COMMISSION DIRECTIVE 2000/33/EC

of 25 April 2000

adapting to technical progress for the 27th time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (*)

(Text with EEA relevance)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances [1], as last amended by European Parliament and Council Directive 1999/33/EC [2], and in particular Article 28 thereof,

Whereas:

(1) Annex V to Directive 67/548/EEC lays down the methods for the determination of the physico-chemical properties, toxicity and ecotoxicity of substances and preparations. It is necessary to adapt that Annex to technical progress.

(2) According to Article 7(2) of Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes [3], an experiment entailing the use of animals shall not be performed if another scientifically satisfactory method to obtain the result sought is reasonably and practicably available.

(3) The Commission intends to introduce into Annex V to Directive 67/548/EEC certain alternative testing methods not entailing the use of animals, in order to make them available for the testing of chemicals according to Article 3(1) of Directive 67/548/EEC.

(4) The measures provided for in this Directive are in accordance with the opinion of the Committee on the Adaptation to Technical Progress of the Directives for the Elimination of Technical Barriers to Trade in Dangerous Substances and Preparations,

HAS ADOPTED THIS DIRECTIVE:

Article 1

The texts in Annexes I and II to this Directive are added to Part B of Annex V to Directive 67/548/EEC.

Article 2

1. Member States shall bring into force the laws, regulations and administrative provisions necessary to comply with this Directive by 1 October 2001 at the latest. They shall forthwith inform the Commission thereof.

When Member States adopt those provisions, they shall contain a reference to this Directive or be accompanied by such a reference on the occasion of their official publication. Member States shall determine how such reference is to be made.

2. Member States shall communicate to the Commission the main provisions of national law which they adopt in the field covered by this Directive and a correlation table between this Directive and the national provisions adopted.

Article 3

This Directive shall enter into force on the third day following its publication in the *Official Journal of the European Communities*.

Article 4

This Directive is addressed to the Member States.

Done at Brussels, 25 April 2000.

For the Commission
Margot WALLSTRÖM
Member of the Commission

(*) Adopted before the 26th adaptation.
[1] OJ 196, 16.8.1967, p. 1.
[2] OJ L 199, 30.7.1999, p. 57.
[3] OJ L 358, 18.12.1986, p. 1.

ANNEX I

'B.40. SKIN CORROSION

1. METHOD

1.1. Introduction

Two *in vitro* test 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). Description 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 (5 kΩ) (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 polytetrafluoroethylene (PTFE) 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 contianing magnesium sulphate solution (154 mM) (Figure 2).

1.5.3. *Test procedure*

1.5.3.1. Application of the test material

Liquid test substances (150 µl) 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 (150 µl) 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 3 ml magnesium sulphate solution (154 mM). 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 20 kΩ, this may be due to the test substance coating the epidermal surface of the sking 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	10 M 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 5 kΩ, 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, 150 µl 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 20 ml glass scintillation vial) containing deionised water (8 ml). 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 5 ml 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 1 ml sample of the supernatant is then diluted 1 in 5 (v/v) (i.e. 1 ml + 4 ml) with 30 % (w/v) SDS in distilled water. The optical density (OD) of the solution is measured at approximately 565 nm.

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 565 nm = $8{,}7 \times 10^4$; 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	10 M 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 5 kΩ 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 essay 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 the international validation standard (2).

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 25 μl/cm^2). 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 (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 0,3 mg/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 wavelenght 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 essay*

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 5 kΩ, then it is non-corrosive. If the TER value is less than or equal to 5 kΩ, 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 5 kΩ, 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 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 (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**

(1) ECVAM (1998), ECVAM News & Views, *ATLA* 26, pp. 275-280.

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

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

(4) Oliver, G.J.A., Pemberton, M.A. & Rhodes, C. (1986), An *in vitro* skin corrosivity test — modifications and validation, *Food & Chemical Toxicology* 24, pp. 507-512.

