ANNEX

DEFINITIONS

1. Skin corrosion *in vivo:* 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 discolouration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

2. Transcutaneous Electrical Resistance (TER): 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.

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27 March 2002

OECD/OCDE

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE 431

In Vitro Skin Corrosion: Human Skin Model Test

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 does not require the use of live animals or animal tissue for the assessment of skin corrosivity.

2. The assessment of skin corrosivity has typically involved the use of laboratory animals (2). Concern for the pain and suffering involved with this procedure has been addressed in the 1992 revision of Guideline 404 that allows for the determination of skin corrosion by using alternative, *in vitro*, methods, avoiding pain and suffering of animals.

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 (9) led to the recommendation of two equivalent tests as replacements for the *in vivo* skin corrosivity test (10)(11): the human skin model test (this Guideline) and the transcutaneous electrical resistance test (see Test Guideline 430).

DEFINITIONS

4. Definitions used are provided in the Annex .

INITIAL CONSIDERATIONS

5. Validation studies have shown that tests employing human skin models (3)(4)(5)(9) are able to reliably discriminate between known skin corrosives and non-corrosives. The test protocol may also enable the distinction between severe and less severe skin corrosives.

6. The test described in this Guideline allows the discrimination between corrosive and non-corrosive chemical substances and mixtures. It does not provide information on skin irritation, nor does it allow the subcategorisation of corrosive substances as permitted in the Globally Harmonised Classification System (GHS) (1).

7. For a full evaluation of local skin effects after single dermal exposure, it is recommended to follow the sequential testing strategy as appended to Test Guideline 404 (2). 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 topically 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 (12)] below defined threshold levels at specified exposure periods. The principle of the human skin model assay is based on the hypothesis that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion, and are cytotoxic to the underlying cell layers.

PROCEDURE

Human skin models

9. Human skin models can be constructed or obtained commercially (e.g., the EpiDerm™ model) (13) (14) or be developed or constructed in the testing laboratory (15)(16). Human skin models used for this test must comply with the following:

General Model Conditions:

10. Human keratinocytes should be used to construct the epithelium. Multiple layers of viable epithelial cells should be present under a functional stratum corneum. The skin model may also have a stromal component layer. Stratum corneum should be multi-layered with the necessary lipid profile to produce a functional barrier. The containment properties of the model should prevent the passage of material around the stratum corneum to the viable tissue. Passage of test chemicals around the stratum corneum will lead to poor modeling of the exposure to skin. The skin model should be free of contamination with bacteria (including mycoplasma) or fungii.

Functional Model Conditions :

11. The magnitude of viability is usually quantified by using MTT or other metabolically converted vital dyes. In these cases the optical density (OD) of the extracted (solubilized) dye from the negative control tissue should be at least 20 fold greater than the OD of the extraction solvent alone [for an overview, see (17)]. The negative control tissue should be stable in culture (provide similar viability measurements) for the duration of the test exposure period. The stratum corneum should be sufficiently robust to resist the rapid penetration of certain cytotoxic marker chemicals (e.g.,1% Triton X-100). This property can be estimated by the exposure time required to reduce cell viability by 50% (ET50) (e.g. for the EpiDerm™ model this is > 2 hours). The tissue should demonstrate reproductivity over time and preferably between laboratories. Moreover it should be capable of predicting the corrosive potential of the reference chemicals (see Table 1) when used in the testing protocol selected.

Application of the test and control substances

12. Two tissue replicates are used for each treatment (exposure time), including controls. For liquid materials, sufficient test substance must be applied to uniformly cover the skin surface; a minimum of 25µL/cm^2 should be used. For solid materials, sufficient test substance must be applied evenly to cover the skin, and it should be moistened with water to ensure good contact with the skin. Where appropriate, solids should be ground to a powder before application. The application method should be appropriate for the test substance (see e.g., reference 5). At the end of the exposure period, the test material must be

carefully washed from the skin surface with saline solution.

13. Concurrent positive and negative controls should be used for each study to ensure adequate performance of the experimental model. The suggested positive control substances are glacial acetic acid or 8N KOH. The suggested negative controls are 0.1 N NaCl, saline or water.

Cell viability measurements

14. Only quantitative, validated, methods can be used to measure cell viability. Furthermore, the measure of viability must be compatible with use in a three-dimensional tissue construct. Non-specific dye binding must not interfere with the viability measurement. Protein binding dyes and those which do not undergo metabolic conversion (e.g. neutral red) are therefore not appropriate. The most frequently used assay is MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1)] reduction, which has been shown to give accurate and reproducible results (5) but others may be used. The skin sample is placed in an MTT solution of appropriate concentration (e.g. 0.3 – 1 mg/mL) at appropriate incubation temperature for 3 hours. The precipitated blue formazan product is then extracted using a solvent (isopropanol), and the concentration of the formazan is measured by determining the OD at a wavelength between 540 and 595 nm.

15. Chemical action by the test material on the vital dye may mimic that of cellular metabolism leading to a false estimate of viability. This has been shown to happen when such a test material is not completely removed from the skin by rinsing (9). If the test material directly acts on the vital dye, additional controls should be used to detect and correct for test substance interference with the viability measurement (9)(18).

