OECD/OCDE 427

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Skin Absorption: *in vivo* Method

INTRODUCTION

1. Exposure to many chemicals occurs mainly *via* the skin whilst the majority of toxicological studies performed in laboratory animals use the oral route of administration. The *in vivo* percutaneous absorption study set out in this guideline provides the linkage necessary to extrapolate from oral studies when making safety assessments following dermal exposure.

2. A substance must cross a large number of cell layers of the skin before it can reach the circulation. The rate-determining layer for most substances is the *stratum corneum* consisting of dead cells. Permeability through the skin depends on the lipophilicity of the chemical and the thickness of the outer layer of epidermis, as well as on factors such as molecular weight and concentration of the substance. In general, the skin of rats and rabbits is more permeable than that of humans, whereas the skin permeability of guinea pigs, pigs and monkeys is more similar to that of humans.

INITIAL CONSIDERATIONS

3. The methods for measuring percutaneous absorption can be divided into two categories; *in vivo* and *in vitro*. The *in vivo* method is capable of providing good information, in various laboratory species, on skin absorption. More recently *in vitro* methods have been developed. These utilise transport across full or partial thickness animal or human skin to a fluid reservoir. The *in vitro* method is described in a separate OECD Test Guideline (1). It is recommended that the OECD Guidance Document for the Conduct of Skin Absorption Studies (2) be consulted to assist in the selection of the most appropriate method in the given situation, as it provides more details on the suitability of both *in vivo* and *in vitro* methods.

4. The *in vivo* method, described in this guideline, allows the determination of the penetration of the test substance through the skin into the systemic compartment. The technique has been widely used for many years (3)(4)(5)(6)(7). Although *in vitro* percutaneous absorption studies may in many cases be appropriate there may be situations in which only an *in vivo* study can provide the necessary data

5. Advantages of the *in vivo* method are that it uses a physiologically and metabolically intact system, uses a species common to many toxicity studies and can be modified for use with other species. The disadvantages are the use of live animals, the need for radiolabelled material to facilitate reliable results, difficulties in determining the early absorption phase and the differences in permeability of the preferred species (rat) and human skin. Animal skin is generally more permeable and therefore may overestimate human percutaneous absorption (6)(8)(9). Caustic/corrosive substances should not be tested in live animals.

PRINCIPLE OF THE TEST

6. The test substance, preferably radiolabelled, is applied to the clipped skin of animals at one or more appropriate dose levels in the form of a representative in-use preparation. The test preparation is

allowed to remain in contact with the skin for a fixed period of time under a suitable cover (non occlusive, semi occlusive, or occlusive) to prevent ingestion of the test preparation. At the end of the exposure time the cover is removed and the skin is cleaned with an appropriate cleansing agent, the cover and the cleansing materials are retained for analysis and a fresh cover applied. The animals are housed prior to, during and after the exposure period in individual metabolism cages and the excreta and expired air over these periods are collected for analysis. The collection of expired air can be omitted when there is sufficient information that little or no volatile radioactive metabolite is formed. Each study will normally involve several groups of animals that will be exposed to the test preparation. One group will be killed at the end of the exposure period. Other groups will be killed at scheduled time intervals thereafter (2). At the end of the sampling time the remaining animals are killed, blood is collected for analysis, the application site removed for analysis and the carcass is analysed for any unexcreted material. The samples are assayed by appropriate means and the degree of percutaneous absorption is estimated (6)(8)(9).

DESCRIPTION OF THE METHOD

Selection of animal species

7. The rat is the most commonly used species, but hairless strains and species having skin absorption rates more similar to those of human, can also be used (3)(6)(7)(8)(9). Young adult healthy animals of a single sex (with males as the default sex) of commonly used laboratory strains should be employed. At the commencement of the study, the weight variation of animals used should not exceed ± 20% of the mean weight. As an example, male rats of 200g –250g are suitable, particularly in the upper half of this range.

Number and sex of animals

8. A group of at least four animals of one sex should be used for each test preparation and each scheduled termination time. Each group of animals will be killed after different time intervals, for example at the end of the exposure period (typically 6 or 24 hours) and subsequent occasions (e.g. 48 and 72 hours). If there are data available that demonstrate substantial differences in dermal toxicity between males and females, the more sensitive sex should be chosen. If there are no such data, then either gender can be used.