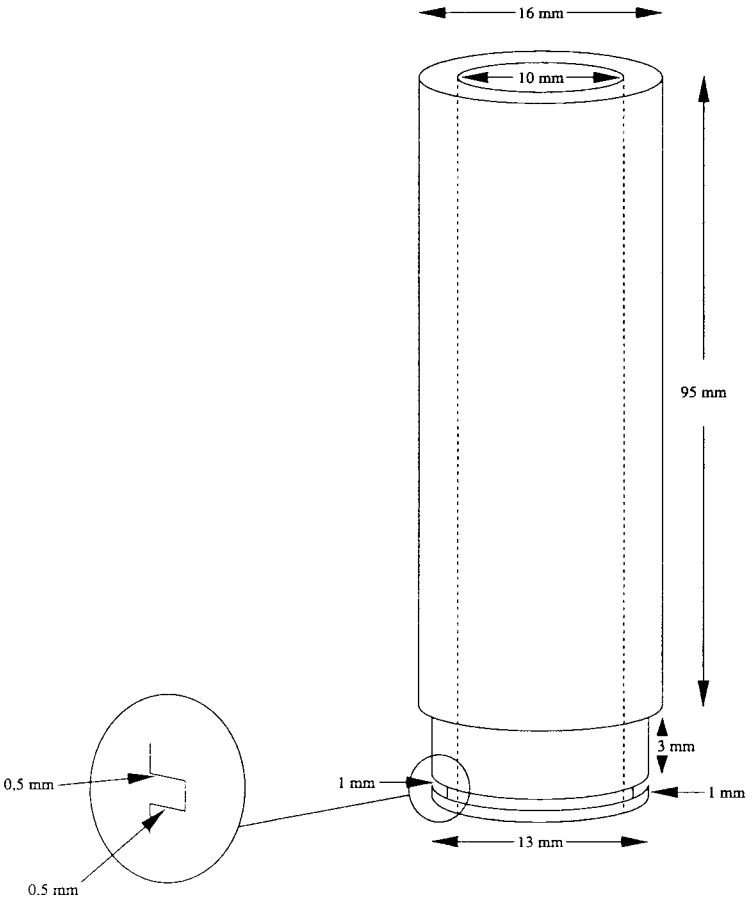
(5) Botham, P.A., Hall, T.J., Dennett, R., McCall, J.C., Basketter, D.A., Whittle, E., Cheeseman, M., Esdaile, D.J. & Gardner, J. (1992), The skin corrosivity test *in vitro*: results of an interlaboratory trial, *Toxicology in Vitro* 6, pp. 191-194.

(6) Worth, A.P., Fentem, J.H., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Esdaile, D.J. & Liebsch, M. (1998), An evaluation of the proposed OECD testing strategy for skin corrosion, *ATLA* 26, pp. 709-720.

(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, pp. 219-255.

Figure 1

PTFE tube dimensions



Electrode dimensions

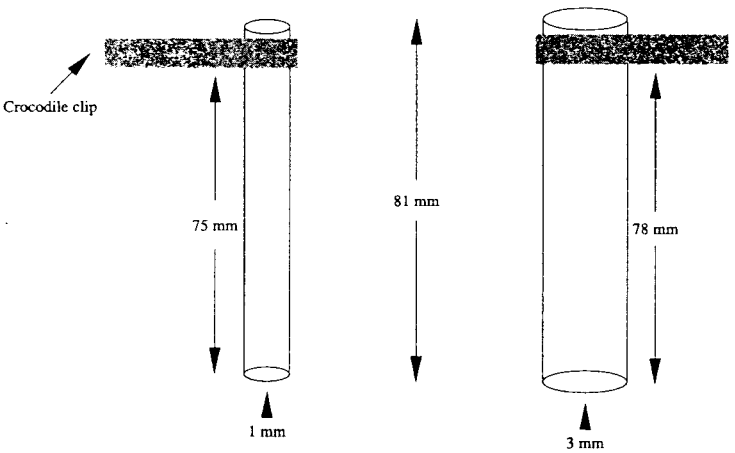
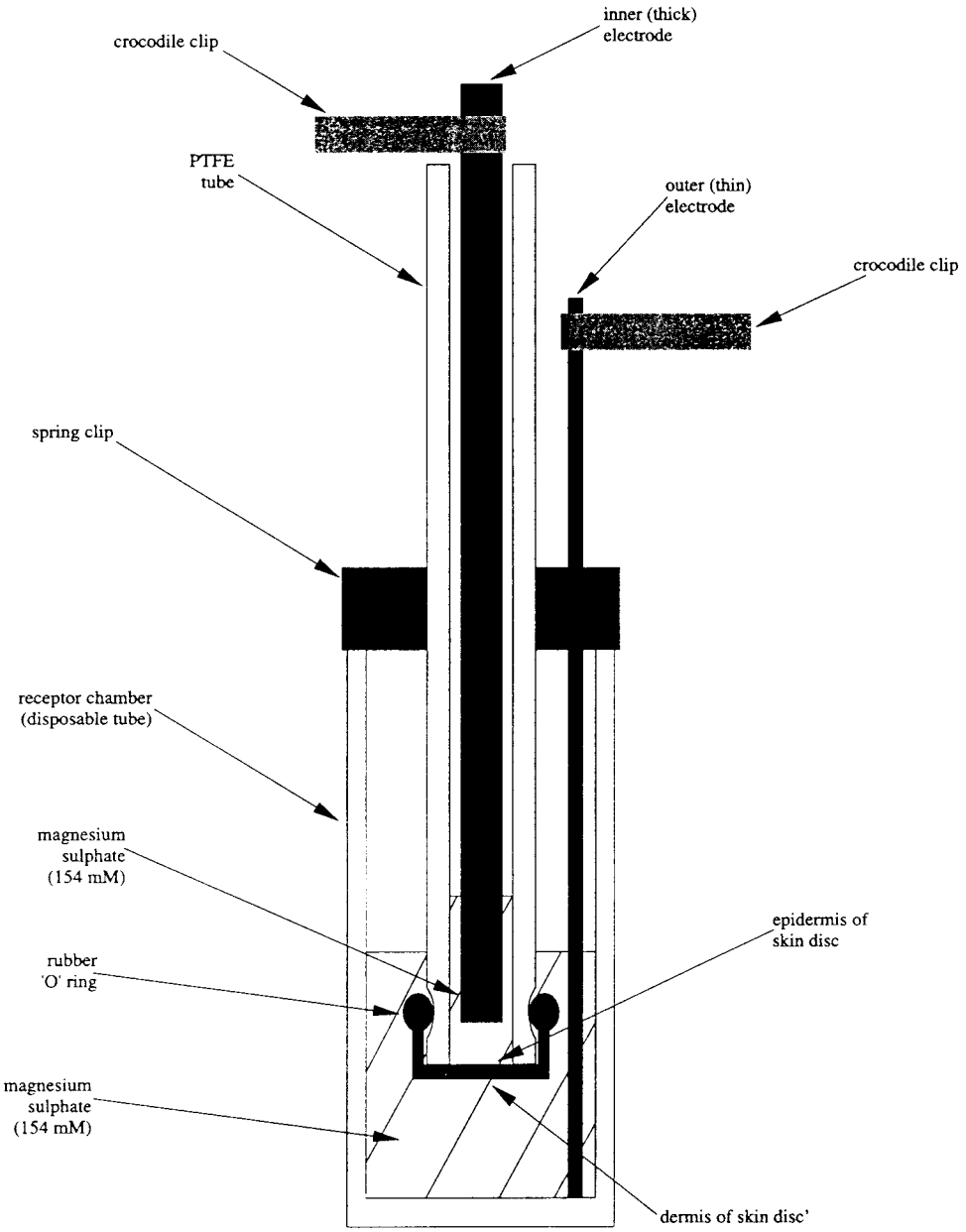


