in or on the skin.

XII. PRESENTATION OF THE RESULTS

Depending on the experimental setup, percent dose, micrograms, micromoles or a absorption/penetration constant can be presented. The determination of absorption/penetration constants can give valuable information under specific test conditions. These conditions are not realised in the majority of our experiments where the substance is washed off after 30 minutes. The presentation of data in % cumulated dose and in micrograms/cm² in the form of tables and graphs is recommended. This software is also equipped with a number of functions for statistical treatment of data. The raw data are presented in a table containing the data for each membrane, including data on tritiated water absorption/penetration and mass balance.

LITERATURE REFERENCES:

1. Bracher M., Faller C. & Noser F.K., *Evaluation of an in vitro percutaneous absorption/penetration model with two oxidative hair dyes*, Int.J. Cosmet. Sci. 9, pp. 223-236, 1987.
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3. Beck H., Bracher M., Faller C. & Hofer H., *Comparison of in vitro and in vivo skin absorption/penetration of dyes in: Prediction of Percutaneous Penetration-Methods, Measurements, Modelling* (Ed. by Scott R.C., Guy R.H., Hadgraft J. and Boddé H.E.), 2, pp. 441-450, IBC Technical Services Ltd., London, 1991.
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5. Beck H., Bracher M. & Spengler J., *Bandrowski's Base: HPLC Analysis and cutaneous absorption/penetration*, Conference Proceedings 16th IFSCC Int. Congress, New York, pp. 250-262, 1990.
6. Beck H., Bracher M. & Bartnik F. G., *Percutaneous absorption of hair dyes: An interlaboratory comparison of in vivo and in vitro data with rat and pig*, In Vitro Toxicology, 7, 4, pp. 305-312, 1994.

STANDARD PROTOCOL

PERCUTANEOUS ABSORPTION/ PENETRATION *IN VITRO* EXCISED PIG SKIN

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CONTENTS	Page
I. Objective	24
II. Abstract	24
III. Methods	25
III.I Preparation of pig skin	
III.II Blending of test samples with radio labelled test substance	
III.II.I Emulsions and gels	
III.II.II Low-viscosity samples	
III.III Application of blended test samples	
III.III.I Application of emulsions and gels	
III.III.II Application of low-viscosity samples	
III.IV Absorption/penetration experiment	
III.V Preparation of analytical samples	
III.V.I Test sample residues on the upper penetration cell wall	
III.V.II Receptor fluid	
III.V.III Non-absorbed test sample	
III.V.IV Horny layer	
III.V.V Epidermis	
III.V.VI Dermis	
III.VI LSC-measurement of radioactivity	
IV. Data evaluation	28
V. Materials	28
VI. Appendix	29
VI.I Data evaluation: calculation example for dermis	
VI.II Calculation sheet (example)	
VI.III Plot of results (example)	
VII. Literature references	31

I. OBJECTIVE

This ex-vivo/in-vitro assay is used for the estimation of dermal absorption / penetration of active respectively inactive ingredients of cosmetics and pharmaceuticals which are being developed for single or repetitive topical administration. It complements our dermato-toxicological test battery for the estimation of compatibility and risk assessment, supports the selection of suitable active and inactive ingredients and rationalizes the optimization of formulations respectively vehicles.

II. ABSTRACT

The experiments are carried out with specially prepared, un-boiled back skin of selected female pigs (about 130 days old, about 100 kg weight, obtained from a local butcher according to our specifications). We prepare skin discs with a gently dry-shaved surface, about 3-4 mm thickness and 5 cm outer diameter.

Pig skin is highly recommended in the relevant literature for the in vitro estimation of the dermal absorption/penetration of substances by human skin in vivo because of its close physiological similarity and comparative ease of availability. The latter is in contrast to human skin obtained from surgery or cadavers, which is normally not available frequently and in sufficient quantities, and which is quite often already damaged. Furthermore, results with human skin are highly variable from batch to batch due to the various and differing sources and anatomical sites.

