

## B.6. SKIN SENSITISATION

### 1. METHOD

#### 1.1 Introduction

*Remarks:*

The sensitivity and ability of tests to detect potential human skin sensitisers are considered important in a classification system for toxicity relevant to public health.

There is no single test method which will adequately identify all substances with a potential for sensitising human skin and which is relevant for all substances.

Factors such as the physical characteristics of a substance, including its ability to penetrate the skin, must be considered in the selection of a test.

Two types of tests using guinea-pigs have been developed: the adjuvant-type tests, in which an allergic state is potentiated by dissolving or suspending the test substance in Freund's Complete Adjuvant (FCA), and the non-adjuvant tests.

Adjuvant-type tests are likely to be more accurate in predicting a probable skin sensitising effect of a substance in humans than those methods not employing Freund's Complete Adjuvant and are thus the preferred methods.

The Guinea-Pig Maximisation Test (GPMT) is a widely used adjuvant-type test. Although several other methods can be used to detect the potential of a substance to provoke skin sensitisation reaction, the GPMT is considered to be the preferred adjuvant technique.

With many chemical classes, non-adjuvant tests (the preferred one being the Buehler test) are considered to be less sensitive.

In certain cases there may be good reasons for choosing the Buehler test involving topical application rather than the intradermal injection used in the Guinea-Pig Maximisation Test. Scientific justification should be given when the Buehler test is used.

The Guinea-Pig Maximisation Test (GPMT) and the Buehler test are described in this method. Other methods may be used provided that they are well-validated and scientific justification is given.

If a positive result is seen in a recognised screening test, a test substance may be designated as a potential sensitiser, and it may not be necessary to conduct a further guinea pig test. However, if a negative result is seen in such a test, the guinea pig test must be conducted using the procedure described in this test method.

See also General Introduction Part B.

#### 1.2 Definitions

**Skin sensitisation:** (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterised by pruritis, erythema, oedema, papules, vesicles, bullae or a combination of these. In other species the reactions may differ and only erythema and oedema may be seen.

**Induction exposure:** an experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state.

**Induction period:** a period of at least one week following an induction exposure during which a hypersensitive state may be developed.

**Challenge exposure:** an experimental exposure of a previously treated subject to a test substance following an induction period, to determine if the subject reacts in a hypersensitive manner.

### 1.3 Reference Substances

The sensitivity and reliability of the experimental technique used should be assessed every six months by use of substances which are known to have mild-to-moderate skin sensitisation properties.

In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in a non-adjuvant test should be expected for mild/moderate sensitisers.

The following substances are preferred.

CAS numbers	EINECS numbers	EINECS names	Common names
101-86-0	202-983-3	$\alpha$ -hexylcinnamaldehyde	$\alpha$ -hexylcinnamaldehyde
149-30-4	205-736-8	benzothiazole-2-thiol (mercaptobenzothiazole)	kaptax
94-09-7	202-303-5	benzocaine	norcaine

There may be circumstances where, given adequate justification other control substances meeting the above criteria may be used.

### 1.4 Principle of the test method

The test animals are initially exposed to the test substance by intradermal injections and/or epidermal application (induction exposure). Following a rest period of 10 to 14 days (induction period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure in the test animals is compared with that demonstrated by control animals which undergo sham treatment during induction and receive the challenge exposure.

### 1.5 Description of the test methods

If removal of the test substance is considered necessary, this should be achieved using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

#### 1.5.1 *Guinea-Pig Maximisation Test (GPMT)*

##### 1.5.1.1 Preparations

Healthy young adult albino guinea-pigs are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by clipping, shaving or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid abrading the skin. The animals are weighed before the test commences and at the end of the test.

##### 1.5.1.2 Test conditions

##### 1.5.1.2.1 Test animals

Commonly used laboratory strains of albino guinea-pigs are used.

##### 1.5.1.2.2 Number and sex

Male and/or female animals can be used. If females are used, they should be nulliparous and non-pregnant.

A minimum of 10 animals is used in the treatment group and at least 5 animals in the control group. When fewer than 20 test and 10 control guinea pigs have been used, and it is not possible to conclude that the test

substance is a sensitiser, testing in additional animals to give a total of at least 20 test and 10 control animals is strongly recommended.