Figure 2

Apparatus for the rat skin TER assay



ANNEX II

'B.41. PHOTOTOXICITY — *IN VITRO* 3T3 NRU PHOTOTOXICITY TEST

1. METHOD

1.1. Introduction

Phototoxicity is defined as a toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after systemic administration of a chemical.

Information derived from the *in vitro* 3T3 NRU phototoxicity test serves to identify the phototoxic potential of a test substance, i.e. the existence or absence of possible hazards likely to arise from a test substance in association with exposure to UV and visible light.

Since the toxicological endpoint of the *in vitro* test is determination of photocytotoxicity, induced by the combined action of a chemical and light, compounds that are phototoxic in vivo after systemic application and distribution to the skin, as well as compounds that act as photoirritants after topical application to the skin, can be identified by the test.

The *in vitro* 3T3 NRU phototoxicity test was developed and validated in a joint EU/COLIPA project from 1992-1997 (1) (2) (3), to establish a valid *in vitro* alternative to the various *in vivo* tests in use. In 1996 an OECD workshop recommended an *in vitro* tier testing approach for phototoxicity assessment (4).

Results from the *in vitro* 3T3 NRU phototoxicity test were compared with acute phototoxicity/photoirritation effects *in vivo* in animals and humans, and the test has been shown to give excellent predictivity for these effects. The test is not designed to predict other adverse effects that may arise from the combined action of a chemical and light, e.g. photogenotoxicity, photoallergy, and photocarcinogenicity, although many chemicals which show these specific properties will react positive in the *in vitro* 3T3 NRU phototoxicity test. In addition, the test is not designed to permit an assessment of phototoxic potency.

A sequential approach to phototoxicity testing of chemicals is set out in the Appendix.

1.2. Definitions

Irradiance: the intensity of ultraviolet (UV) or visible light incident on a surface, measured in W/m^2 or mW/cm^2.

Dose of light: the quantity (= intensity × time) of ultraviolet (UV) or visible radiation incident on a surface, expressed in Joules (= W × s) per surface area, e.g. J/m^2 or J/cm^2.

UV light wavebands: the designations recommended by the CIE (Commission Internationale de L'Eclairage) are: UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm). Other designations are also used: the division between UVB and UVA is often placed at 320 nm, and the UVA may be divided into UV-A1 and UV-A2 with a division made at about 340 nm.

Cell viability: parameter measuring total activity of a cell population (e.g. uptake of the vital dye neutral red into cellular lysosomes) which, depending on the endpoint measured and the test design used, correlates with the total number and /or vitality of the cells.

Relative cell viability: cell viability expressed in relation to negative (solvent) controls which have been taken through the whole test procedure (either + UV or − UV), but not treated with a test chemical.

Prediction model: an algorithm used to transform the results of a toxicity test into a prediction of toxic potential. In the present test guideline, PIF and MPE can be used for transformation of the results of the in vitro 3T3 NRU phototoxicity test into a prediction of phototoxic potential.