Our experiments are carried out in accordance with assay protocols published in the respective and relevant literature (see references). Suitable glass cells (Franz-type), which are kept at 32°C (skin temperature) with a thermostat, are used for these experiments.

On account of the elaborate analytical procedures, radiolabelled (¹⁴C, ³H) test substances are preferred to get proper information about their absorption (residue on skin surface) and distribution between horny layer, epidermis, dermis and receptor fluid. In some cases, relevant results can only be obtained by using radiolabelled substances (e.g. discrimination between endogenous and exogenous water or urea; limited sensitivity and selectivity of conventional analytical methods (HPLC, GC)).

III. METHODS

III.I. Preparation of pig skin

The fresh back skin (about 30 x 40 cm), as obtained from the butcher, is cut in strips of about 10 x 40 cm with a sharp butcher knife. Thereafter, most of the fat layer is removed with an electric household knife. Care has to be taken not to damage the underlying dermis. Finally, residues of fat are carefully scraped off with the broad blade of a sharp butcher knife to yield an even surface and an average skin thickness of about 3-4 mm. The skin is rinsed with warm tap water (about 30°C). The bristles are cut with a special electric clipper for animals (Type GT 102 "Favorita I", Aesculap, Germany; shaver head GH 703 1/10 mm). A punch with 5 cm inner diameter is used to get skin discs which fit into the penetration cells. Only intact skin discs (e.g. no cuts, scars, scratches, injuries) are used for the following experiments. The skin discs are put into plastic bags, which are evacuated, sealed and stored at - 20 °C until use. A maximal storage time of three months under these conditions has proven to be tolerable for maintaining the integrity of the skin.

III.II. Blending of test samples with radiolabelled test substance

III.II.I. EMULSIONS AND GELS

The test samples are blended with radiolabelled test substance. A specific activity of about 0,4 MBq/mg has proven to be suitable for the subsequent radioanalysis. The blended test samples are stored for about 16 hours, normally at room temperature, prior to the penetration experiment, to achieve an equilibrium within the test sample. Only temperature-sensitive samples should be stored in a refrigerator.

III.II.II. LOW-VISCOSITY SAMPLES

The blending of solutions etc. is carried out as described above (III.II.I.). The subsequent storage preceding the penetration experiment can be omitted.

III.III. Application of blended test samples

The skin discs, which have been thawed for about 30 minutes after removal from the freezer, are dabbed dry with wool.

III.III.I. APPLICATION OF EMULSIONS AND GELS

The outer border of the application area is marked with a suitable stamp. For the estimation of the applied sample amount, a piece of aluminium foil and a finger-cot are weighed first without and thereafter with the suitable amount of test sample. The test sample is applied onto the epidermal skin side and evenly distributed within the application area (ca. 2.5 cm diameter, 4.9 cm² area) with the finger-cot. After the application, the aluminium foil and finger-cot are weighed again to calculate the actual applied dose of test sample, which should amount to about 4 mg/cm².