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal. This method can be found in Dir 96/54/EC (OJ L 248 1996).  
A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

#### 1.5.1.2.3 Dose levels

The concentration of the test substance used for each induction exposure should be well-tolerated systemically and should be the highest to cause mild-to-moderate skin irritation. The concentration used for the challenge exposure should be the highest non-irritant dose. The appropriate concentrations should be determined from a pilot study using two or three animals, if other information are not available. Consideration should be given to the use of FCA-treated animals for this purpose.

#### 1.5.1.3 Procedure

##### 1.5.1.3.1 Induction

###### Day 0-treated group

Three pairs of intradermal injections of 0.1 ml volume are given in the shoulder region which is cleared of hair so that one of each pair lies on each side of the midline.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline

Injection 2: the test substance in an appropriate vehicle at the selected concentration

Injection 3: the test substance at the selected concentration formulated in a 1:1 mixture (v/v) FCA/water or physiological saline

In injection 3, water soluble substances are dissolved in the aqueous phase prior to mixing with FCA. Liposoluble or insoluble substances are suspended in FCA prior to combining with the aqueous phase. The final concentration of test substance shall be equal to that used in injection 2.

Injections 1 and 2 are given close to each other and nearest the head, while 3 is given towards the caudal part of the test area.

###### Day 0-control group

Three pairs of intradermal injections of 0.1 ml volume are given in the same sites as in the treated animals.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline

Injection 2: the undiluted vehicle

Injection 3: a 50% w/v formulation of the vehicle in a 1:1 mixture (v/v) FCA/water or physiological saline.

###### Day 5-7-treated and control groups

Approximately twenty-four hours before the topical induction application, if the substance is not a skin irritant, the test area, after close-clipping and/or shaving is treated with 0.5 ml of 10% sodium lauryl sulphate in vaseline, in order to create a local irritation.

###### Day 6-8-treated group

The test area is again cleared of hair. A filter paper (2 x 4 cm) is fully-loaded with test substance in a suitable vehicle and applied to the test area and held in contact by an occlusive dressing for 48 hours. The choice of the vehicle should be justified. Solids are finely pulverised and incorporated in a suitable vehicle. Liquids can be applied undiluted, if appropriate.

###### Day 6-8-control group

The test area is again cleared of hair. The vehicle only is applied in a similar manner to the test area and held in contact by an occlusive dressing for 48 hours.

##### 1.5.1.3.2 Challenge

###### Day 20-22-treated and control groups

The flanks of treated and control animals are cleared of hair. A patch or chamber loaded with the test substance is applied to one flank of the animals and, when relevant, a patch or chamber loaded with the

vehicle only may also be applied to the other flank. The patches are held in contact by an occlusive dressing for 24 hours.

Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal. This method can be found in Dir 96/54/EC (OJ L 248 1996).  
A complete list of Annex V Testing Methods and the corresponding OJ can be downloaded from a previous page in this site.

#### 1.5.1.3.3 Observation and Grading: treated and control groups

- approximately 21 hours after removing the patch the challenge area is cleaned and closely-clipped and/or shaved and depilated if necessary;
- approximately 3 hours later (approximately 48 hours from the start of the challenge application) the skin reaction is observed and recorded according to the grades shown in appendix;
- approximately 24 hours after this observation a second observation (72 hours) is made and once again recorded.

Blind reading of test and control animals is encouraged.

If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. A rechallenge may also be performed on the original control group.

All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded according to the grading scale of Magnusson/Kligman (See appendix). Other procedures, e.g. histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

#### 1.5.2 *Buehler test*

##### 1.5.2.1 Preparations

Healthy young adult albino guinea-pigs are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by clipping, shaving or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid abrading the skin. The animals are weighed before the test commences and at the end of the test.

##### 1.5.2.2 Test conditions

###### 1.5.2.2.1 Test animals

Commonly used laboratory strains of albino guinea-pigs are used.

###### 1.5.2.2.2 Number and sex

Male and/or female animals can be used. If females are used, they should be nulliparous and non-pregnant.

A minimum of 20 animals is used in the treatment group and at least 10 animals in the control group.