Housing and feeding conditions

9. The temperature in the experimental animal room should be 22°C (± 3°C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used and should be freely available together with an unlimited supply of drinking water. During the study, and preferably also during the acclimatisation, the animals are individually housed in metabolism cages. Since food and water spillage would compromise the results, the probability of such events should be minimised.

Preparation of animals

10. The animals are marked to permit individual identification and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

11. Following the acclimatisation period, (and approximately 24 hours prior to dosing), each animal will have an area of skin in the region of the shoulders and the back clipped. The permeation properties of damaged skin are different from intact skin and care should be taken to avoid abrading the skin. Following

the clipping (approximately 24 hrs before the test substance is applied to the skin, see paragraph 14) the skin surface is gently wiped with acetone to remove sebum. An additional soap and water wash is not recommended because any soap residue might promote test substance absorption. The area must be large enough to allow reliable calculation of the absorbed amount of test chemical per cm^2 skin, preferably at least 10 cm^2. This area is practicable with rats of 200 – 250 g bodyweight. After preparation, the animals are returned to metabolism cages.

Test substance

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

Test preparation

13. The test substance preparation (e.g., neat, diluted or formulated material containing the test chemical 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. Where necessary, the test substance is dissolved or suspended in a suitable vehicle. For vehicles other than water the absorption characteristics and potential interaction with the test substance should be known.

Application to the skin

14. An application site of a specific surface area is defined on the skin surface. A known amount of the test preparation is then evenly applied to the site. This amount should normally mimic potential human exposure, typically 1-5 mg/cm^2 for a solid or up to 10 $\mu l/cm^2$ for liquids. Any other quantities should be justified by the expected use conditions, the study objectives or physical characteristics of the test preparation. Following application, the treated site must be protected from grooming. An example of a typical device is shown in Figure 1. Normally, the application site will be protected by a non-occlusive cover (e.g. a permeable nylon gauze cover). However, for infinite applications the application site should be occluded. In case evaporation of semi-volatile test substances reduces the recovery rate of the test substance to an unacceptable extend (see also paragraph 20), it is necessary to trap the evaporated test substance in a charcoal filter covering the application device (see Figure 1). It is important that any device does not damage the skin, nor absorb or react with the test preparation. The animals are returned to individual metabolism cages in order to collect excreta.

Duration of exposure and sampling

15. The duration of exposure is the time interval between application and removal of test preparation by skin washing. A relevant exposure period (typically 6 or 24 hours) should be used, based on the expected human exposure duration. Following the exposure period, the animals are maintained in the metabolism cages until the scheduled termination. The animals should be observed for signs of toxicity/abnormal reactions at regular intervals for the entire duration of the study. At the end of the exposure period the treated skin should be observed for visible signs of irritation.

16. The metabolism cages should permit separate collection of urine and faeces throughout the study. They should also allow collection of ^{14}C-carbon dioxide and volatile ^{14}C-compounds, which should be analysed when produced in quantity (>5%). The urine, faeces and trap fluids (e.g. ^{14}C-carbon dioxide and volatile ^{14}C-compounds) should be individually collected from each group at each sampling time. If there is sufficient information that little or no volatile radioactive metabolite is formed, open cages can be used.

17. Excreta are collected during the exposure period, up to 24 hours after the initial skin contact and then daily until the end of the experiment. Whilst three excreta collection intervals will normally be sufficient, the envisaged purpose of the test preparation or existing kinetic data may suggest more appropriate or additional time points for study.

18. At the end of the exposure period the protective device is removed from each animal and retained separately for analysis. The treated skin of all animals should be washed at least 3 times with a cleansing agent using suitable swabs. Care must be taken to avoid contaminating other parts of the body. The cleansing agent should be representative of normal hygiene practice, for example an aqueous soap solution. Finally, the skin should be dried. All swabs and washings must be retained for analysis. A fresh cover should be applied to protect the treated site of those animals forming the later time point groups prior to their return to individual metabolism cages.