III.III.II. APPLICATION OF LOW-VISCOSITY SAMPLES

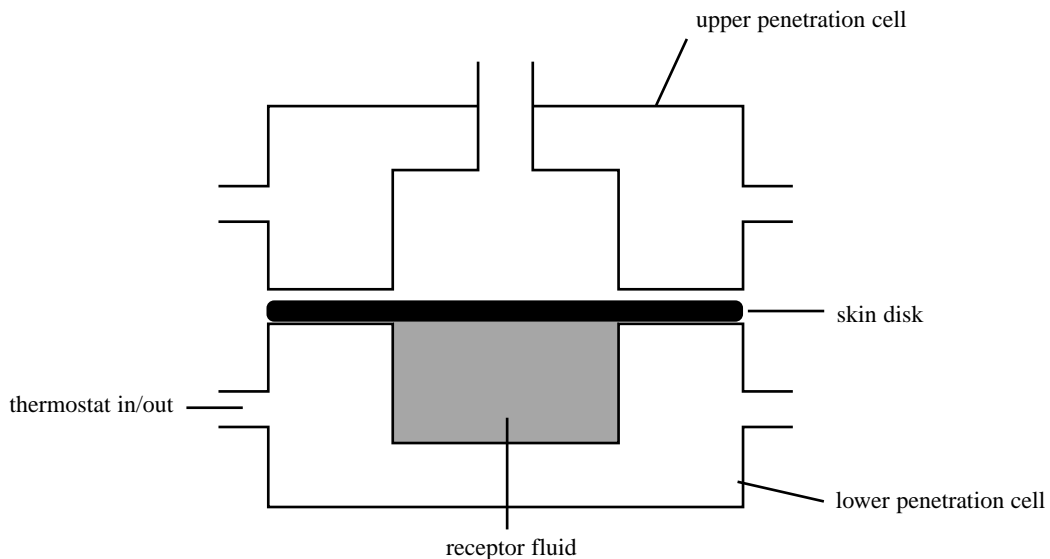
Solutions etc. are applied directly into the mounted cell (see below) with an appropriate syringe. They cover the application area on the skin surface immediately after spreading.

III.IV. Absorption / penetration experiment

The lower penetration cells are positioned onto a multiple electronic magnetic stirrer manifold (Variomag, Germany) and connected by tygon tubes with each other and a thermostat (Lauda C6, Germany). Magnetic stirring bars are put into the cells which are then filled with the appropriate receptor fluid. Test samples are applied to the skin (creams and lotions before and low viscosity samples (solutions etc.) after mounting of the penetration cells, see III.III). The skin discs are positioned onto the lower penetration cells, epidermal side up. Air bubbles between skin bottom side and receptor fluid should be avoided. The upper penetration cell must not come into contact with the applied test substance. The magnetic stirrer is set at 150 rpm.

The experiments are usually carried out in triplicate which has proven to be sufficient due to the relatively small variability between skin discs. The integrity of the skin discs is checked only visually, since any pre-check with tritiated water, as described in the literature, would influence the horny layer hydration and, furthermore, has proven to be dispensable for carefully prepared skin discs.

Schematic diagram of the penetration cell



temperature:	32 ° C (skin temperature in vivo)
receptor fluid:	0.9 % NaCl, 0.1% gentamycin in buffer (pH 7.4) for (ca. 5 ml) water-soluble substances; for other substances 1% bovine serum albumin is added
application area:	circular (diameter ca. 2.5 cm; area ca. 4.9 cm ²)
duration:	24 h (maximum) as standard or shorter periods
number of skin discs per test sample:	n = 3
applied test sample:	ca. 4 mg/cm ² = ca. 20 mg/skin disc resp. ca. 20 µl/skin disc

III.V. Preparation of analytical samples

After termination of the absorption / penetration experiment (normally after 24 hours, shorter periods might be useful for kinetic studies), the analytical samples are prepared as follows:

III.V.I. TEST SAMPLE RESIDUES ON THE UPPER PENETRATION CELL WALL

The upper penetration cell is placed into 15 ml of a suitable solvent (i.e. 2-propanol) which is capable of soluting the test substance out of the emulsion, gel etc. to remove residues sticking to the glass. An aliquot of 1ml blank is added to 10 ml scintillation cocktail in a polyethylene counting vial for radioanalysis.

III.V.II. RECEPTOR FLUID

After removal of the skin disc from the lower penetration cell, the receptor fluid (ca. 5 ml) is pipetted into a 10 ml-volumetric flask. The bottom side of the skin disc and the lower penetration cell are washed each with 1-2 ml distilled water, which are then added to the receptor fluid. Thereafter, the volumetric flask is filled up to 10 ml with distilled water. An aliquot of 1 ml is added to 10 ml scintillation cocktail in a polyethylene counting vial for radioanalysis.

