OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER)

INTRODUCTION

1.   Skin corrosion refers to the production of irreversible tissue damage in the skin following the application of a test material [as defined by the Globally Harmonised System for the Classification and Labelling of Chemical Substances and Mixtures (GHS)] (1). This test Guideline provides a procedure by which the assessment of corrosivity is not carried out in live animals.

2.   The assessment of skin corrosivity has typically involved the use of laboratory animals (2). Concern for the pain and suffering of animals involved with this procedure has been addressed in the 2002 revision of Guideline 404 and in the supplement to Guideline 404 that allows for the determination of skin corrosion by using alternative, *in vitro,* methods, avoiding pain and suffering.

3.   The principal obstacle to completely replacing *in vivo* testing for skin corrosion in Guideline 404 has been the lack of formal, independent, validation of *in vitro* tests. A first step towards defining alternative tests that could be used for skin corrosivity testing for regulatory purposes was the conduct of prevalidation studies (3). Following this, a formal validation study of *in vitro* methods for assessing skin corrosion (4)(5) was conducted (6)(7)(8). The outcome of these studies and other published literature led to the recommendation that the following tests could be used for the assessment of *in vivo* skin corrosivity (9)(10)(11): the human skin model test (see Test Guideline 431) and the transcutaneous electrical resistance test (this Guideline).

DEFINITIONS

4.   Definitions used are provided in the Annex.

INITIAL CONSIDERATIONS

5.   A validation study and other published studies have reported that the rat skin transcutaneous electrical resistance (TER) assay (12)(13) is able to reliably discriminate between known skin corrosives and non-corrosives (5)(9).

6.   The test described in this Guideline allows the identification of corrosive chemical substances and mixtures. It further enables the identification of non-corrosive substances and mixtures when supported by a weight of evidence determination using other existing information (e.g. pH, structure-activity-relationships, human and/or animal data) (1)(2)(11)(14). It does not provide information on skin irritation, nor does it allow the sub-categorisation of corrosive substances as permitted in the Globally Harmonised System for Hazard Classification and Labelling (GHS) (1).

7.   For a full evaluation of local skin effects after a single dermal exposure, it is recommended to follow the sequential testing strategy as appended to Test Guideline 404 (2) and provided in the GHS (1).

This testing strategy includes the conduct of *in vitro* tests for skin corrosion (as described in this guideline) and skin irritation before considering testing in live animals.

PRINCIPLE OF THE TEST

8.   The test material is applied for up to 24 hours to the epidermal surfaces of skin discs in a two-compartment test system in which the skin discs function as the separation between the compartments. The skin discs are taken from humanely killed rats aged 28-30 days. 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 TER below a threshold level (12). For rat TER, a cut-off value of 5kΩ has been selected based on extensive data for a wide range of chemicals where the vast majority of values were either clearly well above (often > 10 kΩ), or well below (often < 3 kΩ) this value (12). Generally, materials that are non-corrosive in animals but are irritating or non-irritating do not reduce the TER below this cut-off value. Furthermore, use of other skin preparations or other equipment may alter the cut-off value, necessitating further validation.

9.   A dye-binding step is incorporated into the test procedure for confirmation testing of positive results in the TER including values around 5 kΩ. The dye-binding step determines if the increase in ionic permeability is due to physical destruction of the stratum corneum. The TER method utilising rat skin has shown to be predictive of *in vivo* corrosivity in the rabbit assessed under OECD guideline 404 (2). It should be noted that the *in vivo* rabbit test is highly conservative with respect to skin corrosivity and skin irritation when compared with the human skin patch test (15).

PROCEDURE

Animals

10.   Rats are the species of choice because the sensitivity of their skin to chemicals in this test has been previously demonstrated (10). The age (when the skin is collected) and strain of the rat is particularly important to ensure that the hair follicles are in the dormant phase before adult hair growth begins.

11.   The dorsal and flank hair from young, approximately 22 day-old, male or female rats (Wistar-derived or a comparable strain), is carefully removed with small clippers. Then, the animals are washed by careful wiping, whilst submerging the clipped 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 of the second wash, when the stratum corneum has recovered from the hair removal.

