

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Skin Absorption: *in vitro* Method

INTRODUCTION

1. This test guideline has been designed to provide information on absorption of a test substance applied to excised skin. It can either be combined with the OECD Test Guideline for Skin Absorption: *In vivo* Method (1), or be conducted separately. It is recommended that the OECD Guidance Document for the Conduct of Skin Absorption Studies (2) be consulted to assist in the design of studies based on this Test Guideline. The OECD Guidance Document has been prepared to facilitate the selection of appropriate *in vitro* procedures for use in specific circumstances, to ensure the reliability of results obtained by this method.

INITIAL CONSIDERATIONS

2. The methods for measuring skin absorption and dermal delivery can be divided into two categories: *in vivo* and *in vitro*. *In vivo* methods on skin absorption are well established and provide pharmacokinetic information in a range of animal species. An *in vivo* method is separately described in another OECD guideline (1). *In vitro* methods have also been used for many years to measure skin absorption. Although formal validation studies of the *in vitro* methods covered by this Test Guideline have not been performed, OECD experts agreed in 1999 that there was sufficient data evaluated to support the *in vitro* Test Guideline (3). Further details that substantiate this support, including a significant number of direct comparisons of *in vitro* and *in vivo* methods, are provided with the Guidance Document (2). There are a number of monographs that review this topic and provide detailed background on the use of an *in vitro* method (4)(5)(6)(7)(8)(9)(10)(11)(12). *In vitro* methods measure the diffusion of chemicals into and across skin to a fluid reservoir and can utilise non-viable skin to measure diffusion only, or fresh, metabolically active skin to simultaneously measure diffusion and skin metabolism. Such methods have found particular use as a screen for comparing delivery of chemicals into and through skin from different formulations and can also provide useful models for the assessment of percutaneous absorption in humans.

3. The *in vitro* method may not be applicable for all situations and classes of chemicals. It may be possible to use the *in vitro* test method for an initial qualitative evaluation of skin penetration. In certain cases, it may be necessary to follow this up with *in vivo* data. The Guidance Document (2) should be consulted for further elaboration of situations where the *in vitro* method would be suitable. Additional detailed information to support the decision is provided in an OECD Expert Meeting report (3).

4. This guideline presents general principles for measuring dermal absorption and delivery of a test substance using excised skin. Skin from many mammalian species, including humans, can be used. The permeability properties of skin are maintained after excision from the body because the principal diffusion barrier is the non-viable *stratum corneum*; active transport of chemicals through the skin has not been identified. The skin has been shown to have the capability to metabolise some chemicals during percutaneous absorption (6), but this process is not rate limiting in terms of actual absorbed dose, although it may affect the nature of the material entering the bloodstream.

PRINCIPLE OF THE TEST

5. The test substance, which may be radiolabelled, is applied to the surface of a skin sample separating the two chambers of a diffusion cell. The chemical remains on the skin for a specified time under specified conditions, before removal by an appropriate cleansing procedure. The receptor fluid is sampled at time points throughout the experiment and analysed for the test chemical and/or metabolites.
6. When metabolically active systems are used, metabolites of the test chemical may be analysed by appropriate methods. At the end of the experiment the distribution of the test chemical and its metabolites are quantified, when appropriate.
7. Using appropriate conditions, which are described in this guideline and accompanying guidance document (2), absorption of a test substance during a given time period is measured by analysis of the receptor fluid and the treated skin. The test substance remaining in the skin should be considered as absorbed unless it can be demonstrated that absorption can be determined from receptor fluid values alone. Analysis of the other components (material washed off the skin and remaining within the skin layers) allows for further data evaluation, including total test substance disposition and percentage recovery.
8. To demonstrate the performance and reliability of the test system in the performing laboratory, the results for relevant reference chemicals should be available and in agreement with published literature for the method used. This requirement could be met by testing an appropriate reference substance (preferably of a lipophilicity close to the test substance) concurrently with the test substance or by providing adequate historical data for a number of reference substances of different lipophilicity (e.g. caffeine, benzoic acid, and testosterone).

DESCRIPTION OF THE METHOD

Diffusion cell

9. A diffusion cell consists of a donor chamber and a receptor chamber between which the skin is positioned (an example of a typical design is provided in Figure 1). The cell should provide a good seal around the skin, enable easy sampling and good mixing of the receptor solution in contact with the underside of the skin, and good temperature control of the cell and its contents. Static and flow-through diffusion cells are both acceptable. Normally, donor chambers are left unoccluded during exposure to a finite dose of a test preparation. However, for infinite applications and certain scenarios for finite doses, the donor chambers may be occluded.

Receptor fluid

10. The use of a physiologically conducive receptor fluid is preferred although others may also be used provided that they are justified. The precise composition of the receptor fluid should be provided. Adequate solubility of the test chemical in the receptor fluid should be demonstrated so that it does not act as a barrier to absorption. In addition, the receptor fluid should not affect skin preparation integrity. In a flow-through system, the rate of flow must not hinder diffusion of a test substance into the receptor fluid. In a static cell system, the fluid should be continuously stirred and sampled regularly. If metabolism is being studied, the receptor fluid must support skin viability throughout the experiment.