III.V.III NON-ABSORBED TEST SAMPLE

Non-absorbed test sample is removed from the skin surface by gentle scraping with a spatula. The residue is then transferred to a polyethylene scintillation vial by cleaning the spatula, assisted by ultrasonication, in 5 ml scintillation cocktail, which are used as such for radioanalysis. Between the following working-up steps the skin samples should be stored at ca. 4°C.

III.V.IV. HORNY LAYER

Horny layer samples are obtained by 18-fold adhesive tape stripping with Tesafilm (Type 4129, width 25 mm, Beiersdorf, Hamburg, Germany). Tesafilm is applied to the horny layer for 10 seconds under light pressure and removed thereafter. After each strip the skin disc is revolved through about 40°. The 18 strips are pooled to yield 6 fractions of 3 strips each. The 6 strip fractions are soluted in 10 ml scintillation cocktail in polyethylene counting vials and subjected to radioanalysis.

III.V.V. EPIDERMIS

Epidermis and dermis are heat-separated by contacting the epidermal side for 45 seconds under light pressure with a 80°C hot plate (CERAN). Thereafter, the epidermis is removed from the dermis with a spatula and/or tweezers and soluted in 2 ml 'Soluene' (Packard) in a glass counting vial for 24 hours at 50°C. After cooling to ambient temperature, 10 ml scintillation cocktail are added to the epidermis solution. Chemiluminescence decays more rapidly after addition of one drop of glacial acid and storage in the dark for 1-2 hours prior to radioanalysis.

III.V.VI. DERMIS

The outer, hard edge of the dermis, which does not contain test substance, is cut off and discarded. The remaining dermis is cut into pieces to increase the surface for dissolution in 12 ml 'Soluene' (Packard) for 48 hours at 50°C in a glass counting vial. After cooling to ambient temperature, 10 ml scintillation cocktail are added to 1 ml dermis solution. Chemiluminescence decays more rapidly after addition of one drop of glacial acid and storage in the dark for 24 hours prior to radioanalysis.

III.VI. LSC-measurement of radioactivity

HIONIC-FLUOR (Packard) scintillation cocktail is used for all samples.

^3H is measured with program # 2 and ^{14}C with program # 3 (see appendix, page 29) in a liquid scintillation counter 'LSC3801' (Beckman Instruments).

IV. DATA EVALUATION

See calculation sheet (example) [appendix page 29].

The radioactivity in the samples is recalculated to yield the total non-absorbed (skin surface + upper cell surface) and absorbed radioactivity (horny layer, epidermis, dermis, receptor fluid). The sum of these values (total recovered radioactivity) was set to 100% (normalized) and the percentages of radioactivity in surface, horny layer etc. were recalculated as percentages of the normalized, recovered total radioactivity (see pages 31/32, upper plot) to yield information about the distribution of the applied test substance (non-absorbed vs. absorbed).

The sum of the normalized, absorbed radioactivity was set to 100% and the horny layer, epidermis, dermis and receptor fluid data were recalculated as percentages of the normalized, absorbed radioactivity (see pages 31/32, lower plot) to yield information about the test substance distribution within the skin.

V. MATERIALS

chemicals:	Commercial suppliers
solvents:	Commercial suppliers
radiochemicals:	Commercial suppliers
finger-cots:	Latex OP Fingerlinge, Vivomed
electric knife:	Type 2762, Condel
electric clipper:	Type GT 102 "Favorita II", Aesculap, shaver head GH 703 1/10 mm
magnetic stirrer:	Multipoint HP and Telesystem (15 Pl.), Variomag
thermostat:	Type CS- C6, Lauda
adhesive tape:	Tesafilm Type 4129, Beiersdorf
scintillation cocktail:	Hionic-Fluor, Packard
tissue solutor:	Soluene 350, Packard
counting vials:	Packard
liquid scintillation counter:	LSC 3801, Beckman

