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SCCP

Memorandum
Classification and categorization of skin sensitisers and grading of test reactions

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1. INTRODUCTION

Animal and human assays for skin sensitisation are used in research and by industry. They are used in hazard identification and risk assessment for regulatory purpose (Directives on Cosmetics 76/768/EEC, Dangerous Substances 67/548/EEC, Dangerous Preparations 1999/45/EC) (ref. 20, 21, 22).

Guideline methods are described for animal assays (ref OECD 406, OECD 429) and international recommendations for human assays (ref Wahlberg and Lindberg, Johansen et al). Deviations from the guideline methods are frequent; interpretation of results and terminology are often not harmonised. Some key issues for interpretation of the quality and results of tests are addressed below.

Allergic contact dermatitis is the clinical disease caused by skin exposure to skin sensitising substances (contact allergens). Skin exposure of humans and test animals to contact allergens may cause *induction* of contact allergy (sensitisation). Re-exposure of a sensitised individual or animal to the substance may result in *elicitation* of a response (allergic contact dermatitis or positive test reaction). The dose sufficient for induction is generally larger than the dose sufficient for elicitation. To avoid allergic contact dermatitis the sensitised individual has to avoid further exposure to the substance at levels causing elicitation.

The scope of this memorandum is to clarify issues essential for interpretation of data and dossier results in risk assessment of potential skin sensitisers.

2. ANIMAL ASSAYS

The animal test methods used in harmonised classification of substances, according to their potential to cause skin sensitisation, are the guinea pig maximization test (GPMT), the Buehler test, and the local lymph node assay (LLNA) (ref OECD 406, OECD 429, EU B.6, EU B.42, UNECE GHS).

2.1. Guinea pig maximization test (GPMT)

The test method is described in OECD guideline 406 (ref OECD 406, EU B.6).

Principle: The test animals (guinea pig) are repeatedly exposed to the test substance by intradermal injection, with and without Freund's Complete Adjuvant, and topical application by occlusion (induction exposure). Following a rest period of 14 days (induction period), the animals are exposed to a challenge dose by occlusion. The skin reaction to the challenge exposure in the test animals is compared with the reaction in control animals.

It should be noted that it is essential to use the dose levels prescribed in the guideline.

Dose levels and number of animals, according to OECD guideline 406:

- The concentration of test substance used for each induction exposure (intradermal and topical) should be well-tolerated systemically and should be the highest to cause mild-to-

moderate skin irritations. If the substance is not a skin irritant, the test area is pre-treated with sodium lauryl sulfate (SLS) (10% in petrolatum) in order to create local irritation.

- The concentration used for the challenge exposure should be the highest non-irritant dose.
- A minimum of 10 animals in the treatment group and at least 5 animals in the control group.
- It should be noted that it is essential to use the dose levels prescribed. To perform a pilot study is mandatory. The test report must include information on the vehicle and concentrations used for induction and challenge exposures, the result of the pilot study for determination of concentrations, the number of animals, the results, etc. according to guideline 406.

A development of the GPMT with multiple dose design has been described, suitable for dose-response studies (ref Andersen et al.); this provides additional information.

Deviations from the OECD guideline 406 should be justified.

The guideline grading scale for challenge patch test reactions is shown in Table 1.

Table 1: Grading scale for the evaluation of challenge patch test reactions in the GPMT and Buehler test (ref OECD 406)

Morphology grade	*Morphology*
0	No visible change
1	Discrete or patchy erythema
2	Moderate and confluent erythema
3	Intense erythema and swelling

The original description of grading and classes of skin sensitisers, based on the sensitisation rate shown by challenge, after intradermal and topical induction in the GPMT, is shown in Table 2.

Table 2: Original categorisation in grades and classes based on sensitisation rate in the GPMT (Ref Magnusson and Kligman 1969, Magnusson and Kligman 1970, Wahlberg and Boman)

Sensitisation rate (%)	*Sensitisation grade*	*Sensitisation class*
0-8	I	Weak
9-28	II	Mild
29-64	III	Moderate
65-80	IV	Strong
81-100	V	Extreme

It should be noted that the GPMT cannot prove that a substance is a non-sensitiser.

2.2. Buehler test

The OECD guideline 406 describes the test method (ref OECD 406, EU B.6).

Principle: The test animals (guinea pig) are repeatedly exposed to the test substance by topical application by occlusion (induction exposure). Following a rest period of 12 days (induction period), the animals are exposed to a challenge dose by occlusion. The skin reaction to the challenge exposure in the test animals is compared with the reaction in control animals. (The method does not use adjuvant or intradermal injections.)