PIF (photo irritation factor): a factor generated by comparing two equally effective cytotoxic concentrations (EC_{50}) of the test chemical obtained in the absence (− UV) and in the presence (+ UV) of a non-cytotoxic irradiation with UVA/vis light.

MPE (mean photo effect): a novel measure derived from mathematical analysis of the complete shape of two concentration response curves obtained in the absence (− UV) and in the presence (+ UV) of a noncytotoxic irradiation with UVA/vis light.

Phototoxicity: an acute toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after the systemic administration of a chemical.

Photoirritation: a sub-species of the term "phototoxicity", which is used to describe only those phototoxic reactions which are produced at the skin after exposure to chemicals (topically or orally). These photoxic reactions lead always to non-specific cell damage (sunburn like reactions).

Photoallergy: an acquired immunological reactivity, which does not occur on first treatment with chemical and light, and needs an induction period of one or two weeks before skin reactivity can be demonstrated.

Photogenotoxicity: a genotoxic response observed with a genetic endpoint, which is elicited after the exposure of cells to a non-genotoxic dose of UV/visible light and a non-genotoxic chemical.

Photocarcinogenicity: carcinogenicity induced by repeated application of light and a chemical. The term "photo co-carcinogenesis", is used if UV induced tumorigenesis is enhanced by a chemical.

1.3. **Reference substances**

Besides the positive control chemical chlorpromazine which should be concurrently tested in each assay, for newly establishing the 3T3 NRU phototoxicity test it is recommended to use as reference chemicals a subset from the chemicals used in interlaboratory trials with the present test (1) (3) (13).

1.4. **Initial considerations**

Many types of chemicals have been reported to induce phototoxic effects (5) (6) (7) (8). The only common feature is their ability to absorb light energy within the sunlight region. According to the first law of photochemistry (Grotthaus-Draper's Law) photoreaction requires sufficient absorption of light quanta. Thus, before biological testing according to the present test guideline is considered, a UV/vis absorption spectrum of the test chemical should be determined (e.g. according to OECD Test Guideline 101). If the molar extinction /absorption coefficient is less than 10 litre $\times$ mol^{-1} $\times$ cm^{-1}, the chemical has no photoreactive potential and does not need to be tested in the *in vitro* 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (Appendix).

1.5. **Principle of the test method**

Four mechanisms have been identified by which absorption of light by a (chemical) chromophore can result in a phototoxic response (7). All of them result in cell damage. Therefore, the *in vitro* 3T3 NRU phototoxicity test is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of UVA/vis light. Cytotoxicity in this test is expressed as a concentration dependent reduction of the uptake of the vital dye neutral red (NR) (9) 24 hours after treatment with the test chemical and irradiation.

Balb/c 3T3 cells are maintained in culture for 24 h for the formation of monolayers. Two 96-well plates per test chemical are then preincubated with eight different concentrations of the chemical for 1 h. Thereafter one of the two plates is exposed to a non-cytotoxic UVA/vis light dose of 5 J/cm^2 UVA (+UV experiment), whereas the other plate is kept in the dark (−UV experiment). In both plates, the treatment medium is then replaced by culture medium and after another 24 h of incubation, cell viability is determined by neutral red uptake (NRU) for 3 h. Relative cell viability, expressed as percentage of untreated negative controls, is calculated for each of the eight test concentrations. To predict the phototoxic potential, the concentration responses obtained in the presence (+UV) and in the absence (−UV) of irradiation are compared, usually at the EC_{50} level, i.e. at the concentration inhibiting cell viability by 50 % cf. untreated controls.

1.6. **Quality criteria**

UVA sensitivity of the cells, historical data: cells should be regularly checked for sensitivity to UVA. Cells are seeded at the density used in the *in vitro* 3T3 NRU phototoxicity test, irradiated the next day with UVA doses from 1-9 J/cm^2, and cell viability is determined one day later using the NRU assay. Cells meet the quality criteria, if their viability after irradiation with 5 J/cm^2 UVA is not less than 80 % of the viability of dark controls. At the highest UVA dose of 9 J/cm^2, viability should not be less than 50 % of that of dark controls. This check should be repeated about every 10th passage of the cells.

UVA sensitivity of the negative control cells, current test: the test meets the quality criteria if negative controls (cells in Earl's balanced salt solution (EBSS) with or without 1 % dimethylsulfoxide (DMSO) or 1 % ethanol (EtOH)) in the +UVA experiment show a viability of not less than 80 % of that of non-irradiated cells in the same solvent of the concurrent dark experiment (−UVA).

Viability of negative controls: the absolute optical density ($OD_{540\ NRU}$) measured in the NR extract of the negative controls indicates whether the 1×10^4 cells seeded per well have grown with normal doubling time during the two days of the assay. A test meets the acceptance criteria if the mean $OD_{540\ NRU}$ of untreated controls is ≥ 0,2.