VI. APPENDIX

VI.I. Data evaluation : calculation example for dermis

total applied activity **TA:** 500000 dpm
 activity in dermis **DA:** 6000 dpm
 total recovered activity **RA:** 450000 dpm
 blank **BL:** 28 dpm

total volume: 12 ml Soluene + 4 ml volume of dermis

a. total activity dermis TAD:

$$\frac{(DA - BL) * \text{total volume}}{\text{aliquot}} = \frac{(6000\text{dpm} - 28\text{dpm}) * 16\text{ml}}{1\text{ml}} = 95552 \text{ dpm}$$

b. % applied activity

$$\frac{TAD * 100\%}{TA} = \frac{95552\text{dpm} * 100\%}{500000\text{dpm}} = 19.1\%$$

c. % applied activity (normalized)

$$\frac{TAD * 100\%}{RA} = \frac{95552\text{dpm} * 100\%}{450000\text{dpm}} = 21.2\%$$

d. % absorbed activity

activity surface + upper cell **SC:** 100000 dpm

$$\frac{TAD * 100\%}{(RA - SC)} = \frac{95552\text{dpm} * 100\%}{450000\text{dpm} - 100000\text{dpm}} = 27.3\%$$

VI.II. CALCULATION SHEET (EXAMPLE)

Absorption/Penetration

Experiment # 2968

PEN.048

Date:

Skin lot #

Test substance:

16.06.1994

94 E 1

C14-Urea

Test sample: Laurar 14

(5% Urea)

appl. sample weight	22.7	20.6	21.5	21.6	
[mg]					
pen. cell / skin disc	#1	#2	#3	mean	cov
	[dpm]	[dpm]	[dpm]	[dpm]	[%]
upper cell (15ml)	135.6	155.5	197.1	2021	19
skin surface	73287	49750	29881	50945	43
horny layer strips (sum)	133701	176871	113253	141107	23
epidermis	64886	57188	53984	58658	10
dermis (16ml)	6478.1	5210.7	6123.7	94552	11
receptor fluid (10ml)	3835.8	3321.3	4705.5	39262	18
sum	282323.5	292496.5	208144.3	386545	18

VI.II. CALCULATION SHEET (EXAMPLE) contd.

horny layer				
strip # 1-3	34026	62368	34174	43495
strip # 4-6	30967	44953	25377	33738
strip # 5-9	25168	24148	23509	24247
strip # 10-12	20702	23518	15529	19888
strip # 13-15	13940	13668	8224	11916
strip # 16-18	8898	8216	6440	7823

random sample of test sample					
=> estimation of spec.activ.	1#	#2	#3	mean	
weight	27.7	26.9	23.1	26	
dpm	579248	563152	489752	544051	
dpm/weight	20911	20935	21201	21006	1