Terminal procedures

19. For each group, the individual animals should be killed at the scheduled time and blood collected for analysis. The protective device or cover should be removed for analysis. The skin from the application site and a similar area of non-dosed, clipped skin should be removed from each animal for separate analysis. The application site may be fractionated to separate the *stratum corneum* from the underlying epidermis to provide more information on the test chemical disposition. The determination of this deposition over a time course after the exposure period should provide some indication of the fate of any test chemical in the *stratum corneum.* To facilitate skin fractionation (following the final skin wash and killing the animal) each protective cover is removed. The application site skin, with an annular ring of surrounding skin, is excised from the rat and pinned on a board. A strip of adhesive tape is applied to the skin surface using gentle pressure and the tape removed together with part of the *stratum corneum.* Successive strips of tape are applied until the tape no longer adheres to the skin surface, when all of the *stratum corneum* has been removed. For each animal, all of the tape strips may be combined in a single container to which a tissue digestant is added to solubilise the *stratum corneum.* Any potential target tissues may be removed for separate measurement before the residual carcass is analysed for absorbed dose. The carcasses of the individual animals should be retained for analysis. Usually analysis of the total carcass content will be sufficient. Target organs may be removed for separate analysis (if indicated by other studies). Urine present in the bladder at scheduled kill should be added to the previous urine collection. After collection of the excreta from the metabolism cages at the time of scheduled kill, the cages and their traps should be washed with an appropriate solvent. Other potentially contaminated equipment should likewise be analysed.

Analysis

20. In all studies adequate recovery (i.e., mean of 100 ± 10% of the radioactivity) should be achieved. Recoveries outside this range must be justified. The amount of the administered dose in each sample should be analysed by suitably validated procedures.

21. Statistical considerations should include a measure of variance for the replicates for each application.

DATA AND REPORTING

Data

22. The following measurements should be made for each animal, at each sampling time for test chemical and/or metabolites. In addition to individual data, data grouped according to sampling times should be reported as means.

- quantity associated with the protective appliances;
- quantity that can be dislodged from the skin;
- quantity in/on skin that cannot be washed from the skin;
- quantity in the sampled blood;
- quantity in the excreta and expired air (if appropriate);
- quantity remaining in the carcass and any organs removed for separate analysis.

23. The quantity of test substance and/or metabolites in the excreta, expired air, blood and in the carcass will allow determination of the total amount absorbed at each time point. A calculation of the amount of test chemical absorbed per cm^2 of skin exposed to the test substance over the exposure period can also be obtained.

Test report

24. 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:

- identification data [e.g., CAS number, if available; source; purity (radiochemical purity); known impurities; lot number];
- physical nature, physicochemical properties (e.g., pH, volatility, solubility, stability, molecular weight, and log P_{ow});

Test preparation:

- formulation and justification of use;
- details of test preparation, amount applied, achieved concentration, vehicle, stability and homogeneity;

Test animals:

- species/strain used;
- number, age and sex of animals;
- source of animals, housing conditions, diet, etc.;
- individual animal weights at start of test;

Test conditions:

- details of the administration of the test preparation (site of application, assay methods, occlusion/non-occlusion, volume, extraction, detection);
- details of food and water quality.

Results:

- any signs of toxicity;
- tabulated absorption data (expressed as rate, amount or percentage);
- overall recoveries of the experiment;
- interpretation of the results, including comparison with any available data on percutaneous absorption of the test compound.

Discussion of results.

Conclusions.

LITERATURE

(1) OECD (2004). Test Guideline 428: Skin absorption: *in vitro* Method. OECD, Paris.

(2) OECD (2004). Guidance Document for the Conduct of Skin Absorption Studies. OECD, Paris.

(3) ECETOC (1993) Percutaneous Absorption. European Centre for Ecotoxicology and Toxicology of Chemicals, Monograph No. 20.

(4) Zendzian RP (1989) Skin Penetration Method suggested for Environmental Protection Agency Requirements. *J. Am. Coll. Toxicol*. 8 (5), 829-835.

(5) Kemppainen BW, Reifenrath WG (1990) Methods for skin absorption. CRC Press Boca Raton, FL, USA.