Preparation of the skin discs

12.   Animals are humanely killed when 28-30 days old; this age is critical. The dorso-lateral skin of each animal is then removed and stripped of excess subcutaneous fat by carefully peeling it away from the skin. Skin discs, with a diameter of approximately 20-mm each, are removed. The skin may be stored before disks are used where it is shown that positive and negative control data are equivalent to that obtained with fresh skin.

13.   Each skin disc is placed over one of the ends 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 2. 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 $MgSO_4$ solution (154 mM) (Figure 1). The skin disc should be fully submerged in the $MgSO_4$ solution. As many as 10-15 skin discs can be obtained from a single rat skin.

14.   Before testing begins, the electrical resistance of two skin discs is measured as a quality control procedure for each animal skin. Both discs should give resistance values greater than 10 kΩ for the remainder of the discs to be used for the test. If the resistance value is less than 10 kΩ, the remaining discs from that skin should be discarded.

Application of the test and control substances

15.   Concurrent positive and negative controls should be used for each study to ensure adequate performance of the experimental model. Skin discs from a single animal should be used. The suggested positive and negative control substances are 10M hydrochloric acid and distilled water, respectively.

16.   Liquid test substances (150 μL) are applied uniformly to the epidermal surface inside the tube. When testing solid materials, a sufficient amount of the solid is applied evenly to the disc to ensure that the whole surface of the epidermis is covered. Deionised water (150 μL) is added on top of the solid and the tube is gently agitated. In order to achieve maximum contact with the skin, solids may need to be warmed to 30° C to melt or soften the test substance, or ground to produce a granular material or powder.

17.   Three skin discs are used for each test and control substance. Test substances are applied for 24 hours at 20-23° C. 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.

TER measurements

18.   The skin impedance is measured as TER by using a low-voltage, alternating current Wheatstone bridge (13). General specifications of the bridge are 1-3 Volt operating voltage, a sinus or rectangular shaped alternating current of 50 – 1000 Hz, and a measuring range of at least 0.1 -30 kΩ. The databridge used in the validation study measured inductance, capacitance and resistance up to values of 2000H, 2000μF, and 2MΩ, respectively at frequencies of 100Hz or 1kHz, using series or parallel values. For the purposes of the TER corrosivity assay measurements are recorded in resistance, at a frequency of 100Hz and using series values. 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 from the tube and the tissue is then hydrated by the addition of 3mL $MgSO_4$ solution (154mM). The databridge electrodes are placed on either side of the skin disc to measure the resistance in kΩ/skin disc (Figure 1). Electrode dimensions and the length of the electrode exposed below the crocodile clips are shown in Figure 2. The clip attached to the inner electrode is rested on the top of the PTFE tube during resistance measurement to ensure that a consistent length of electrode is submerged in the $MgSO_4$ solution. The outer electrode is positioned inside the receptor chamber so that it rests on the bottom of the chamber. The distance between the spring clip and the bottom of the PTFE tube is maintained as a constant (Figure 2), because this distance affects the resistance value obtained. Consequently, the distance between the inner electrode and the skin disc should be constant and minimal (1-2 mm).

19.   If the measured resistance value is greater than 20 kΩ, this may be due to the remains of the test

substance coating the epidermal surface of the skin disc. Further 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 $MgSO_4$ solution is discarded and the resistance measurement is repeated with fresh $MgSO_4$.

20.   The properties and dimensions of the test apparatus and the experimental procedure used may influence the TER values obtained. The 5 kΩ corrosive threshold was developed from data obtained with the specific apparatus and procedure described in this Guideline. Different threshold and control values may apply if the test conditions are altered or a different apparatus is used. Therefore, it is necessary to calibrate the methodology and resistance threshold values by testing a series of reference standards chosen from the chemicals used in the validation study (4)(5), or from similar chemical classes to the chemicals being investigated. A set of suitable reference chemicals is shown in Table 1.

Dye Binding Methods

21.   Exposure of certain non-corrosive materials can result in a reduction of resistance below the cut-off of 5 kΩ allowing the passage of ions through the stratum corneum, thereby reducing the electrical resistance (5). For example, neutral organics and chemicals that have surface-active properties (including detergents, emulsifiers and other surfactants) can remove skin lipids making the barrier more permeable to ions. Thus, if the TER values of test substances are less than or around 5 kΩ in the absence of visual damage, an assessment of dye penetration should be carried out on the control and treated tissues to determine if the TER values obtained were the result of increased skin permeability, or skin corrosion (3)(5). In case of the latter where the stratum corneum is disrupted, the dye sulforhodamine B, when applied to the skin surface rapidly penetrates and stains the underlying tissue. This particular dye is stable to a wide range of chemicals and is not affected by the extraction procedure described below.

