OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE

In Vitro Skin Irritation: Human Skin Model Test

INTRODUCTION

1. Skin irritation refers to the production of reversible damage to the skin following the application of a test substance for up to 4 hours (1).

The assessment of skin irritation has typically involved the use of laboratory animals (1). Concern for the pain and suffering involved with this procedure has been addressed in the revision of Test Guideline 404 that allows for the determination of skin corrosion/irritation by using alternative, *in vitro* methods, avoiding pain and suffering of animals.

3. The Test Guideline presented here does not require the use of live animals or animal tissue for the assessment of skin irritation. It is based on human reconstructed tissue models which in their overall design (the use of human skin-derived keratinocytes as cell source, representative tissue and cytoarchitecture) closely mimic the biochemical and physiological properties of the upper parts of the human skin, i.e. the epidermis. The high relevance of the model to the human situation avoids the problem of inter-species (animal/human) differences encountered with the traditional animal test.

DEFINITIONS

4. Definitions used are provided in the Annex.

INITIAL CONSIDERATIONS

5. Prevalidation and validation studies (2, 3, 4, 5, 6, 7, 8, 9, 10) have reported that in vitro tests employing reconstructed human skin models are able to reliably discriminate between known skin irritants and non-irritants according to the EU classification system; R38, no label (11).

6. The test described in this method allows the hazard identification of irritant substances of high purity (10). It does not provide adequate information on skin corrosion, nor does it allow the sub-categorization of irritating substances as defined in the Globally Harmonized Classification System (GHS).

7. For a full evaluation of local skin effects after single dermal exposure, it is recommended to follow the sequential testing strategy as appended to Test Guideline 404 (1) and provided in the Globally Harmonized System (12). This testing strategy includes the conduct of in vitro tests for skin corrosion and skin irritation (as described in this document) before considering the necessity of any exceptional testing in living animals. It should be noted that the test method based on the EPISKIN™ assay allows the prediction of both irritant and non-irritant substances and can thus be considered as a stand alone method to be used as replacement for the animal test. (13).

PRINCIPLE OF THE TEST

8. The principle of the in vitro skin model irritation assay is based on the premise that irritant chemicals are able to penetrate the stratum corneum by diffusion and are cytotoxic to the cells in the underlying layers. Moreover, if the cytotoxic effect is absent or weak, a quantifiable amount of inflammatory mediators is released by the epidermis and may be used in a tiered approach to increase the sensitivity of the test.

9. The test material is applied topically to a three-dimensional human epidermal model, comprised of at least a reconstructed epidermis with several epidermal cell layers and a stratum corneum with barrier function. Irritant materials are identified by their ability to decrease cell viability below defined threshold levels (e.g. 50%). As an additional measure of skin irritation, release of inflammatory mediators (e.g. Interleukin 1 alpha) may be determined.

10. In vitro human skin model systems for skin irritation testing may be used to test solids, liquids, semi-solids and waxes. The liquids may be aqueous or non aqueous; solids may be soluble or insoluble in water. Solids should be ground to a fine powder before application. Since 58 carefully selected chemicals representing a wide spectrum of chemical classes were included in the validation of the in vitro human skin model test system for skin irritation, the method is expected to be generally applicable across chemical classes except for gases and aerosols.

PROCEDURE

Human skin models

11. Human skin models can be obtained commercially (e.g. EpiDerm™ and EPISKIN™ models) or be developed or constructed in the testing laboratory. Any new model should be validated and at least comply with the following performance standards:

General model conditions:

12. Human keratinocytes should be used to construct the epithelium. Multiple layers of viable epithelial cells (basal layer, stratum spinosum, stratum granulosum) should be present under a functional stratum corneum. Stratum corneum should be multilayered containing the essential lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic markers chemicals, e.g. Sodium Lauryl Sulphate (SLS)] or Triton X-100. This property may be estimated by the determination of IC_{50} or ET_{50} after application of an established cytotoxic marker chemical. The containment properties of the model should prevent the passage of material around the stratum corneum to the viable tissue, which would lead to poor modelling of the exposure to skin. The skin model should be free of contamination by bacteria, mycoplasma, or fungi.

Functional model conditions*:*

13. Viability: The magnitude of viability is usually quantified by using MTT (14) or other metabolically converted vital dyes. In these cases the optical density (OD) of the extracted (solubilised) dye from the negative control tissue should be at least 20 fold greater than the OD of the extraction solvent alone. It should be documented that the negative control tissue is stable in culture (provide similar viability measurements) for the duration of the test exposure period.