Positive control: a known phototoxic chemical shall be tested concurrently with each *in vitro* 3T3 NRU phototoxicity test. Chlorpromazine (CPZ) was used as positive control in the EU/COLIPA validation study and is therefore recommended. For CPZ tested with the standard protocol in the *in vitro* 3T3 NRU phototoxicity test, the following test acceptance criteria were defined: CPZ irradiated (+UVA): EC_{50} = 0,1 to 2,0 μg/ml, CPZ non-irradiated (−UVA): EC_{50} = 7,0 to 90,0 μg/ml. The photo irritation factor (PIF), i.e. the shift of EC_{50} should be at least 6.

Other known phototoxic chemicals, suitable for the chemical class or solubility characteristics of the test chemical being evaluated, may be used as the concurrent positive controls, in place of CPZ. In this case, based on historical data, the ranges of EC_{50} values and PIF or MPE (mean photo effect) should be adequately defined as acceptance criteria for the test.

1.7. **Description of the test method**

1.7.1. *Preparations*

1.7.1.1. Cells

A permanent mouse fibroblast cell line — Balb/c 3T3, clone 31 — either from ATCC or from ECACC was used in the validation study, and is therefore recommended. Other cells or cell lines may be successfully used with the same test protocol, if the culture conditions are adapted to the specific needs of the cells, but equivalency must be demonstrated.

Cells should be checked regularly for the absence of mycoplasma contamination and should only be used if the results of such checking was satisfactory.

Since the UVA sensitivity of cells may increase with the number of passages, Balb/c 3T3 cells of the lowest obtainable passage number should be used, preferably less than 100. It is important that UVA sensitivity of the Balb/c 3T3 cells is regularly checked according to the quality control procedure described in this guideline.

1.7.1.2. Media and culture conditions

Appropriate culture media and incubation conditions should be used for routine cell passage and during the test procedure. For Balb/c 3T3 cells, these are DMEM supplemented with 10% new-born calf serum, 4 mM glutamine, penicillin and streptomycin; and humidified incubation at 37°C /7,5% CO_2. It is particularly important that cell culture conditions ensure a cell cycle time within the normal historical range of the cells or cell line used.

1.7.1.3. Preparation of cultures

Cells from frozen stock cultures are seeded in culture medium at an appropriate density and subcultured at least once before they are used in the *in vitro* 3T3 NRU phototoxicity test.

For the phototoxicity test cells are seeded in culture medium at a density such that cultures will not reach confluence by the end of the test, i.e. when cell viability is determined 48 h after the seeding of the cells. For Balb/c 3T3 cells grown in 96-well plates, 1×10^4 cells per well is the recommended cell density.

For each test chemical, cells are seeded identically in two separate 96-well plates, which are then taken concurrently through the whole test procedure under identical culture conditions, except for the time period where one of the plates is irradiated (+UVA/vis) and the other one is kept in the dark (−UVA/vis).

1.7.1.4. Metabolic activation

Whereas the use of metabolising systems is a general requirement for all *in vitro* tests for the prediction of genotoxic and carcinogenic potential, up to now, in the case of phototoxicology, no chemical is known for which metabolic transformation is needed for the chemical to act as a phototoxin *in vivo* or *in vitro*. Thus, it is neither considered necessary nor scientifically justified for the present test to be performed with a metabolic activation system.

1.7.1.5. Test chemical/preparation

Test chemicals must be freshly prepared immediately prior to use, unless stability data demonstrate the acceptability of storage. Preparation under red light may be required when rapid photodegradation is likely to occur.

Test chemicals should be dissolved in buffered salt solutions, e.g. Earl's balanced salt solution, (EBSS) or phosphate buffered saline (PBS), which, to avoid interference during irradiation, must be free from protein components and light absorbing pH indicator colours.

Test chemicals of limited solubility in water should be dissolved in appropriate solvents at 100-fold the desired final concentration and then diluted 1:100 with the buffered salt solution. If a solvent is used it must be present at a constant volume of 1% (v/v) in all cultures, i.e. in the negative controls as well as in all concentrations of the test chemical.

Dimethylsulphoxide (DMSO) and ethanol (EtOH) are the recommended solvents. Other solvents of low cytotoxicity (e.g. acetone) may be appropriate, but they should carefully be assessed for specific properties, e.g. reaction with the test chemical, quenching of the phototoxic effect, radical catching properties.

Vortex mixing and/or sonication and/or warming to 37 °C may be used, if necessary, to aid solubilisation.

1.7.1.6. UV irradiation/preparation

Light source: the choice of an appropriate light source and appropriate filtering is the most crucial factor in phototoxicity testing. UVA and visible regions are usually associated with photosensitisation (7) (10), whereas UVB is of less relevance and is directly highly cytotoxic, increasing its cytotoxicity through 1000 fold from 313 to 280 nm (11). Criteria for the choice of an appropriate light source should include the essential requirement that the light source emits wavelengths absorbed by the test chemical and that the dose of light (achievable in a reasonable time) should be sufficient for the detection of known photosensitisers. Furthermore, the wavelengths and doses employed should not be unduly deleterious to the test system, which includes the emission of heat (infra-red region).