Sulforhodamine B dye application and removal

22.   Following TER assessment, the magnesium sulfate is discarded from the tube and the skin is carefully examined for obvious damage. If there is no obvious major damage, sulforhodamine B dye (Acid Red 52; C.I. 45100; CAS number 3520-42-1), 150μL of a 10% (w/v) dilution in distilled water, is applied to the epidermal surface of each skin disc for 2 hours. These skin discs are then 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 20-mL glass scintillation vial) containing deionised water (8mL). The vials are agitated gently for 5 minutes to remove any additional 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.

23.   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 x g). A 1mL sample of the supernatant is 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 565nm.

Calculation of dye content

24.   The sulforhodamine B dye content per disc is calculated from the OD values (5) (sulforhodamine B dye molar extinction coefficient at 565nm = 8.7×10^4; molecular weight = 580). The dye content is determined for each skin disc by the use of an appropriate calibration curve and a mean dye content is then calculated for the replicates.

Interpretation of results

25.   The mean TER results are accepted if the concurrent positive and negative control values fall within the acceptable ranges for the method in the testing laboratory. The acceptable resistance ranges for the methodology and apparatus described above are given in the following table:

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

26.   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 above are given below:

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

27.   The test substance is considered to be <u>non-corrosive</u> to skin:

   i)   if the mean TER value obtained for the test substance is greater than 5 kΩ, or

   ii) the mean TER value is less than or equal to 5 kΩ, and

        • the skin disc is showing no obvious damage, and
        • the mean disc dye content is well below the mean disc dye content of the 10M HCl positive control obtained concurrently (see paragraph 26 for acceptable ranges).

28.   The test substance is considered to be <u>corrosive</u> to skin:

   i)   if the mean TER value is less than or equal to 5 kΩ and the skin disk is obviously damaged, or

   ii) the mean TER value is less than or equal to 5 kΩ, and
        • the skin disc is showing no obvious damage, but
        • the mean disc dye content is greater than or equal to the mean disc dye content of the 10M HCl positive control obtained concurrently (see paragraph 26 for positive control values).

DATA AND REPORTING

Data

29.   Resistance values (kΩ) and mean dye content values (µg/disc), where appropriate, for the test material, as well as for positive and negative controls should be reported in tabular form (individual trial data and means ± S.D.), including data for replicates/repeat experiments, mean and individual values.

Test report

30.   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 nature and purity;
- – physico-chemical properties such as physical state, 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.

Test Animals:
- – strain and sex used;
- – age of the animals when used as donor animals;
- – source, housing condition, diet, etc.;
- – details of the skin preparation.

Test Conditions:
- – calibration curves for test apparatus;
- – calibration curves for dye binding test performance;
- – details of the test procedure used for TER measurements;
- – details of the test procedure used for the dye binding assessment; if appropriate;
- – description of any modification of the test procedure;
- – description of evaluation criteria used.

Results:
- – tabulation of data from the TER and dye binding assay (if appropriate) for individual animals and individual skin samples;
- – description of any effects observed.

Discussion of the results.

Conclusions.

LITERATURE

(1) OECD (2001) Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures. OECD Series on Testing and Assessment Number 33. ENV/JM/MONO(2001)6, Paris
http://www.olis.oecd.org/olis/2001doc.nsf/LinkTo/env-jm-mono(2001)6

(2) OECD (2002). OECD Guideline for Testing of Chemicals. No. 404: Acute Dermal Irritation, Corrosion, revised version, as adopted on 24 April 2002, 7 pp plus Annex and Supplement.

(3) 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., and Balls, M. (1995). A prevalidation study on *in vitro* skin corrosivity testing. The report and recommendations of ECVAM Workshop 6. ATLA 23, 219-255.

(4) Barratt, M.D., Brantom, P.G., Fentem, J.H., Gerner, I., Walker, A.P., and Worth, A.P. (1998). The ECVAM international validation study on *in vitro* tests for skin corrosivity. 1. Selection and distribution of the test chemicals. Toxic. in Vitro 12, 471-482.