14. Barrier function: The stratum corneum (SC) and its lipid composition should be sufficient to resist the rapid penetration of cytotoxic marker chemicals, e.g. SDS or Triton X-100. This property can be estimated either by determination of the concentration at which a marker chemical reduces the viability of the tissues by 50% (IC_{50}) after a fixed exposure time, or by determination of the exposure time required to reduce cell viability by 50% (ET_{50}) upon application of the marker chemical at a specified, fixed concentration.

15. Morphology: An on-going histological examination of the reconstructed skin/epidermis should be performed, showing human skin/epidermis-like structure (including functional stratum corneum).

16. Reproducibility: The results of the method using a specific model should demonstrate reproducibility over time and between laboratories. The model must be capable to demonstrate correct prediction of Reference Chemicals over an extended time period.

17. Quality controls (QC) of the model: Each batch of the epidermal model used must meet defined production release criteria, among which those for *viability* (cf. 13) and for *barrier function* (cf.14) are the most relevant. An acceptability range (upper and lower limit) for the IC_{50} or the ET_{50} must be established by the skin model supplier (or investigator when using an in-house model). Only results produced with qualified tissues can be accepted for reliable prediction of irritation effects. As an example, the acceptability ranges for EPISKIN and EpiDerm are given below:

Table 1: Examples of QC batch release criteria

	lower acceptance limit	**mean of acceptance range**	**upper acceptance limit**
EPISKIN (18 h SLS)	IC50 = 1.0 mg/ml	IC50 = 2.32 mg/ml	IC50 = 3.0 mg/ml
EpiDerm (1% Triton X100)	ET50 = 4.8 hr	ET50 = 6.7 hr	ET50 = 8.7 hr

Application of the test and control substances

18. A sufficient number of tissue replicates should be used for each treatment and for controls (at least two if demonstrated statistically significant and if compliant with the method performance). For liquid as well as solid materials, sufficient amount of test substance must be applied to uniformly cover the skin surface, i.e., a minimum of 25 •L/cm^2 or (25 mg/cm^2) should be used. For solid substances, the epidermis surface should be moistened with deionised or distilled water before application, to ensure good contact with the skin. If appropriate, solids should be ground to a powder before application. At the end of the exposure period, the test material must be carefully washed from the skin surface with an appropriate buffer, or 0.9% NaCl.

19. Concurrent negative controls (NC) and positive controls (PC) should be used for each study to demonstrate that viability (NC), barrier function and resulting tissue sensitivity (PC) of the tissues are within a defined historical acceptance range. The suggested positive control substance is 5% SLS. The suggested negative control substances are water or PBS.

Cell viability measurements

20. The most important element of the test procedure is that viability measurements are not performed immediately after the exposure to the test chemicals, but after a sufficiently long post-treatment incubation period of the rinsed tissues in fresh medium. This period allows both for recovery from weakly irritant effects and for appearance of clear cytotoxic effects. During the test optimisation phase (3-6), a 42 hr post-incubation period proved to be optimal and was therefore used in the ECVAM SIVS.

21. Only quantitative, validated methods can be used to measure cell viability. Furthermore, the measure of viability must be compatible with use in a three-dimensional tissue construct. Non-specific phenomena (e.g. dye binding, protein binding, reagent interaction, etc.) must not interfere with the viability measurement process.

22. The most frequently used assay is MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; EINECS number 206-069-5, CAS number 298-93-1)] reduction (14), which has been shown to give accurate and reproducible results. The skin sample is placed in MTT solution of appropriate concentration (e.g. 0.3 – 1 mg/mL) for 3 hours. The precipitated blue formazan product is then extracted using a solvent (e.g. isopropanol, acidic isopropanol), and the concentration of formazan is measured by determining the Optical Density (OD) at a wavelength between 540 and 595 nm (preferably 570 nm).

23. Optical properties of the test material or its chemical action on the vital dye may mimic the effect of cellular metabolism leading to a false estimate of viability (because the reaction may prevent or reverse the colour generation as well as causing it). This may occur when a specific test material is not completely removed from the skin by rinsing or when it penetrates the epidermis. If the test material acts directly on the vital dye or is naturally coloured, additional controls should be used to detect and correct for test substance interference with the viability measurement technique. Non specific colour (NSC) due to these interferences should not exceed 30% of the negative control (for corrections), if NSC > 30%, the test chemical is considered as incompatible with the test.