The simulation of sunlight with solar simulators is considered the optimal light source. Both, Xenon arcs and (doped) mercury-metal halide arcs are used in solar simulators. The latter have the advantage of emitting less heat and of being cheaper, but the match to sunlight is not perfect. Since all solar simulators emit significant quantities of UVB, they should be suitably filtered to attenuate the highly cytotoxic UVB wavelengths.

For the *in vitro* 3T3 NRU phototoxicity test an irradiance spectrum practically devoid of UVB should be used (UVA:UVB $\sim$ 1:20). An example of the spectral irradiance distribution of the filtered solar simulator used in the validation study of the *in vitro* 3T3 NRU phototoxicity test has been published (3).

Dosimetry: the intensity of light (irradiance) should be regularly checked before each phototoxicity test, by using a suitable broadband UV-meter. The UV-meter must have been calibrated to the source. The performance of the UV-meter should be checked, and for this purpose, the use of a second, reference UV-meter of the same type and identical calibration is recommended. Ideally, at greater intervals, a spectroradiometer should be used to measure the spectral irradiance of the filtered light source and to check the calibration of the broadband UV-meter, but such instruments require skilled operation by appropriately trained persons.

A dose of 5 J/cm^2 (UVA) was determined in the validation study to be non-cytotoxic to Balb/c 3T3 cells and sufficiently potent to excite even weak phototoxic chemicals. To achieve 5 J/cm^2 within a time period of 50 min, irradiance has to be adjusted to 1,666 mW/cm^2. If another cell line or a different light source are used, the UVA dose may have to be slightly adapted, by using the criteria of being non-deleterious to the cells and sufficient to detect standard phototoxins. The time of light exposure is calculated in the following way:

$$t(\text{min}) = \frac{\text{Strahlungsdosis (J/cm}^2) \times 1000}{\text{Strahlung (mW/cm}^2) \times 60} \qquad (1\ \text{J} = 1\ \text{W sec})$$

1.7.2. *Test conditions*

The maximum concentration of a test chemical should not exceed 100 µg/ml, since all phototoxic chemicals were detected at lower concentrations, whereas at higher concentrations the incidence of false positives (overpredictions) increases (13). The pH of the highest concentration of the test chemical should be satisfactory (pH range: 6,5 - 7,8).

The ranges of concentrations of a chemical tested in the presence (+UVA) and in the absence (−UVA) of light should be adequately determined in preceding range-finder experiments. Range and intercept of a concentration series shall be adjusted in such a way that concentration-response curves are sufficiently supported by experimental data. Geometric concentration series (with a constant dilution factor) should be used.

1.7.3. *Test procedure* ([1])

1.7.3.1. First day

Prepare a cell suspension of 1×105^5 cells/ml in culture medium and dispense 100 µl culture medium only into the peripheral wells of a 96-well tissue culture microtiter plate (= blanks). In the remaining wells, dispense 100 µl of a cell suspension of 1×10^5 cells/ml (= 1×10^4 cells/well). For each test chemical, prepare two plates: one for determination of cytotoxicity (−UVA), and the other for determination of photocytotoxicity (+UVA).

Incubate the cells for 24 h (7,5% CO_2, 37 °C) until they form a half-confluent monolayer. This incubation period allows for cell recovery and adherence, and for exponential growth.

1.7.3.2. Second day

After incubation, decant the culture medium from the cells and wash twice with 150 µl EBSS/PBS per well. Add 100 µl of EBSS/PBS containing the appropriate concentration of test chemical or just solvent (negative control). Apply 8 different concentrations of the test chemical. Incubate cells with the test chemical in the dark for 60 minutes (7,5% CO_2, 37 °C).

To perform the (+UVA) part of the assay, irradiate the cells at room temperature for 50 minutes through the lid of the 96-well plate with 1,7 mW/cm^2 UVA (= 5 J/cm^2). Ventilate with a fan to prevent H_2O condensation under the lid. Keep duplicate plates (−UVA) at room temperature in a dark box for 50 min (= UVA exposure time).

Decant test solution and wash twice with 150 µl EBSS/PBS. Replace EBSS/PBS with culture medium and incubate (7,5% CO_2, 37 °C) overnight (18-22 h).

1.7.3.3. Third day

Microscopic evaluation

Examine the cells under a phase-contrast microscope. Record changes in morphology of the cells due to cytotoxic effects of the test chemical. This check is recommended, to exclude experimental errors, but these records are not used for evaluation of cytotoxicity or phototoxicity

Neutral red uptake test

Wash the cells with 150 µl prewarmed EBSS/PBS. Remove the washing solution by gentle tapping. Add 100 µl NR medium and incubate at 37 °C, in a humidified atmosphere of 7,5% CO_2, for 3 h.