Dose levels and number of animals, according to OECD guideline 406:

- The concentration of test substance used for each induction exposure (topical) should be the highest to cause mild irritation.
- The concentration used for the challenge exposure should be the highest non-irritant dose.
- A minimum of 20 animals in the treatment group and at least 10 animals in the control group.
- It should be noted that it is essential to use the dose levels prescribed. To perform a pilot study is mandatory. The test report must include information on the vehicle and concentrations used for induction and challenge exposures, the result of the pilot study for determination of concentrations, the number of animals, the results, etc. according to guideline 406.

Deviations from OECD guideline 406 should be justified.

Morphology grading scale for the evaluation of challenge patch test reactions is the same as for GPMT, see Table 1 (ref OECD 406).

It should be noted that the Buehler test cannot prove that a substance is a non-sensitiser.

2.3. Local lymph node assay (LLNA)

The OECD guideline 429 describes the test method (ref OECD 429, EU B.42).

Principle: The test is performed in mice. The test substance is applied to the dorsum of the ears for 3 days. On day 5, tritiated thymidine is injected i.v. for radioactive labelling to measure cell proliferation; 5 h later, the regional lymph nodes are excised and the incorporated radioactivity is measured. An increase in lymph node cell proliferative activity compared to vehicle treated control animals, indicates sensitisation. The LLNA focuses on the induction of skin sensitisation, not elicitation.

Dose levels, vehicles and number of animals, according to OECD guideline 429:

- 3 consecutive concentrations shall be selected, based on recommendations in (ref Kimber et al).
- Vehicle: The vehicle should be selected on the basis of maximising the test concentrations and solubility whilst producing a solution/suspension suitable for application of the test substance. In order of preference, recommended vehicles are acetone/olive oil (4:1 v/v),

dimethylformamide, methyl ethyl ketone, propylene glycol, dimethyl sulphoxide. Others may be used if sufficient scientific rationale is provided. Hydrophilic materials have to be incorporated into a vehicle system which wets the skin. Wholly aqueous vehicles are to be avoided.

- A minimum of four animals per dose group, with a minimum of three concentrations plus a negative control group treated with the vehicle and a positive control group.
- It should be noted that it is essential to use the dose levels and the most suitable vehicle prescribed. To perform pilot studies is mandatory. The test report must include information on the vehicle and concentrations, the number of animals, the results, etc. according to guideline 429.

According to OECD guideline 429 a positive response in the LLNA includes a stimulation index (SI) $\geq$ 3, together with considerations of dose-response and, where appropriate, statistical significance. The estimated concentration of a substance, necessary to give a 3-fold increase in lymph node cell proliferative activity compared to vehicle-treated controls, is the EC3 value.

2.4. Use of animal test results in European legislation

Animal test results are used for regulatory purpose such as classification with R43, limitations, and approval to use. The general classification and labelling requirements for dangerous substances and preparations specifies the application of test results for this purpose (Annex VI of Directive 67/548/EEC).

According to Directives 67/548/EEC and 1999/45/EC (ref. 21, 22), substances and preparations shall be classified as sensitising and assigned the symbol “Xi”, and the risk phrase “R43 May cause sensitisation by skin contact”:

- if practical experience shows the substance or preparation to be capable of inducing a sensitisation by skin contact in a substantial number of persons (see 3.4.)
- where there are positive results from an appropriate animal test.

The default concentration value for labelling of preparations with R43 is 1%. Preparations containing >0.1% of a classified sensitiser must have the warning phrase "Contains xxx (name of the substance). May cause an allergic reaction".

533 substances are classified with R43 in Annex I of Directive 67/548/EEC. A specific concentration limit for labelling with R43 below 1% has been set for 22 of these substances. Approx. 4,000 substances have been identified as a skin sensitiser (ref De Groot). Producers and importers are obliged to classify and label substances not listed in Annex I, if they fulfil the classification criteria. The Cosmetics Directive is not using labelling with the risk phrases of Directive 67/548/EEC, while the Detergents Directive 648/2004/EC (ref. 25) does.

Positive results from animal tests sufficient to classify a substance with R43 are:

- GPMT: if at least 30% of the animals have a positive response (Grade III-V, Table 2).
- Buehler test: if at least 15% of the animals have a positive response
- LLNA: if at least a 3-fold increase in proliferative counts is induced, compared to vehicle-treated controls (stimulation index SI $\geq$ 3), together with consideration of dose-response.

In notification of new substances, the LLNA is the preferred method for measuring skin sensitisation potential in animals. When LLNA is not used, justification of alternative method (GPMT or Buehler test) should be provided (ref ECB SNIF).