Quality criteria

24. For each assay using valid batches, negative control (NC) tissues should exhibit OD reflecting the quality of the tissues that followed all shipment and receipt steps and all the irritation protocol process. Control OD values should not be below historical established lower boundaries. Similarly positive control (PC) tissues treated with 5% aq. SLS should reflect the sensitivity retained by tissues and their ability to respond to an irritant chemical in the conditions of each individual assay (e.g. viability < 40% for EPISKIN and < 20% for EpiDerm). Associated standard deviations should be defined (e.g. SD $\leq$ 18% for EPISKIN and EpiDerm).

Interpretation of results

25. The optical density (OD) values obtained with each test sample can be used to calculate the percentage of viability compared to the negative control, which is set at 100%. The cut-off value of percentage cell viability distinguishing irritant from non-irritant test materials and the statistical procedure(s) used to evaluate the results and identify irritant materials, must be clearly defined and documented, and proven to be appropriate. For example, the cut-off values for the prediction of irritation associated with the EPISKIN and EpiDerm models were established during prevalidation and test optimisation studies. These were confirmed by the ECVAM SIVS and are given below:

26. The test substance is considered to be irritant to skin:

i) if the tissue viability after exposure and post-treatment incubation is less than or equal ($\leq$) to 50%.

Complementary endpoints

27. In response to physical or chemical stress, keratinocytes produce and release inflammatory cytokines interleukins [IL-1α, tumor necrosis factor α (TNF-a)], chemotactic cytokines [IL-8, interferon, e.g. induced protein 10 (IP-10)], growth-promoting factor [IL-6, IL-7, IL-15, granulocyte/macrophage colony-stimulating factor GM-CSF)], transforming growth factor [TGF], cytokines regulating humoral versus cellular immunity [IL-10, IL-12] and other signalling factors, which rapidly generate cutaneous inflammation, suggesting that measurement of such keratinocyte responses may allow the evaluation of toxicological properties of chemicals in order to identify irritants (15).

28. In the first and second phases of the ECVAM SIVS, IL-1α release into the assay medium was evaluated as a promising complementary endpoint to the classic MTT cytotoxicity test (14). It was proven during the study that MTT is a more robust endpoint than IL-1 alpha (8). Although IL-1 alpha proved to be useful to acquire additional information on the irritant potential of chemicals, only results from the MTT assay are currently used for classification and labelling according to the EU classification system. Further investigations are on-going to determine the reproducibility of the IL-1 alpha assay to allow combination of two endpoints for more reliable prediction of irritancy.

Example of Interleukin 1 alpha (IL-1α) measurements in the EPISKIN model

29. For epidermis tissues showing a cell viability > 50%, the amount of IL-1α released into the tissue culture medium at the end of the post-treatment incubation period (after 42h post-treatment incubation) is measured in the medium (immediately or frozen) using ELISA (16, 17).

30. The test substance is considered to be an irritant to skin**:**

i) if the viability after 15 minutes of exposure and 42 hours of post incubation is more (>) than 50%, and the amount of IL-1α release is more (>) than 9.18 IU/ml. (If the negative control value is more (>) than 1,6 IU/ml, it is recommended to subtract the negative control. In that case, the cut off value is set to 7.65 IU/ml). These values are specific to the EPISKIN model and can differ for other models.

31. The test substance is considered to be non irritant to skin:

i) if the viability after 15 minutes of exposure and 42 hours of post incubation is more (>) than 50%, and the amount of IL-1α release is less or equal (≤) to 9.18 IU/ml. (If the negative control value is more (>) than 1,6 IU/ml, it is recommended to subtract the negative control. In that case, the cut off value is set to 7.65 IU/ml). These values are specific to the EPISKIN model and can differ for other models.

DATA AND REPORTING

Data

32. For each treatment, data from individual replicate test samples (e.g., OD values and calculated percentage cell viability data for each test chemical, including positive and negative classification) must be reported in tabular form, including data from repeat experiments as appropriate. In addition means ± standard deviation for each trial should be reported. Observed interactions with MTT reagent and eventually IL-1α values, if appropriate, must be reported for each tested chemical.

Test report

33. The test report must include the following information:

Test and Control Substances:

Chemical name(s) such as IUPAC or CAS name and CAS number, if known;

Purity and composition of the substance or preparation (in percentage(s) by weight);

-Physical-chemical properties such as physical state, volatility, pH, stability, water solubility relevant to the conduct of the study;

-Treatment of the test/control substances prior to testing, if applicable (e.g., warming, grinding);

Stability, if known.

