B.40. SKIN CORROSION

1. METHOD

1.1 INTRODUCTION

Two *in vitro* tests for skin corrosivity, the rat skin transcutaneous electrical resistance (TER) assay and a test employing a human skin model, have been endorsed as scientifically valid by the European Centre for the Validation of Alternative Methods (ECVAM, Joint Research Centre, European Commission) (1)(2)(3). The ECVAM validation study demonstrated that both tests were able to reliably discriminate between known skin corrosives and non-corrosives. Furthermore, the test protocol based on a human skin model enabled correct distinction between degrees of corrosive effects (known severe skin corrosives, R35, and other skin corrosives, R34) (2). Descriptions and procedures for both tests are given; the choice of which test to use depends on the specific requirements and preferences of the user.

See also General Introduction Part B.

1.2 DEFINITIONS

Skin corrosion: the production of irreversible tissue damage in the skin following the application of a test material.

1.3 REFERENCE SUBSTANCES

None specified, but see points 1.5.3.4 and 1.7.2.3.

1.4 PRINCIPLE OF THE TEST METHOD - RAT SKIN TER ASSAY

The test material is applied for up to 24 hours to the epidermal surfaces of skin discs taken from the pelts of humanely killed young rats. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the inherent TER below a threshold level (5kΩ) (4)(5). Irritant and non-irritant materials do not reduce the TER below the threshold level. A dye-binding step can be incorporated into the test procedure for surfactants and neutral organics (for definition see reference (6)) to reduce the number of false positive results obtained specifically with these chemical types (2) (7).

1.5 DESCRIPTION OF THE TEST METHOD - RAT SKIN TER ASSAY

1.5.1 Animals

Young (20-23 days) rats (Wistar or a comparable strain) are required for the preparation of skin discs. The dorsal and flank hair is carefully removed with small animal clippers. The animals are then washed by careful wiping, whilst submerging the area in antibiotic solution (containing, for example, streptomycin, penicillin, chloramphenicol and amphotericin at concentrations effective in inhibiting bacterial growth). Animals are washed with antibiotics again on the third or fourth day after the first wash, and are used within 3 days (animals must not be older than 31 days for pelt preparation).

1.5.2 Preparation of the skin discs

Animals are humanely killed. The dorsal skin of each animal is then removed and stripped of excess fat by carefully peeling it away from the skin. The pelt is placed over the end of a PTFE (polytetrafluoroethylene) tube, ensuring that the epidermal surface is in contact with the tube. A rubber 'O' ring is press-fitted over the end of the tube to hold the skin in place and excess tissue is trimmed away. Tube and 'O' ring dimensions are shown in Figure 1. The rubber 'O' ring is then carefully sealed to the end of the PTFE tube with petroleum jelly. The tube is supported by a spring clip inside a receptor chamber containing magnesium sulphate solution (154mM) (Figure 2).

1.5.3 **Test procedure**

1.5.3.1 *Application of the test material*

Liquid test substances (150µl) are applied to the epidermal surface inside the tube (Figure 2). When testing solid materials, a sufficient amount of the solid is applied to the disc to ensure that the whole surface of the epidermis is covered. Deionised water (150µl) is then added on top of the solid and the tubes are gently agitated. Test substances should have maximum contact with the skin. For some solids this may be achieved by warming up to 30°C to melt the test substance, or by grinding to produce a granular material or powder.

Three skin discs are used for each test substance. Test substances are applied for 24 hours (see also 1.5.3.4). The test substance is removed by washing with a jet of tap water at up to 30°C until no further material can be removed. The removal of test substances which have solidified in the tube can be facilitated by jet washing with warm water at approximately 30°C.

1.5.3.2 *TER measurements*

The TER is measured by using a low-voltage, alternating current databridge (e.g. AIM 401 or 6401, or equivalent). Prior to measuring the electrical resistance, the surface tension of the skin is reduced by adding a sufficient volume of 70% ethanol to cover the epidermis. After a few seconds the ethanol is removed by inverting the tube, and the tissue is then hydrated by the addition of 3ml magnesium sulphate solution (154mM). The databridge electrodes are placed on either side of the skin disc to take the resistance measurement in kΩ/skin disc (Figure 2). Electrode dimensions and the length of the electrode exposed below the crocodile clips are shown in Figure 1. The inner (thick) electrode clip is rested on the top of the PTFE tube during resistance measurement, to ensure that a consistent length of electrode is submerged in the magnesium sulphate solution. The outer (thin) electrode is positioned inside the receptor chamber so that it rests on the bottom of the chamber. The distance between the bottom of the spring clip and the bottom of the PTFE tube is maintained as a constant (Figure 1), since this distance affects the resistance value obtained.