Skin preparations

11. Skin from human or animal sources can be used. It is recognised that the use of human skin is subject to national and international ethical considerations and conditions. Although viable skin is preferred, non-viable skin can also be used provided that the integrity of the skin can be demonstrated. Either epidermal membranes (enzymically, heat or chemically separated) or split thickness skin (typically 200–400 μm thick) prepared with a dermatome, are acceptable. Full thickness skin may be used but excessive thickness (*ca.* > 1 mm) should be avoided unless specifically required for determination of the test chemical in layers of the skin. The selection of species, anatomical site and preparative technique must be justified. Acceptable data from a minimum of four replicates per test preparation are required.

Skin preparation integrity

12. It is essential that the skin is properly prepared. Inappropriate handling may result in damage to the *stratum corneum*, hence the integrity of the prepared skin must be checked. When skin metabolism is being investigated, freshly excised skin should be used as soon as possible, and under conditions known to support metabolic activity. As a general guidance, freshly excised skin should be used within 24 hrs, but the acceptable storage period may vary depending on the enzyme system involved in metabolism and storage temperatures (13). When skin preparations have been stored prior to use, evidence should be presented to show that barrier function is maintained.

Test substance

13. The test substance is the entity whose penetration characteristics are to be studied. Ideally, the test substance should be radiolabelled.

Test preparation

14. The test substance preparation (e.g., neat, diluted or formulated material containing the test substance which is applied to the skin) should be the same (or a realistic surrogate) as that to which humans or other potential target species may be exposed. Any variation from the 'in-use' preparation must be justified.

Test substances concentrations and formulations

15. Normally more than one concentration of the test substance is used in typical formulations, spanning the realistic range of potential human exposures. Likewise, testing a range of typical formulations should be considered.

Application to the skin

16. Under normal conditions of human exposure to chemicals, finite doses are usually encountered. Therefore, an application that mimics human exposure, normally 1–5 mg/cm^2 of skin for a solid and up to 10 $\mu\text{l}/\text{cm}^2$ for liquids, should be used. The quantity should be justified by the expected use conditions, the study objectives or physical characteristics of the test preparation. For example, applications to the skin surface may be infinite, where large volumes per unit area are applied.

Temperature

17. The passive diffusion of chemicals (and therefore their skin absorption) is affected by temperature. The diffusion chamber and skin should be maintained at a constant temperature close to

normal skin temperature of $32 \pm 1^\circ\text{C}$. Different cell designs will require different water bath or heated block temperatures to ensure that the receptor/skin is at its physiological norm. Humidity should preferably be between 30 and 70%.

Duration of exposure and sampling

18. Skin exposure to the test preparation may be for the entire duration of the experiment or for shorter times (i.e., to mimic a specific type of human exposure). The skin should be washed of excess test preparation with a relevant cleansing agent, and the rinses collected for analysis. The removal procedure of the test preparation will depend on the expected use condition, and should be justified. A period of sampling of 24 hours is normally required to allow for adequate characterisation of the absorption profile. Since skin integrity may start to deteriorate beyond 24 hours, sampling times should not normally exceed 24 hours. For test substances that penetrate the skin rapidly this may not be necessary but, for test substances that penetrate slowly, longer times may be required. Sampling frequency of the receptor fluid should allow the absorption profile of the test substance to be presented graphically.

Terminal procedures

19. All components of the test system should be analysed and recovery is to be determined. This includes the donor chamber, the skin surface rinsing, the skin preparation and the receptor fluid/chamber. In some cases, the skin may be fractionated into the exposed area of skin and area of skin under the cell flange, and into *stratum corneum*, epidermis and dermis fractions, for separate analysis.

Analysis

20. In all studies adequate recovery should be achieved (the aim should be a mean of $100 \pm 10\%$ of the radioactivity and any deviation should be justified). The amount of test substance in the receptor fluid, skin preparation, skin surface washings and apparatus rinse should be analysed, using a suitable technique.

DATA AND REPORTING

Data

21. The analysis of receptor fluid, the distribution of the test substance chemical in the test system and the absorption profile with time, should be presented. When finite dose conditions of exposure are used, the quantity washed from the skin, the quantity associated with the skin (and in the different skin layers if analysed) and the amount present in the receptor fluid (rate, and amount or percentage of applied dose) should be calculated. Skin absorption may sometimes be expressed using receptor fluid data alone. However, when the test substance remains in the skin at the end of the study, it may need to be included in the total amount absorbed (see Guidance Document, paragraph 66). When infinite dose conditions of exposure are used the data may permit the calculation of a permeability constant (Kp). Under the latter conditions, the percentage absorbed is not relevant.