Justification of the skin model and protocol used.

Test Conditions

- Cell system used;
- Calibration information for measuring device used for measuring cell viability (e.g., spectrophotometer);
- Complete supporting information for the specific skin model used including its validity. This should include, but is not limited to:
  - i) Viability
  - ii) Barrier function
  - iii) Morphology
  - iv) Reproducibility
  - v) Quality controls (QC) of the model
- Details of the test procedure used;
- Test doses used;
- Description of any modifications of the test procedure;
- Reference to historical data of the model. This should include, but is not limited to:
  - i) acceptability of the QC data with reference to historical batch data
  - ii) acceptability of the positive and negative control values with reference to positive and negative control means and ranges.
- Description of evaluation criteria used including the justification for the selection of the cut-off point(s) for the prediction model

Results:

- Tabulation of data from individual test samples;
- Description of other effects observed.

Discussion of the results.

Conclusion.

LITERATURE

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287 **Table 2: Reference Chemicals**

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Chemical Name	CAS Number	EINECS No	EU label
1-bromo-4-chlorobutane	6940-78-9	230-089-3	**no**
diethyl phthalate	84-66-2	201-550-6	**no**
di-propylene glycol	25265-71-8	246-770-3	**no**
naphthalene acetic acid	86-87-3	201-705-8	**no**
allyl phenoxy-acetate	7493-74-5	231-335-2	**no**
isopropanol	67-63-0	200-661-7	**no**
4-methyl-thio-benzaldehyde	3446-89-7	222-365-7	**no**
methyl stearate	112-61-8	203-990-4	**no**
allyl heptanoate	142-19-8	205-527-1	**no**
heptyl butyrate	5870-93-9	227-526-5	**no**
hexyl salicylate	6259-76-3	228-408-6	**R38**
terpinyl acetate	80-26-2	201-265-7	**R38**
tri-isobutyl phosphate	126-71-6	204-798-3	**R38**
1-decanol	112-30-1	203-956-9	**R38**
cyclamen aldehyde	103-95-7	203-161-7	**R38**
1-bromohexane	111-25-1	203-850-2	**R38**
a-terpineol	98-55-5	202-680-6	**R38**
di-n-propyl disulphide	629-19-6	211-079-8	**R38**
butyl methacrylate	97-88-1	202-615-1	**R38**
heptanal	111-71-7	203-898-4	**R38**

The chemicals listed in table 1 provide a representative distribution of the 58 chemicals used in the ECVAM international skin irritation validation study (10, 18). Their selection is based on the following criteria:

1. the chemicals are commercially available

2. they are representative of the range of irritant responses (from non-irritant to strong irritant) that the validated *in vitro* test method is capable of predicting

3. they have a well-defined chemical structure

4. they are representative of the validated method's reproducibility and predictive capacity as determined in the ECVAM validation study

5. they include classification based on both endpoints (MTT and IL-1α release)

6. they are representative of the chemical functionality used in the validation process

7. they are not associated with an extremely toxic profile (e.g. carcinogenic or toxic to the reproductive system)

8. and they are not associated with prohibitive disposal costs.

Because the Reference Chemicals are a sub-set of the chemicals used in the SIVS, several additional selection criteria were applied by the ECVAM Chemical Selection Sub Committee (CSSC) in the selection process of test chemicals used in the ECVAM SIVS (10). These comprise e.g. exclusion of rapidly polymerizing and hydrolyzing chemicals, chemical gases and aerosols.

ANNEX

DEFINITIONS

Skin irritation is the production of reversible damage to the skin following the application of a test substance for up to 4 hours.

Skin irritation is a locally arising, non-immunogenic reaction, which appears shortly after stimulation (19). Its main characteristic is its reversible process involving inflammatory reactions and most of the clinical characteristic signs of irritation (erythema, oedema, redness, itching and pain) related to an inflammatory process.

Cell viability: parameter measuring total activity of a cell population e.g., as ability of cellular mitochondrial dehydrogenases to reduce the vital dye MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue;)**,** which depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

Interleukin 1 alpha release: parameter measuring the release of Interleukin 1 alpha**,** a vertebrate cytokine that is especially important in inducing inflammatory responses Human keratinocytes express and release large amounts of IL-1α.