After incubation, remove the NR medium, and wash the cells with 150 µl EBSS/PBS. Decant and blot EBSS/PBS totally. (Optionally: centrifuge reversed plate.)

Add exactly 150 µl NR desorb solution (freshly prepared ethanol/acetic acid)

Shake microtiter plate rapidly on a microtiter plate shaker for 10 min, until the NR has been extracted from the cells and has formed a homogeneous solution.

Measure the optical density of NR extract at 540 nm in a spectrophotometer, using blanks as a reference. Save the data in appropriate file format (e.g. ASCII) for subsequent analysis.

([1]) Additional details can be found in reference 12.

2. DATA

2.1. Quality and quantity of data

The data should permit a meaningful analysis of the concentration-response obtained in the presence and in the absence of UVA/vis irradiation. If cytotoxicity is found, both the concentration range and the intercept of individual concentrations should be set in such a way as to allow the fit of a curve to experimental data. Due to the fact that a test chemical might not be cytotoxic up to the defined limit concentration of 100 μg/ml in the dark experiment (−UVA), but highly cytotoxic when irradiated (+UVA), the concentration ranges to be tested in both parts of the experiment may need to differ by orders of magnitude to fulfil the requirement of adequate data quality. If no cytotoxicity is found in both parts of the experiment (−UVA and +UVA), testing with a great intercept between single doses up to the highest concentration is sufficient.

There is no requirement for verification of a clear positive result by performing a repeat experiment. In addition, clear negative results need not to be verified, provided the test chemical was tested at sufficiently high concentrations. In such cases, one main experiment, supported by one or more range-finding preliminary experiments, is sufficient.

Tests with borderline results near to the cut-off line of the prediction model should be repeated for verification.

If repeat testing is considered necessary, then variation of the experimental conditions may be important to achieve a clear result. A key variable in this test is preparation of solutions of the test chemical. Hence, variation of these conditions (co-solvent, trituration, sonication) may be most relevant in the repetition of a test. Alternatively, variation of the pre-irradiation incubation time may be considered. A shorter time can be relevant for water-unstable chemicals.

2.2. Treatment of results

Where possible, the concentration of a test chemical reflecting a 50% inhibition of the cellular NRU (EC_{50}) is determined. This can be done by applying any appropriate non-linear regression procedure (preferably a Hill function or logistic regression) to the concentration-response data, or by using other fitting procedures (14). Before using an EC_{50} for further calculations, the quality of the fit should be appropriately checked. Alternatively, graphical fitting methods can be used to calculate the EC_{50}. In this case, the use of probability paper is recommended (x-scale: log, y-scale: probit), as in many cases the concentration response function will become almost linear after this transformation.

2.3. Evaluation of results (prediction models)

2.3.1. *Prediction model version 1: photo-irritation-factor (PIF)*

If both, in the presence (+UVA) and in the absence (−UVA) of light, complete concentration response curves are obtained, a photo-irritation-factor (PIF) is calculated by means of the following formula:

(a) $$PIF = \frac{EC_{50}\,(-\,UV)}{EC_{50}\,(+\,UV)}$$

A PIF < 5, predicts no phototoxic potential, whereas a PIF ≥ 5 predicts phototoxic potential.

If a chemical is only cytotoxic +UVA and is not cytotoxic when tested −UVA, the PIF cannot be calculated, although this is a result that indicates phototoxic potential. In such cases, a "> PIF" can be calculated if the (−UV) cytotoxicity test is performed up to the highest test concentration (C_{max}) and this value is used for calculation of the "> PIF";

(b) $$> PIF = \frac{C_{max}\ (-\,UV)}{EC_{50}\ (+\,UV)}$$

If only a "> PIF" can be obtained, then any value > 1 predicts phototoxic potential.

If both EC_{50} (−UV) and EC_{50} (+UV) cannot be calculated due to the fact that a chemical does not show any cytotoxicity up to the highest test concentration, this indicates no phototoxic potential. In such cases, a formal "PIF = *1" is used to characterise the result;

(c) $$PIF = *1 = \frac{C_{max}\ (-\,UV)}{C_{max}\ (+\,UV)}$$

If only a "PIF = *1" can be obtained, this predicts no phototoxic potential.

In cases (b) and (c), concentrations achieved in the *in vitro* 3T3 NRU phototoxicity test should be carefully taken into consideration when predicting phototoxic potential.

2.3.2. *Prediction model version 2: mean-photo effect (MPE)*

Alternatively, a novel version of the model for predicting phototoxic potential can be applied, which has been developed by using data of the EU/COLIPA validation study (15) and tested under blind conditions in a subsequent study on the *in vitro* phototoxicity of UV filter chemicals (13). This model overcomes the limitation of the PIF model in cases where an EC_{50} cannot be obtained. The model uses the "mean photo effect" (MPE), a measure which is based on comparison of the complete concentration response curves. For application of the MPE model, a special computer software was developed at the Humboldt University (Berlin), which can be obtained free of charge.