The European Commission Technical Committee on Classification and Labelling nominated an expert group on skin sensitisation. The group investigated the possibility for potency categorization of classified skin sensitisers (R43), and proposed how to use data derived from the current guideline test methods (ref Basketter et al., ECB report Apr 2002, ECB report Nov 2002). The following has been proposed by the expert group (but not yet, to date of this memorandum, adopted by the regulatory authorities):

- Categorisation of substances classified with R43 into three groups according to allergen potency (extreme, strong and moderate). Such categorization is based on EC3 values in the LLNA (Table 3), on intradermal induction concentration in the GPMT, and topical induction concentration in the Buehler test (ref Basketter et al., ECB report Apr 2002, ECB report Nov 2002).
- Two additional default concentration values for labelling with R43 were proposed: >0.1% for preparations containing strong sensitisers, and >0.001% for extreme sensitisers respectively.
- Moderate and strong sensitisers should be listed on the label of preparations under relevant directives, when present in a concentration of 10 ppm or greater, and extreme skin sensitisers when present in a concentration of 1 ppm or greater.
- Where multiple animal data sets lead to different categorization of the same substance, the higher potency category should be applied. When human data indicate the need to change the potency categorization derived from animal experiments, this should be used for re-categorization into a higher potency category only.

Table 3: Potency categorization of substances classified with R43, based on LLNA (ref Basketter et al., ECB report Apr 2002, ECB report Nov 2002)

Category	*EC3 value (%)* [a]
Extreme	≤ 0.2
Strong	> 0.2 - ≤ 2
Moderate	> 2

[a] EC3 value = the estimated concentration of a chemical necessary to give a 3-fold increase in lymph node cell proliferative activity compared to vehicle-treated controls (SI ≥ 3).

3. HUMAN ASSAYS

3.1. Diagnostic patch testing

Diagnostic patch testing is the procedure used for detection of contact allergy to substances in humans (ref Wahlberg and Lindberg). Patch testing is performed in patients with dermatitis, in experimental and epidemiological studies. The test procedure is standardised.

Test substances in the European standard series for patch testing (ref Wahlberg and Lindberg) are well established concerning concentration and vehicle. Other test substances used may include commercially available screening series, other appropriately diluted substances and formulations and solid materials. Patch testing requires experience.

Exposure: 2 days

Reading: recommended on 2 days and 3 or 4 days after application of the test patches; or on 3 or 4 days and 5 or 7 days after application. The grading scale and criteria are shown in Table 4.

Table 4: Grading scale for the evaluation of diagnostic patch test reactions (ref Wahlberg and Lindberg)

Grading	*Grading symbol*	*Morphological criteria*
Negative	- or N	No visible reaction
Positive, weak	+	Erythema, infiltration, possibly papules
Positive, strong	++	Erythema, infiltration, papules, vesicles
Positive, extreme	+++	Intense erythema. Infiltration, coalescing vesicles
Doubtful	?+	Erythema only
Irritant	IR	Different appearances

3.2. Elicitation studies

A range of tests, attempting to simulate normal exposure, are used for the elicitation of allergic contact dermatitis and they are useful in risk assessment (ref Johansen et al.). Standardisation of the test methods and grading of the reactions is ongoing. The methods most frequently used are: repeated open application test (ROAT), axillary exposure test, shampoo use test, finger immersion test.

3.3. Predictive tests

Predictive human sensitisation tests involve attempts to induce a long lasting or permanent immunologic sensitisation in the individual. Due to serious ethical considerations, the SCCP shares the opinion of the former SCCNFP that predictive human sensitisation tests of potentially cutaneous sensitising cosmetic ingredients or mixtures of ingredients should not be undertaken (ref SCCNFP). However, a range of human test methods for forecasting skin sensitisation potential has been developed and have been widely used by contract laboratories (ref SCCNFP, Marzulli et al 1998).

3.4. Use of human test results in European legislation

Human test results are used for regulatory purpose such as classification with R43 (see 2.4), limitations (substances is cosmetics, chromium in cement, nickel in certain items etc.), and approval to use (ref. 23, 24). The general classification and labelling requirements for dangerous substances and preparations specifies application of test results (Annex VI of Directive 67/548/EEC).

The following evidence (practical experience) is sufficient to classify a substance with R43:

- positive data from appropriate patch testing, normally in more than one dermatological clinic, or
- epidemiological studies showing allergic contact dermatitis caused by the substance or preparation. Situations in which a high proportion of those exposed exhibit characteristic symptoms are to be looked at with special concern, even if the number of cases is small, or
- positive data from experimental studies in man.

The following is sufficient to classify a substance with R43 when there is supportive evidence:

- isolated episodes of allergic contact dermatitis, or
- epidemiological studies where chance, bias or confounders have not been ruled out fully with reasonable confidence.

Supportive evidence may include:

- data from animal tests performed according to existing guidelines, with a result that does not meet the criteria given in the section on animal studies but is sufficiently close to the limit to be considered significant, or
- data from non-standard methods, or
- appropriate structure-activity relationships.

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Dr. C. Chambers
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Prof. V. Kapoulas
Prof. J. Krutmann
Prof. C. Lidén (Rapporteur)
Prof. J.-P. Marty
Dr. S.C. Rastogi
Prof. J. Revuz
Prof. V. Rogiers
Prof. T. Sanner
Dr. I.R. White (Chairman)