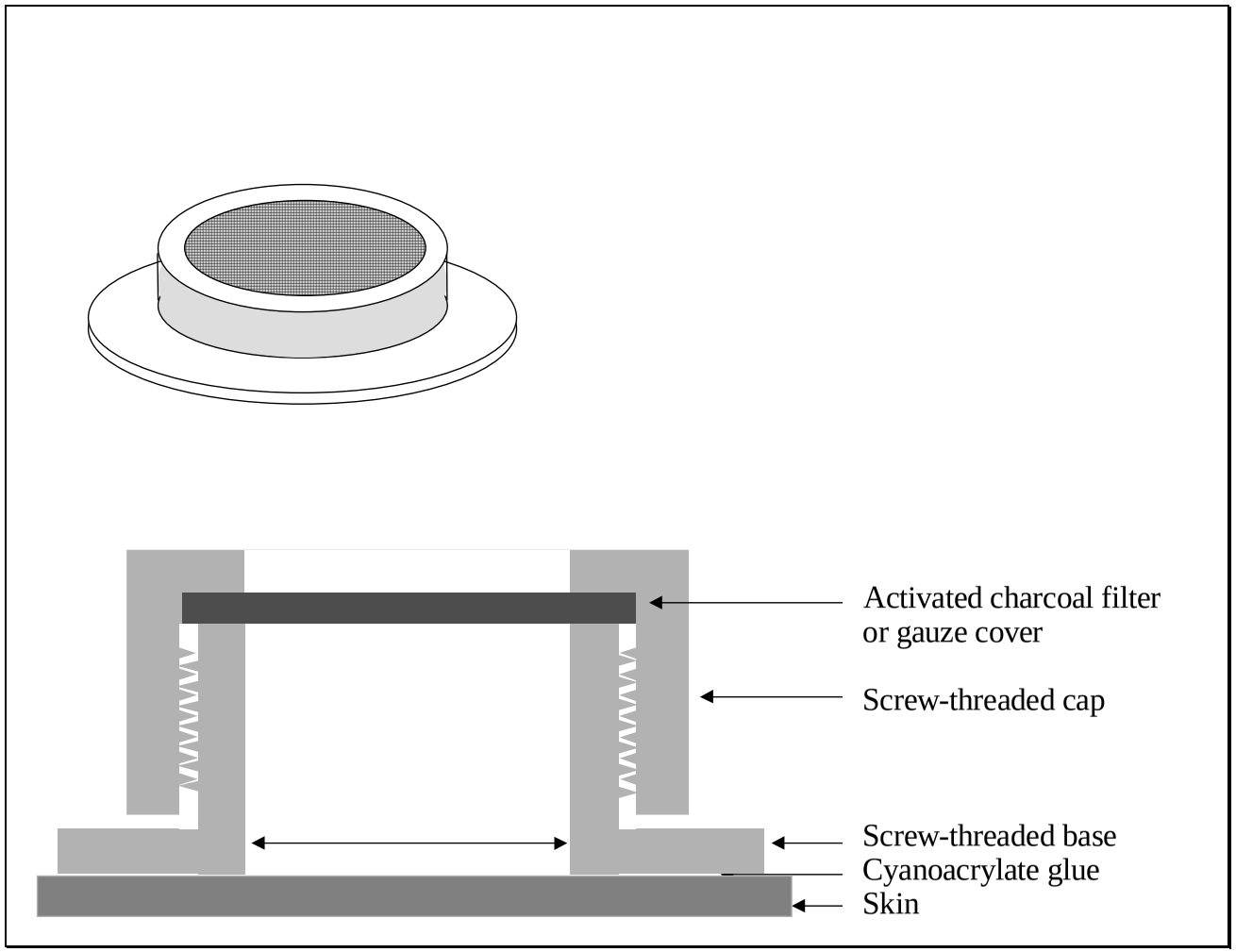
(6) EPA (1992) Dermal Exposure Assessment: Principles and Applications. Exposure Assessment Group, Office of Health and Environmental Assessment.

(7) EPA (1998) Health Effects Test Guidelines, OPPTS 870-7600, Dermal Penetration. Office of Prevention, Pesticides, and Toxic Substances,

(8) Bronaugh RL, Wester RC, Bucks D, Maibach HI and Sarason R (1990) In vivo percutaneous absorption of fragrance ingredients in rhesus monkeys and humans. Fd. Chem. Toxic. 28, 369-373.

(9) Feldman RJ and Maibach HI (1970) Absorption of some organic compounds through the skin in man. J. Invest. Dermatol. 54, 399-404.

Figure 1: An Example of a Design of a Typical Device used to Define and Protect Dermal Application Site during *in vivo* Percutaneous Absorption Studies



ANNEX

DEFINITIONS

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 vivo***) comprises that present in urine, cage wash, faeces, expired air (if measured), blood, tissues (if collected) and the remaining carcass, following removal of application site skin.

The absorbable dose: represents that present on or in the skin following washing.