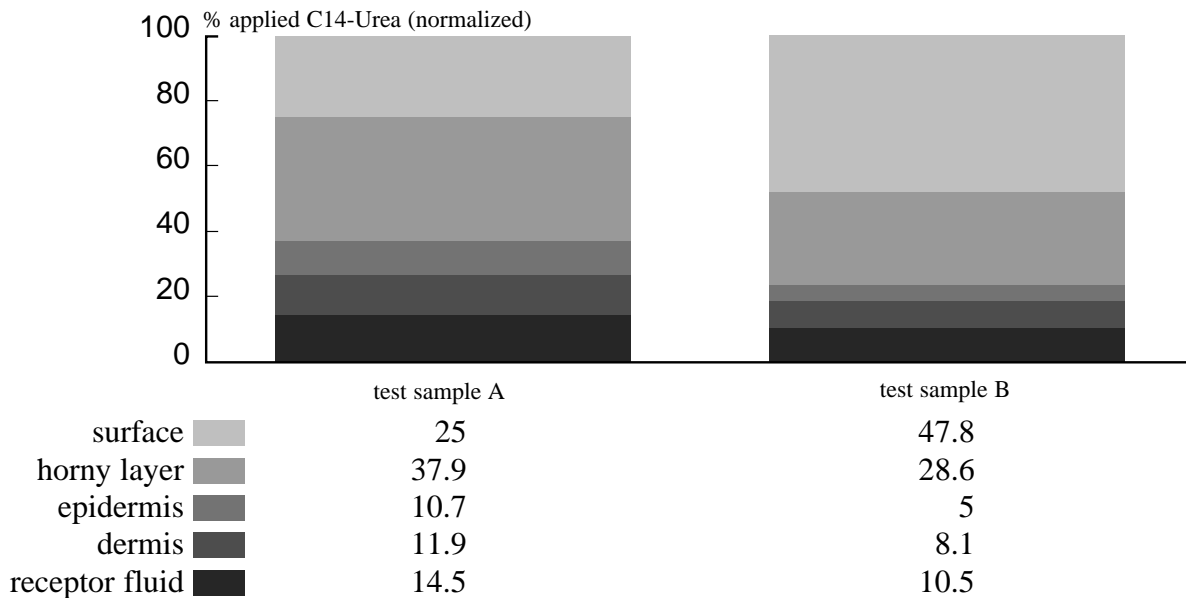
100% [dpm] =453726

analyt. samples (means)	dpm	% applied	% normal.	% absorbed
skin surface	52966	11.7	13.7	xxxxxx
horny layer	141107	31.1	36.5	42.3
epidermis	58658	12.9	15.2	17.6
dermis	94552	20.8	24.5	28.3
receptor fluid	39262	8.7	10.2	11.8
sum	386545	85.2	100.0	100.0

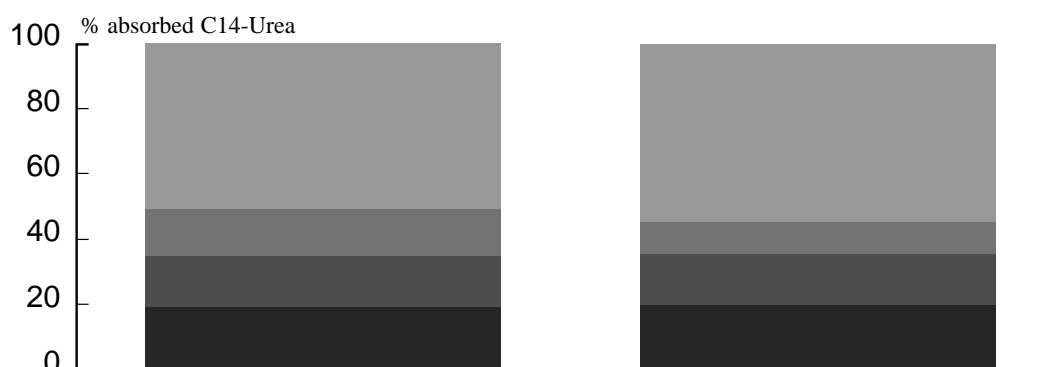
VI.III. PLOT OF RESULTS (EXAMPLE)

Distribution of C14-Urea

pig skin, lot #94 i 1, n = 3.24 h



VI.III. PLOT OF RESULTS (contd.)



	test sample A	test sample B
horny layer	50.6	54.8
epidermis	14.3	9.5
dermis	15.8	15.6
receptor fluid	19.3	20.1

VII. LITERATURE REFERENCES

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STANDARD PROTOCOL

PERCUTANEOUS ABSORPTION/ PENETRATION *IN VITRO* EXCISED RAT SKIN

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CONTENTS		Page
I.	General Aspects	33
II.	Purpose	33
III.	Materials	33
	III.I Equipment	
	III.II Reagents	
IV.	Preparatory Work	34
	IV.I Test Compound	
	IV.II Housing of Test Animals	
	IV.III Preparation of Rat Skin	
	IV.IV Preparation of Test Substance	
V.	Penetration Cells	34
VI.	Receptor Solution	35
VII.	Control of Skin Integrity	35
VIII.	Application of Test Compound	35
IX.	Sampling	36
	IX.I Static System	
	IX.II Dynamic System	
X.	Calculation and Report	36

I. GENERAL ASPECTS

This Standard Protocol describes the procedure of the testing of percutaneous absorption/penetration in an in vitro system. The protocol is adapted to the principles of the American Food and Drug Administration (FDA) ¹.