###### 1.5.2.2.3 Dose levels

The concentration of test substance used for each induction exposure should be the highest possible to produce a mild but not excessive irritation. The concentration used for the challenge exposure should be the highest non-irritating dose. If necessary, the appropriate concentration can be determined from a pilot study using two or three animals.

For water soluble test materials, it is appropriate to use water or a dilute non-irritating solution of surfactant as the vehicle. For other test materials 80% ethanol/water is preferred for induction and acetone for challenge.

##### 1.5.2.3 Procedure

###### 1.5.2.3.1 Induction

###### Day 0-treated group

One flank is cleared of hair (closely-clipped). The test patch system should be fully loaded with test substance in a suitable vehicle (the choice of the vehicle should be justified; liquid test substances can be applied undiluted, if appropriate).

The test patch system is applied to the test area and held in contact with the skin by an occlusive patch or chamber and a suitable dressing for 6 hours.

The test patch system must be occlusive. A cotton pad is appropriate and can be circular or square, but should approximate 4-6 cm<sup>2</sup>. Restraint using an appropriate restrainer is preferred to assure occlusion. If wrapping is used, additional exposures may be required.

#### Day 0-control group

One flank is cleared of hair (closely-clipped). The vehicle only is applied in a similar manner to that used for the treated group. The test patch system is held in contact with the skin by an occlusive patch or chamber and a suitable dressing for 6 hours. If it can be demonstrated that a sham control group is not necessary, a naive control group may be used.

#### Days 6-8 and 13-15-treated and control group

The same application as on day 0 is carried out on the same test area (cleared of hair if necessary) of the same flank on day 6-8, and again on day 13-15.

#### 1.5.2.3.2 Challenge

##### Day 27-29-treated and control group

The untreated flank of treated and control animals is cleared of hair (closely-clipped). An occlusive patch or chamber containing the appropriate amount of test substance is applied, at the maximum non-irritant concentration, to the posterior untreated flank of treated and control animals.

When relevant, an occlusive patch or chamber with vehicle only is also applied to the anterior untreated flank of both treated and control animals. The patches or chambers are held in contact by a suitable dressing for 6 hours.

#### 1.5.2.3.3 Observation and grading

- approximately 21 hours after removing the patch the challenge area is cleared of hair;
- approximately three hours later (approximately 30 hours after application of the challenge patch) the skin reactions are observed and recorded according to the grades shown in the appendix;
- approximately 24 hours after the 30 hour observation (approximately 54 hours after application of the challenge patch) skin reactions are again observed and recorded.

Blind reading of the test and control animals is encouraged.

If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. A rechallenge may also be performed on the original control group.

All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded according to the Magnusson/Kligman grading scale (See appendix). Other procedures, e.g. histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

## 2. DATA (GPMT AND BUEHLER TEST)

Data should be summarised in tabular form, showing for each animal the skin reactions at each observation.

3. **REPORTING (GPMT AND BUEHLER TEST)**

If a screening assay is performed before the guinea pig test the description or reference of the test (e.g. Local Lymph Node Assay (LLNA), Mouse Ear Swelling Test (MEST)), including details of the procedure, must be given together with results obtained with the test and reference substances.

**Test report (GMPT and Buehler test)**

The test report shall, if possible, include the following information:

*Test animals:*

- strain of guinea-pig used;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test.

*Test conditions:*

- technique of patch site preparation;
- details of patch materials used and patching technique;
- result of pilot study with conclusion on induction and challenge concentrations to be used in the test;
- details of test substance preparation, application and removal;
- justification for choice of vehicle;
- vehicle and test substance concentrations used for induction and challenge exposures and the total amount of substance applied for induction and challenge.

*Results:*

- a summary of the results of the latest sensitivity and reliability check (see 1.3) including information on substance, concentration and vehicle used;
- on each animal including grading system;
- narrative description of the nature and degree effects observed;
- any histopathological findings.

*Discussion of results.*

*Conclusions.*

4. **REFERENCES**

This method is analogous to OECD TG 406.

*Appendix*

**TABLE:**

**Magnusson/Kligman grading scale for the evaluation of challenge patch test reactions**

- 0 = no visible change
- 1 = discrete or patchy erythema
- 2 = moderate and confluent erythema
- 3 = intense erythema and swelling