(5) Fentem, J.H., Archer, G.E.B., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Esdaile, D.J., Holzhütter, H.-G., and Liebsch, M. (1998). The ECVAM international validation study on *in vitro* tests for skin corrosivity. 2. Results and evaluation by the Management Team. Toxic. in Vitro 12, 483- 524.

(6) OECD (1996). Final Report of the OECD Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods, 62pp.

(7) Balls, M., Blaauboer, B.J., Fentem. J.H., Bruner. L., Combes, R.D., Ekwall, B., Fielder. R.J., Guillouzo, A., Lewis, R.W., Lovell, D.P., Reinhardt, C.A., Repetto, G., Sladowski. D., Spielmann, H., and Zucco, F. (1995). Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshops. ATLA 23, 129-147.

(8) ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). (1997). Validation and Regulatory Acceptance of Toxicological Test Methods. NIH Publication No. 97-3981. National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA. http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf.

(9) ECVAM (1998). ECVAM News & Views. ATLA 26, 275-280.

(10) ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). (2002). ICCVAM evaluation of EpiDerm™, EPISKIN™ (EPI-200), and the Rat Skin Transcutaneous Electrical Resistance (TER) assay: In Vitro test methods for assessing dermal corrosivity potential of chemicals. NIH Publication No. 02-4502. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA. http://iccvam.niehs.nih.gov/methods/epiddocs/epis_brd.pdf

(11)   OECD (2002) Extended Expert Consultation Meeting on The In Vitro Skin Corrosion Test Guideline Proposal, Berlin, 1st –2nd November 2001, Secretariat's Final Summary Report, 27th March 2002, OECD ENV/EHS, available upon request from the Secretariat

(12)   Oliver, G.J.A., Pemberton, M.A., and Rhodes, C. (1986). An *in vitro* skin corrosivity test - modifications and validation. Fd. Chem. Toxicol. 24, 507-512.

(13)   Botham, P.A., Hall, T.J., Dennett, R., McCall, J.C., Basketter, D.A., Whittle, E., Cheeseman, M., Esdaile, D.J., and Gardner, J. (1992). The skin corrosivity test *in vitro:* results of an interlaboratory trial. Toxic. in Vitro 6, 191-194.

(14)   Worth AP, Fentem JH, Balls M, Botham PA, Curren RD, Earl LK, Esdaile DJ, Liebsch M (1998). An Evaluation of the Proposed OECD Testing Strategy for Skin Corrosion. ATLA 26: 709-720.

(15)   Basketter, D.A., Chamberlain, M., Griffiths, H.A., Rowson, M., Whittle, E., York, M. (1997). The classification of skin irritants by human patch test. Fd. Chem. Toxicol. 35, 845-852.

(16)   Oliver G.J.A, Pemberton M.A and Rhodes C. (1988). An In Vitro model for identifying skin-corrosive chemicals. I. Initial Validation. Toxic. in Vitro. 2, 7-17.

<u>Table 1: Reference Chemicals</u>

1,2-Diaminopropane	CAS-No. 78-90-0	Severely Corrosive
Acrylic Acid	CAS-No. 79-10-7	Severely Corrosive
2-tert. Butylphenol	CAS-No. 88-18-6	Corrosive
Potassuim hydroxide (10%)	CAS-No. 1310-58-3	Corrosive
Sulphuric acid (10%)	CAS-No. 7664-93-9	Corrosive
Octanoic acid (caprylic acid)	CAS-No. 124-07-02	Corrosive
4-Amino-1,2,4-triazole	CAS-No. 584-13-4	Not corrosive
Eugenol	CAS-No. 97-53-0	Not corrosive
Phenethyl bromide	CAS-No. 103-63-9	Not corrosive
Tetrachloroethylene	CAS-No. 127-18-4	Not corrosive
Isostearic acid	CAS-No. 30399-84-9	Not corrosive
4-(Methylthio)-benzaldehyde	CAS-No. 3446-89-7	Not corrosive

Most of the chemicals listed are taken from the list of chemicals selected for the ECVAM international validation study (4). Their selection is based on the following criteria:

    i)   equal number of corrosive and non-corrosive substances;

    ii) commercially available substances covering most of the relevant chemical classes;

    iii) inclusion of severely corrosive as well as less corrosive substances in order to enable discrimination based on corrosive potency;

    iv) choice of chemicals that can be handled in a laboratory without posing **other serious** hazards than corrosivity.