II. PURPOSE

The general intention of this assay is to assess the percutaneous absorption/penetration of a test compound through rat skin in an in vitro test system. For this purpose, a definitive dose of the test substance is applied to the epidermis side of the excised rat skin.

For many compounds, not only for cosmetic ingredients, the absorption/penetration through the skin is an important aspect to evaluate safety and toxicological risk. In this sense, the exact absorption/penetration rate is useful and necessary for any calculations of safety margins of compounds, which come into contact with the human skin. In special cases, it could be important if the kinetics of this passage are analysed.

The detection of the translocated compound is based on the analysis of radiolabelled compounds in the receptor fluid or, if non-radiolabelled test substance was applied, on their recovery by sufficient analytical methods (e.g. HPLC).

III. MATERIALS

III.I. Equipment

Skin penetration/evaporation system e.g. Model LG 1084 LPC (Laboratory Glass Apparatus, Inc.) incl. Franz-type two-chamber glass cells, fraction collector, peristaltic pump, water bath, balances, pipettes, liquid scintillation counter, dessicator, pH-meter, silicon tubes, rubber fittings, general laboratory equipment.

III.II. Reagents

Scintillation liquid, solubilisation liquid, DMSO, saline.

¹Pharmaceutical Res. 4, 265-267 (1987)

IV. PREPARATORY WORK

IV.I. Test Compound

Normally the test compound is radiolabelled as [¹⁴C]. In other cases the test compound could be non radiolabelled if there exists any sufficient analytical method (e.g. HPLC) for its detection in the receptor fluid.

IV.II. Housing of Test Animals

Female Wistar rats are kept under standard housing conditions. They are shaved approx. 20 hours before their killing at a body weight of 120 - 140 g. The hair cycle is in the telogen phase within this weight range. For the skin preparation, it must be guaranteed that the shaved skin is intact, without any scratches and injuries.

IV.III. Preparation of Rat Skin

The shaved back skin area of rats is used in the absorption/penetration test. For the preparation of skin discs the rats are killed by an overdose of carbon dioxide. After the excision of the back skin, the subcutaneous fat is carefully removed, avoiding any damage of the skin. If split thickness skin is required, slices are cut at 400 µm using a dermatome. The relevant skin discs, with a diameter which is dependent on the penetration cell, are punched out of the prepared back skin. The slices are stored temporarily in ice-cold saline solution. The skin may be used fresh or stored at -20°C until required. Prior to freezing, the skin is placed flat between aluminium foil. When frozen skin is required for use it is allowed to thaw at room temperature for at least one hour.

IV.IV. Preparation of Test Substance

The individual application dose as well as the specific formulation of the test compound has to be defined in the study protocol. The pH-value of the test article has to be determined and has to be adjusted in order to mimic the recommended application condition of, for example, a consumer product. It is very important to check the homogeneity of the test article especially if radiolabelled test substances are used in a formulation.

V. PENETRATION CELLS

Temperature-controlled Franz-type two-chamber (donor and receptor) glass cells are used. The typical situation in a static system is that aliquots of the receptor fluid are taken at intervals throughout the experiment and after termination.

In contrast to this system, the receptor fluid in the dynamic system is exchanged continuously using a peristaltic pump. The exchanged receptor fluid is collected in a fraction collector for any further analysis.