Test report

22. The test report must include the requirements stipulated in the protocol, including a justification for the test system used and should, comprise the following:

Test substance:

- physical nature, physicochemical properties (at least molecular weight and log P_{ow}), purity (radiochemical purity);
- identification information (e.g. batch number);
- solubility in receptor fluid.

Test preparation:

- formulation and justification of use;
- homogeneity.

Test conditions:

- sources and site of skin, method of preparation, storage conditions prior to use, any pre-treatment (cleaning, antibiotic treatments, etc.), skin integrity measurements, metabolic status, justification of use;
- cell design, receptor fluid composition, receptor fluid flow rate or sampling times and procedures;
- details of application of test preparation and quantification of dose applied;
- duration of exposure;
- details of removal of test preparation from the skin, for example, skin rinsing;
- details of analysis of skin and any fractionation techniques employed to demonstrate skin distribution;
- cell and equipment washing procedures;
- assay methods, extraction techniques, limits of detection and analytical method validation.

Results:

- overall recoveries of the experiment (Applied dose \equiv Skin washings + Skin + Receptor fluid + Cell washings);
- tabulation of individual cell recoveries in each compartment;
- absorption profile;
- tabulated absorption data (expressed as rate, amount or percentage).

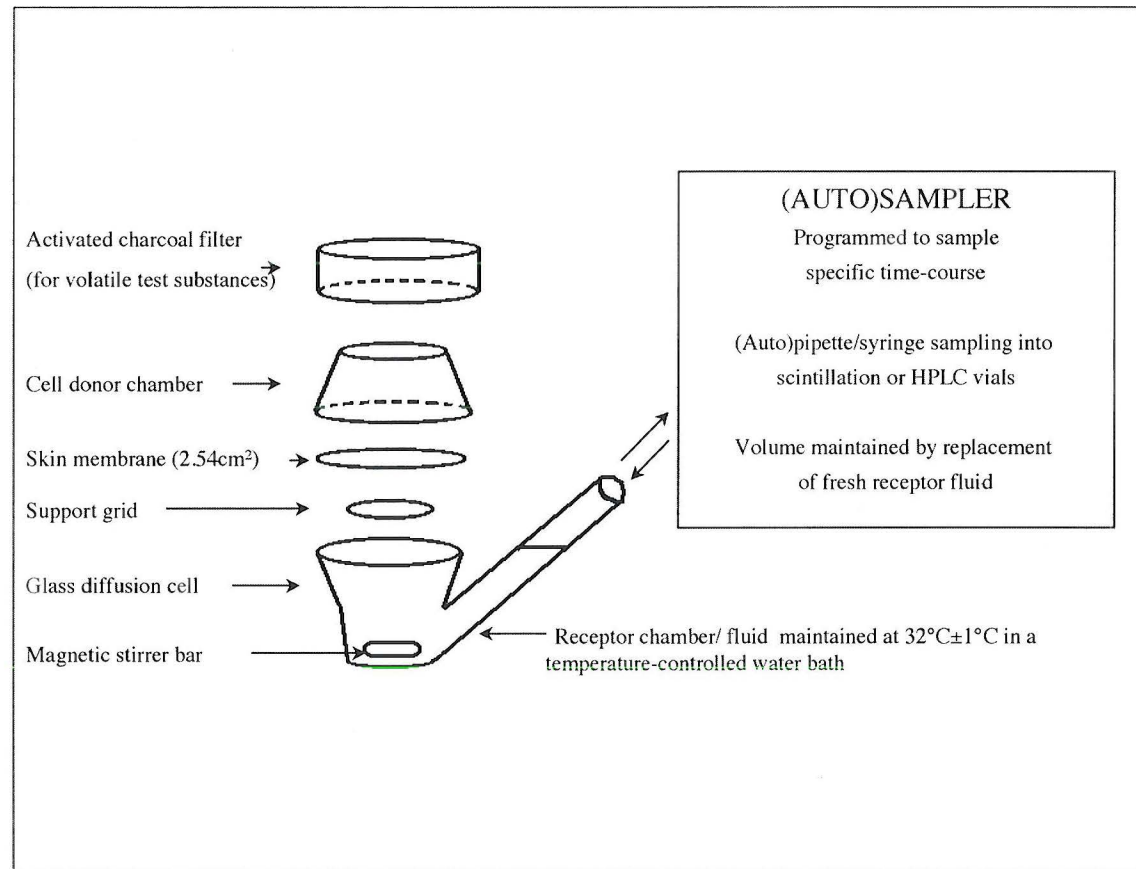
Discussion of results.

Conclusions.

LITERATURE

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Figure 1: An example of a Typical Design of a Static Diffusion Cell for *in vitro* Percutaneous Absorption Studies



ANNEXDEFINITIONS

Unabsorbed dose: represents that washed from the skin surface after exposure and any present on the non-occlusive cover, including any dose shown to volatilise from the skin during exposure.

Absorbed dose: (*in vitro*): mass of test substance reaching the receptor fluid or systemic circulation within a specified period of time.

The absorbable dose: (*in vitro*) represents that present on or in the skin following washing.