Note that if the measured resistance value is greater than 20kΩ, this may be due to the test substance coating the epidermal surface of the skin disc. Removal of this coating can be attempted, for example, by sealing the PTFE tube with a gloved thumb and shaking it for approximately 10 seconds; the magnesium sulphate solution is discarded and the resistance measurement is repeated with fresh magnesium sulphate.

The mean TER results are accepted on condition that concurrent positive and negative control values fall within the acceptable ranges for the method. The suggested control substances and their associated acceptable resistance ranges for the methodology and apparatus described are:

Control	Substance	Resistance range (kΩ)
Positive	10M Hydrochloric acid (36%)	0.5 - 1.0
Negative	Distilled water	10 - 25

1.5.3.3 *Modified procedure for surfactants and neutral organics*

If the TER values of test substances which are either surfactants or neutral organics are less than or equal to 5kΩ, an assessment of dye penetration can be carried out on the tissues. This procedure will determine whether the results are false positives (2).

1.5.3.3.1 *Sulforhodamine B dye application and removal*

Following initial treatment with the test substance, 150µl of a 10% (w/v) dilution of sulforhodamine B dye in distilled water is applied to the epidermal surface of each skin disc for 2 hours. The skin discs are then jet washed with tap water at up to room temperature for approximately 10 seconds to remove any excess/unbound dye. Each skin disc is carefully removed from the PTFE tube and placed in a vial (e.g. a 20ml glass scintillation vial) containing deionised water (8ml). The vials are agitated gently for 5 minutes to remove any further excess/unbound dye. This rinsing procedure is then repeated, after which the skin discs are removed and placed into vials containing 5ml of 30% (w/v) sodium dodecyl sulphate (SDS) in distilled water and are incubated overnight at 60°C. After incubation, each skin disc is removed and discarded and the remaining solution is centrifuged for 8 minutes at 21°C (relative centrifugal force ~175). A 1ml sample of the supernatant is then diluted 1 in 5 (v/v) [i.e. 1ml + 4ml] with 30% (w/v) SDS in distilled water. The optical density (OD) of the solution is measured at approximately 565nm.

1.5.3.3.2 *Calculation of dye content*

The sulforhodamine B dye content per disc is calculated from the OD values (sulforhodamine B dye molar extinction coefficient at 565nm = 8.7 x 10^4; molecular weight = 580). The sulforhodamine B dye content is determined for each skin disc and a mean dye content is then calculated for the replicates. The mean dye binding results are accepted on condition that concurrent control values fall within the acceptable ranges for the method. Suggested acceptable dye content ranges for the control substances for the methodology and apparatus described are:

Control	Substance	Dye content range (µg/disc)
Positive	10M Hydrochloric acid (36%)	40 - 100
Negative	Distilled water	15 - 35

1.5.3.4 *Additional information*

Test substances can also be applied to the skin discs for shorter periods (e.g. 2 hours) to identify those materials which are severely corrosive. However, in the validation study, the TER assay was found to overestimate the corrosive potential of several test chemicals following their application to the skin discs for 2 hours (2), although it enabled the correct identification of corrosives and non-corrosives after a 24-hour application.

The properties and dimensions of the test apparatus and the experimental procedure used may influence the TER values obtained. The 5kΩ corrosive threshold was developed from data obtained with the specific apparatus and procedure described in this method. Different threshold and control values may apply if the test conditions are altered significantly. Therefore, it is recommended that the methodology and resistance threshold value are calibrated by testing a series of reference standards chosen from the chemicals used in the validation study (3).

1.6 PRINCIPLE OF THE TEST METHOD - HUMAN SKIN MODEL ASSAY

The test material is applied topically for up to 4 hours to a three-dimensional human skin model, comprising a reconstructed epidermis with a functional stratum corneum. Corrosive materials are identified by their ability to produce a decrease in cell viability (as determined, for example, by using the MTT reduction assay) below defined threshold levels at specified exposure periods. The principle of the assay is in accordance with the hypothesis that chemicals which are corrosive are those which are able to penetrate the stratum corneum (by diffusion or erosion) and are sufficiently cytotoxic to cause cell death in the underlying cell layers.