2.4. **Interpretation of results**

A positive result in the *in vitro* 3T3 NRU phototoxicity test (PIF ≥ 5 or MPE ≥ 0,1) indicates that the test substance has phototoxic potential. If this result is obtained at concentrations below 10 μg/ml, the test chemical is also likely to act as phototoxin also under various exposure conditions *in vivo*. If a positive result is obtained only at the highest test concentration of 100 μg/ml, further considerations may be necessary for the assessment of hazard or phototoxic potency. These may include data on penetration, absorption and possible accumulation of the chemical in the skin, or testing of the chemical in a confirmatory alternative test, e.g. using a human *in vitro* skin model.

A negative result from the *in vitro* 3T3 NRU phototoxicity test (PIF < 5 or MPE < 0,1) indicates that the test substance was not phototoxic to the cultured mammalian cells under the conditions used. In cases where the chemical could be tested up to the highest concentration of 100 μg/ml, a negative result indicates that the chemical has no phototoxic potential, and phototoxicity *in vivo* may be considered unlikely. In cases where identical concentration-toxicity responses (EC_{50} +UV and EC_{50}−UV) were obtained at lower concentrations, the interpretation of data would be the same. In contrast, if no toxicity was demonstrated (+UV and −UV) and if aqueous solubility limited concentrations to values less than 100 μg/ml, then compatibility of the test substance with the assay may be questioned and confirmatory testing should be considered (e.g. using an *in vitro* skin model, or an *ex vivo* skin model or an *in vivo* test).

3. **REPORTING**

Test report

The test report must include the following information:

Test chemical:

— identification data and CAS No, if known,

— physical nature and purity,

— physicochemical properties relevant to conduct of the study,

— stability and photostability, if known.

Solvent:

— justification for choice of solvent,

— solubility of the test chemical in this solvent,

— percentage of solvent present in treatment medium (EBSS or PBS).

Cells:

— type and source of cells,

— absence of mycoplasma,

— number of cell passages, if known,

— UVA sensitivity of cells, determined with the irradiation equipment, used in the *in vitro* 3T3 NRU phototoxicity test.

Test conditions (a) — incubation before and after treatment:

— type and composition of culture medium,

— incubation conditions (CO_2 concentration, temperature, humidity),

— duration of incubation (pre-treatment, post-treatment).

Test conditions (b) — treatment with the chemical:

— rationale for selection of concentrations of the test chemical used both in the presence and in the absence of UV/vis irradiation,

— in case of limited solubility of the test chemical and absence of cytotoxicity, rationale for the highest concentration tested,

— type and composition of treatment medium (buffered salt solution),

— duration of the chemical treatment.

Test conditions (c) — irradiation:

— rationale for selection of the light source used,

— spectral irradiance characteristics of the light source,

— transmission/absorption characteristics of the filter(s) used,

— characteristics of the radiometer and details on its calibration,

— distance of the light source from the test system,

— UVA irradiance at this distance, expressed in mW/cm^2,

— duration of the UV/vis light exposure,

— UVA dose (irradiance × time), expressed in J/cm^2,

— temperature employed to cell cultures during irradiation, and for cell cultures concurrently kept in the dark.

Test conditions (d) — NRU test:

— composition of NR medium,

— duration of NR incubation,

— incubation conditions (CO_2 concentration, temperature, humidity),

— NR extraction conditions (extractant, duration),

— wavelength used for spectrophotometric reading of NR optical density,

— second wavelength (reference), if used,

— content spectrophotometer blank, if used.

Results:

— cell viability obtained at each concentration of the test chemical, expressed in percent mean viability of controls,

— concentration-response curves, (test chemical concentration v. relative cell viability), obtained in concurrent +UVA and −UVA experiments,

— data analysis of the concentration response curves: if possible, computation/calculation of EC_{50} (+UVA) and EC_{50} (−UVA),

— comparison of the two concentration response curves, obtained in the presence and in the absence of UVA/vis irradiation, either by calculation of the photo irritation factor (PIF), or by calculation of the mean photo effect (MPE),

— classification of phototoxic potential,

— test acceptance criteria (a) — concurrent negative control:

  — absolute viability (optical density of NR extract) of irradiated and non irradiated cells,

  — historical data of negative control, mean and standard deviation.

— test acceptance criteria (b) — concurrent positive control:

  — EC_{50} (+UVA) and EC_{50} (−UVA) and PIF of positive control chemical,

  — historical data of positive control chemical: EC_{50} (+UVA) and EC_{50} (−UVA) and PIF, mean and standard deviation.

Discussion of the results.

Conclusions.

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Appendix

Role of the 3T3 NRU PT in a sequential approach to the phototoxicity testing of chemicals