In both systems, the skin disc is clamped between the chambers with the epidermis facing the donor compartment. The receptor chamber is filled with receptor solution and a teflon-covered magnetic stirrer is added. Care must be taken to avoid any air bubbles in this chamber. The diffusion chamber is temperature-controlled (37°C, skin temperature should be 32°C) in a water bath. Six cells are used per test compound.

Conditions in the donor compartment should be adjusted for the required experiment. For example, the dose of material may be finite in an unoccluded donor compartment, or infinite with the compartment occluded.

VI. RECEPTOR SOLUTION

Usually physiological saline is used in the receptor chamber, the addition of phosphate buffer and the addition of antibiotics could be necessary, depending on the duration of the study and the chemical characteristics of the test substance.

For special purposes, the supplementation of five per cent (w/v) bovine serum albumin could also be recommended.

To guarantee the solubility of lipophilic test compounds in the receptor liquid, the addition of up to 50 % ethanol is allowed. The receptor solutions must be degassed prior to use by vacuum. Solubility of the penetrant in the receptor solution must not be a limiting factor in the experiment.

VII. CONTROL OF SKIN INTEGRITY

To ensure that the skin membranes are intact, a radiolabelled marker having known penetration characteristics, e.g. tritiated water or [³H]-sucrose, should be included in the donor solution. The marker should not affect the diffusion of the test substance.

VIII. APPLICATION OF TEST COMPOUND

Depending on the dimension of the Franz-type chamber, up to approx. 100 mg of the relevant test solution/cream are applied topically to the rat skin. The exact amount of the exposed test article must be determined using an analytical balance.

IX. SAMPLING

IX. I. Static System

As described in section V, the receptor solution may be sampled at intervals, e.g., at hourly intervals for six hours and at the termination of the experiment (approx. 20h). To calculate the non-absorbed/penetrated portion of the test article, residuals are washed off. The quantitative washing solution (e.g. DMSO, shampoo) must be collected and analysed for the mass balance.

The absorbed amount which represents the tissue-bound test article should be analysed after dissolving the skin disc in a solubilisation cocktail for 24 hours at 50°C. Prior to the solubilisation, the skin disc is removed and the outer clamped region is cut away from the exposed region. If any information on the portion of test article which is fixed at the stratum corneum is requested, the exposed skin could be tape-stripped. The tape-strips should be analysed in the same way as the residual tissue.

IX.II. Dynamic System

Samples of receptor solution are taken at hourly intervals throughout the experiment. At the termination of the experiment, the donor chamber is rinsed with a suitable solvent as mentioned in paragraph IX.I. The preparation of the skin is done in the same manner as in the static system.

X. CALCULATION AND REPORT

All analytical data must be compiled in a computer system for the mass balance in the representative protocol. The individual ratio of the absorbed/penetrated test article could be presented in cumulative percentage of the applied quantity or as flux ($\mu\text{g}/\text{cm}^2/\text{h}$).



Colipa, the European Cosmetic, Toiletry and Perfumery Association, was established in 1962. Its objectives are:

- ☞ To provide expertise and support to a range of working groups dealing in scientific, economic, fiscal, legal, consumer and environmental issues.
- ☞ To assist members in complying with European Union legislation affecting cosmetic industry products and operations.
- ☞ To act as an industry voice working with both international authorities and organisations. Additionally, Colipa provides a world-wide perspective to its members through its relationships with equivalent organisations in the USA and Japan.
- ☞ To serve as a communication and information centre for the European cosmetic industry, strengthening the industry's position through continuous interaction with its members.

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by actively contributing to the shaping of workable and fair policy frameworks regulating the industry. To this end, proactive and effective networking and communication are of the essence. Opportunities for achieving alignment on an international scale should be created and optimised.

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by addressing members' needs in an efficient and transparent way, through timely information and decision making processes and focusing on the issues and activities which are important to them.

Best use should be made of members' expertise and dedication to optimise both efficiency and one-voice positions.



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