1.7 DESCRIPTION OF THE TEST METHOD - HUMAN SKIN MODEL ASSAY

1.7.1 **Human skin models**

Human skin models can come from various sources, but they must meet certain criteria. The model must have a functional stratum corneum with an underlying layer of living cells. The barrier function of the stratum corneum must be adequate. This can be shown by demonstrating the model's resistance to cytotoxicity following the application of substances which are known to be cytotoxic to cells, but which do not normally pass through the stratum corneum. The model must be shown to give reproducible results under defined experimental conditions.

The viability of the living cells in the model must be sufficiently high to discriminate well between the positive and negative control substances. Cell viability (for example, as measured by the amount of MTT reduction, i.e. an OD value) following exposure to the negative control substance must fall within acceptable limits for the particular model. Similarly, cell viability values with the positive control substance (relative to those for the negative control) must fall within specified limits. Most importantly, the prediction model used must have been shown to meet international validation standards.

1.7.2 **Test procedure**

1.7.2.1 *Application of the test material*

For liquid materials, sufficient test substance must be applied to cover the skin surface (a minimum of 25µl/cm²). For solid materials, sufficient test substance must be applied to cover the skin, and it should then be moistened to ensure good contact with the skin; where appropriate, solids should be ground to a powder before application. The application method must be shown to be adequate for a wide range of chemical types (e.g. see reference 2). At the end of the exposure period, the test material must be carefully washed from the skin surface with saline solution.

1.7.2.2 *Cell viability measurements*

Any quantitative, validated, method can be used to measure cell viability. The most frequently used assay is MTT reduction, which has been shown to give accurate and reproducible results in various laboratories (2). The skin disc is placed in an MTT solution of 0.3mg/ml at 20-28°C for 3 hours. The precipitated blue formazan product is then extracted (solvent extraction) and the concentration of the formazan is measured by determining the OD at a wavelength between 545 and 595 nm.

1.7.2.3 *Additional information*

The skin model used, and the exact protocol of exposure time and washing procedures, etc. will have a major impact on the cell viability results. It is recommended that the methodology and prediction model are calibrated by testing a series of reference standards chosen from the chemicals used in the ECVAM validation study (3). It is critical that the method used has been shown to be reproducible within and between laboratories for a wide range of chemicals, in accordance with international standards. As a minimum, the method should meet the criteria for scientific validity defined previously (2), and the results of such a validation study must be published in a peer-reviewed scientific journal.

2. DATA

2.1 TREATMENT OF RESULTS

2.1.1 Rat skin TER assay

Resistance values (kΩ) for the test material, positive and negative controls, and any standard reference chemicals should be reported in tabular form, including data for replicates/repeat experiments, mean values and the classification derived.

2.1.2 Human skin model assay

OD values and calculated percentage cell viability data for the test material, positive and negative controls, and any standard reference chemicals should be reported in tabular form, including data for replicates/repeat experiments, mean values and the classification derived.

2.2 EVALUATION AND INTERPRETATION OF RESULTS

2.2.1 Rat skin TER assay

If the mean TER value obtained for the test substance is greater than 5kΩ, then it is non-corrosive. If the TER value is less than or equal to 5kΩ, and the test substance is not a surfactant or neutral organic, then it is corrosive.

For surfactants or neutral organics which give TER values less than or equal to 5kΩ, dye penetration can be carried out. If the mean disc dye content is greater than or equal to the mean disc dye content of the 36% HCl positive control obtained concurrently, then the test substance is a true positive and is therefore corrosive. If the mean disc dye content is less than the mean disc dye content of the 36% HCl positive control obtained concurrently, then the test substance is a false positive and is therefore non-corrosive.

2.2.2 Human skin model assay

The negative control OD value represents 100% cell viability; hence, the OD values obtained for each test sample can be used to calculate a percentage viability relative to the negative control. The cut-off percentage cell viability value distinguishing corrosive from non-corrosive test materials (or discriminating between different corrosive classes) must be clearly defined in the prediction model before the method is validated, and the subsequent validation study must show that the cut-off value is appropriate (e.g. see reference 2).