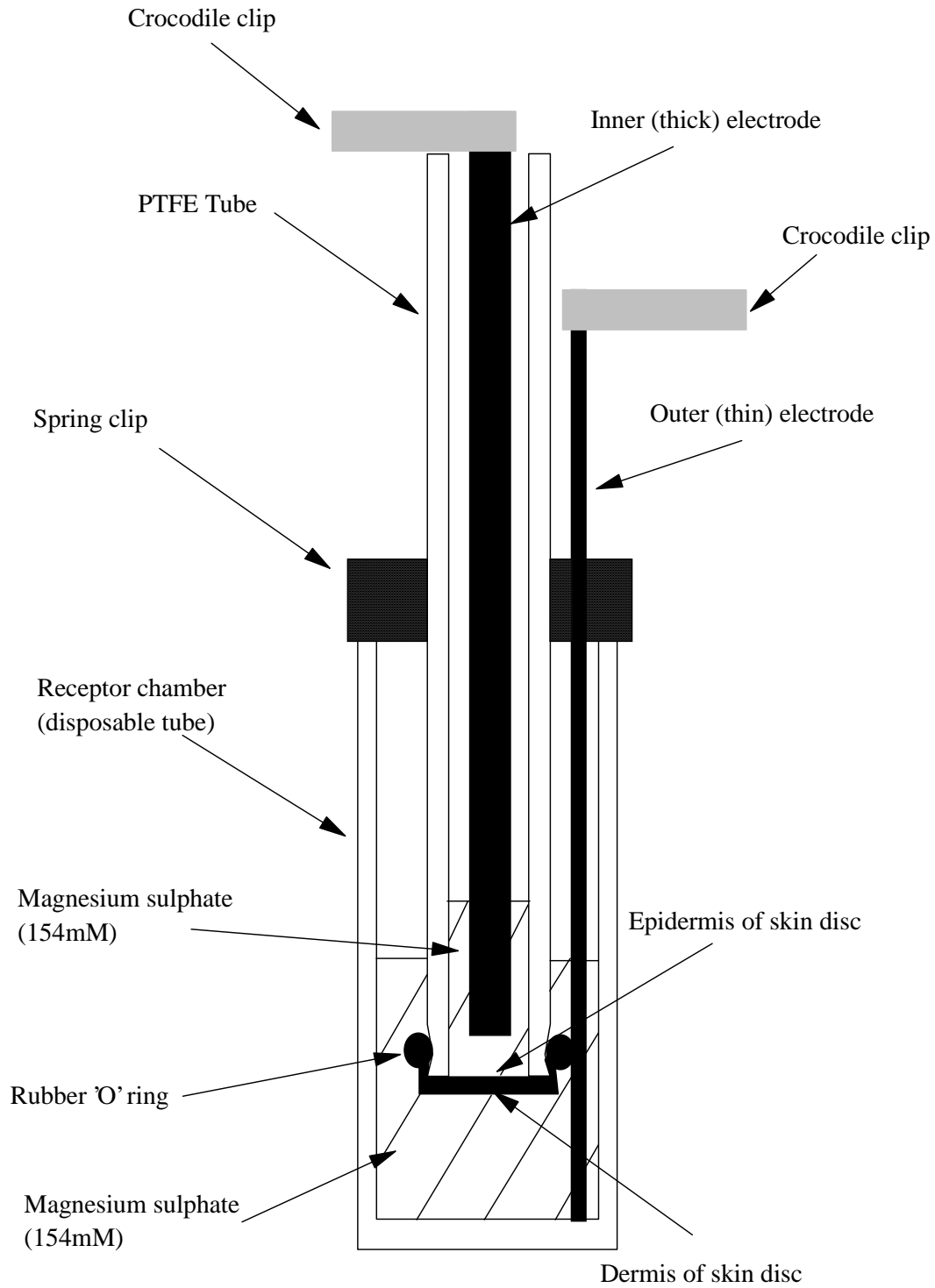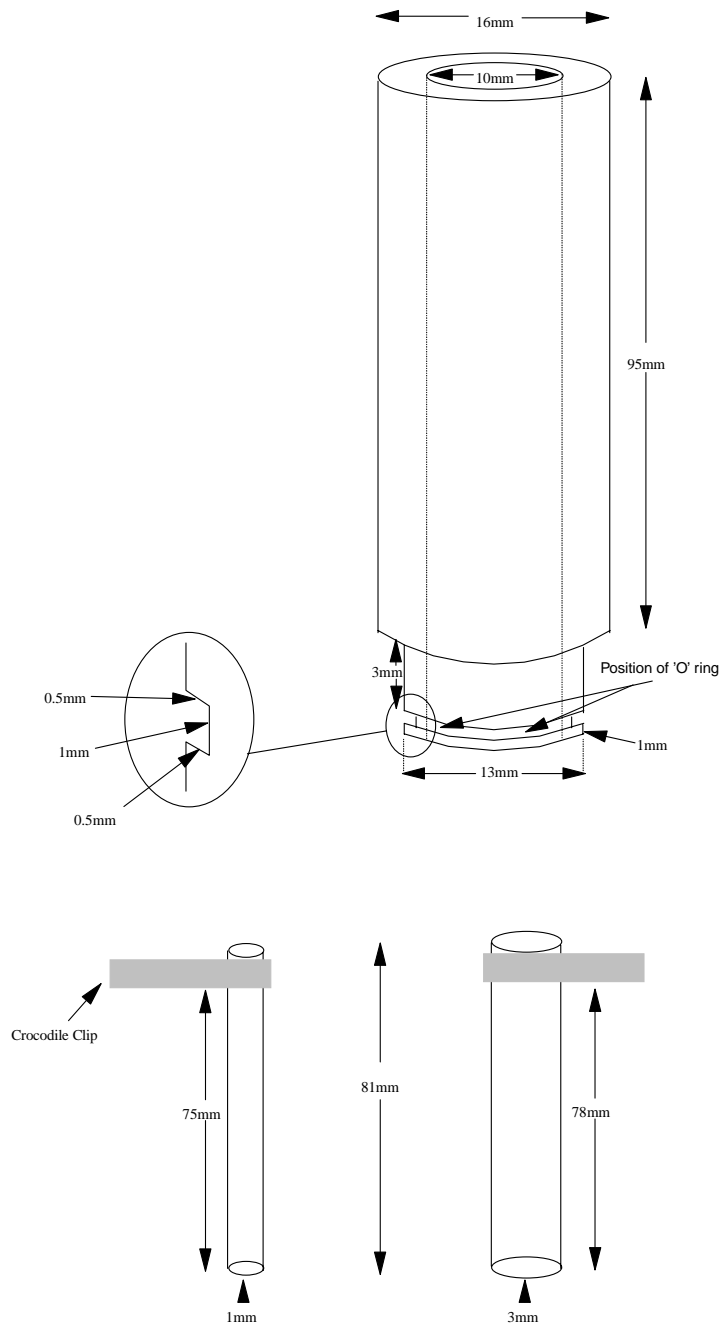**Figure 1: Apparatus for the rat skin TER assay**

Figure 2: Dimensions of the polytetrafluoroethylene (PTFE) and receptor tubes and electrodes used

Critical factors of the apparatus shown above:

- the inner diameter of the PTFE tube,
- the length of the electrodes relative to the PTFE tube and receptor tube, such that the skin disc is not touched by the electrodes and that a standard length of electrode is in contact with the $MgSO_4$ solution,
- the amount of $MgSO_4$ solution in the receptor tube should give a depth of liquid, relative to the level in the PTFE tube, as shown in Figure 1,
- the skin disk should be fixed well enough to the PTFE tube, such that the electrical resistance is a true measure of the skin properties.

ANNEX

DEFINITIONS

<u>Skin corrosion *in vivo:*</u> is the production of irreversible damage of the skin; namely, visible necrosis through the epidermis and into the dermis, following the application of a test substance for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discoloration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

<u>Transcutaneous Electrical Resistance (TER)</u>: is a measure of the electrical impedance of the skin, as a resistance value in kilo Ohms. A simple and robust method of assessing barrier function by recording the passage of ions through the skin using a Wheatstone bridge apparatus