3. REPORTING

TEST REPORT

The test report must include at least the following information:

Test substance:

- identification data, physical nature and, where relevant, physicochemical properties. Similar information should be provided for reference substances, if used.

Test conditions:

- details of test procedure used;
- description and justification of any modifications.

Results:

- tabulation of resistance values (TER assay) or percentage cell viability values (human skin model assay) for the test material, positive and negative controls and any standard reference chemicals, including data for replicates/repeat experiments and mean values;
- description of any other effects observed.

Discussion of the results.

Conclusions

4. REFERENCES

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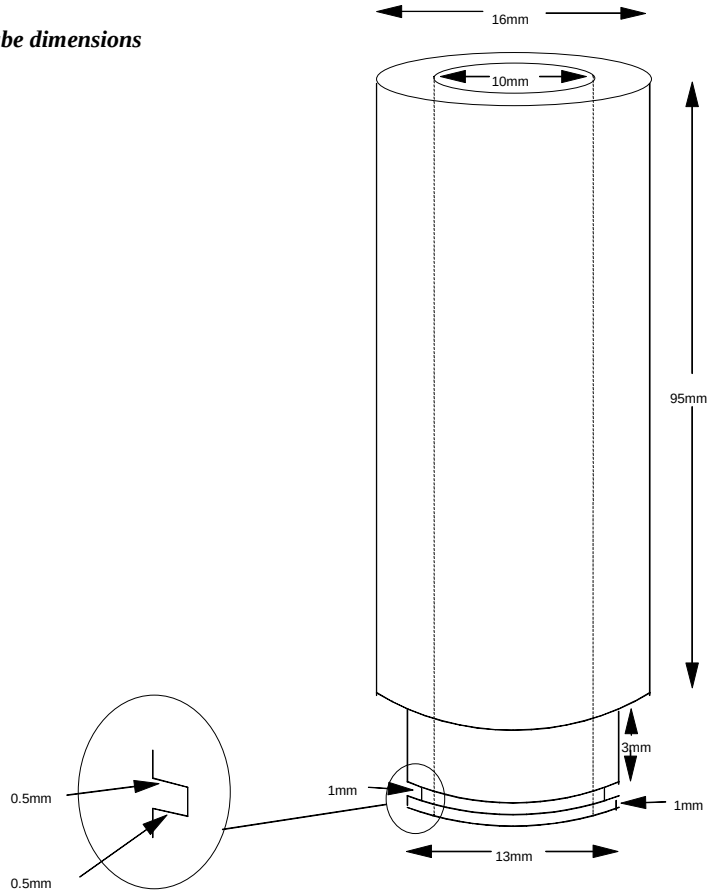
7. Botham, P.A., Chamberlain, M., Barratt, M.D., Curren, R.D., Esdaile, D.J., Gardner, J.R., Gordon, V.C., Hildebrand, B., Lewis, R.W., Liebsch, M., Logemann, P., Osborne, R., Ponec, M., Regnier, J.F., Steiling, W., Walker, A.P. & Balls, M. (1995). A prevalidation study on *in vitro* skin corrosivity testing. The report and recommendations of ECVAM workshop 6. *ATLA* **23**, 219-255.

Figure 1

PTFE tube dimensions



Electrode dimensions

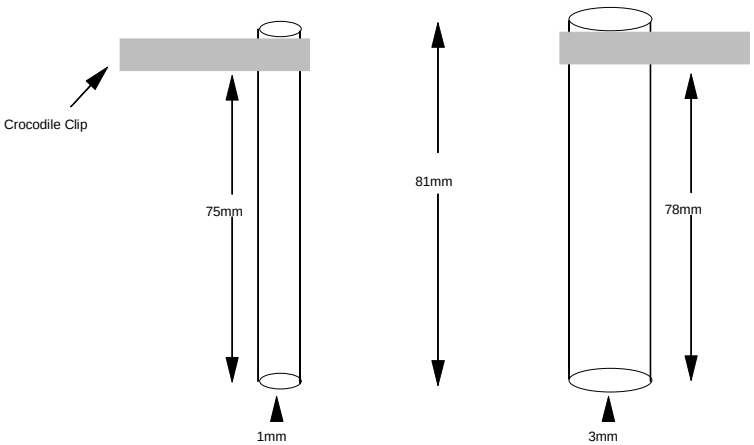


Figure 2

Apparatus for the rat skin TER assay