Interpretation of results

16. The OD values obtained for each test sample can be used to calculate a percentage viability relative to the negative control, which is arbitrarily set at 100%. The cut-off percentage cell viability value distinguishing corrosive from non-corrosive test materials (or discriminating between different corrosive classes), or the statistical procedure(s) used to evaluate the results and identify corrosive materials, must be clearly defined and documented, and be shown to be appropriate. In general, these cut-off values are established during test optimisation, tested during a prevalidation phase, and confirmed in a validation study. As an example, the prediction of corrosivity associated with the EpiDerm™ model is (9):

17. The test substance is considered to be corrosive to skin:

i) if the viability after 3 minutes exposure is less than 50%, or

ii) if the viability after 3 minutes exposure is greater than or equal to 50 % and the viability after 1 hour exposure is less than 15%.

18. The test substance is considered to be non-corrosive to skin:

i) if the viability after 3 minutes exposure is greater than or equal to 50% and the viability after 1 hour exposure is greater than or equal to 15%.

DATA AND REPORTING

Data

19. For each tissue, OD values and calculated percentage cell viability data for the test material, positive and negative controls, should be reported in tabular form, including data from replicate repeat experiments as appropriate, mean and individual values.

Test report

20. The test report must include the following information:

Test and Control Substances:
- identification data and CAS number, if known;
- physical nature and purity;
- physico-chemical properties 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.

Justification of the skin model and protocol used.

Test Conditions:
- cell system used;
- calibration information for measuring device used for measuring cell viability (e.g. Spectrophotometer);
- supporting information for the specific skin model used including its validity.
- details of test procedure used;
- test doses used;
- description of any modifications of the test procedure;
- reference to historical data of the model;
- description of evaluation criteria used.

Results:
- tabulation of data from individual test samples;
- description of other effects observed.

Discussion of the results.

Conclusion

LITERATURE

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Table 1: Reference Chemicals

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
Sulfuric 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.

ANNEX

DEFINITIONS

1. Skin corrosion *in vivo:* 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 discolouration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

2. Cell viability: parameter measuring total activity of a cell population (e.g., as ability of cellular mitochonrial dehidrogenases to reduce the vital dye MTT), which, depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of the cells.

OECD/OCDE

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GUIDELINE FOR TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE: 432

In Vitro 3T3 NRU phototoxicity test

INTRODUCTION

1. Phototoxicity is defined as a toxic response from a substance applied to the body which is either elicited or increased (apparent at lower dose levels) after subsequent exposure to light, or that is induced by skin irradiation after systemic administration of a substance.

2. The *in vitro* 3T3 NRU phototoxicity test is used to identify the phototoxic potential of a test substance induced by the excited chemical after exposure to light. The test evaluates photo-cytotoxicity by the relative reduction in viability of cells exposed to the chemical in the presence versus absence of light. Substances identified by this test are likely to be phototoxic *in vivo,* following systemic application and distribution to the skin, or after topical application.

3. Definitions used are provided in Annex 1.

INITIAL CONSIDERATION

4. Many types of chemicals have been reported to induce phototoxic effects (1)(2)(3)(4). Their common feature is their ability to absorb light energy within the sunlight range. According to the first law of photochemistry (Grotthaus-Draper Law), photoreaction requires sufficient absorption of light quanta. Thus, before biological testing is considered, a UV/vis absorption spectrum of the test chemical must be determined according to OECD Test Guideline 101. If the molar extinction/absorption coefficient is less than 10 litre x mol^{-1} x cm^{-1} the chemical has no photoreactive potential and may not need to be tested in the *in vitro* 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (1)(5). See also Annex 2.

5. The reliability and relevance of the *in vitro* 3T3 NRU phototoxicity test was recently evaluated (6)(7)(8). The *in vitro* 3T3 NRU phototoxicity test was shown to be predictive of acute phototoxicity effects in animals and humans *in vivo*. The test is not designed to predict other adverse effects that may arise from combined action of a chemical and light, e.g., it does not address photogenotoxicity, photoallergy, or photocarcinogenicity, nor does it allow an assessment of phototoxic potency. In addition, the test has not been designed to address indirect mechanisms of phototoxicity, effects of metabolites of the test substance, or effects of mixtures.

6. 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, there are only rare examples where 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.

PRINCIPLE OF THE TEST METHOD

7. 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 simulated solar light. Cytotoxicity in this test is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red when measured 24 hours after treatment with the test chemical and irradiation (9).

8. Balb/c 3T3 cells are maintained in culture for 24 h for formation of monolayers. Two 96-well plates per test chemical are pre-incubated with eight different concentrations of the test substance for 1 h. Thereafter one of the two plates is exposed to the highest non-cytotoxic irradiation dose whereas the other plate is kept in the dark. 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. Cell viability is expressed as percentage of untreated solvent controls and is calculated for each test concentration. To predict the phototoxic potential, the concentration responses obtained in the presence and in the absence of irradiation are compared, usually at the IC_{50} level, i.e., the concentration reducing cell viability to 50 % compared to the untreated controls.

DESCRIPTION OF THE TEST METHOD

Preparations

Cells

9. A permanent mouse fibroblast cell line, Balb/c 3T3, clone 31, either from the American Type Culture Collection (ATCC), Manassas, VA, USA, or from the European Collection of Cell Cultures (ECACC), Salisbury, Wiltshire, UK, was used in the validation study, and therefore is recommended. Other cells or cell lines may be used with the same test procedure if culture conditions are adapted to the specific needs of the cells, but equivalency must be demonstrated.

10. Cells should be checked regularly for the absence of mycoplasma contamination and only used if none is found (10).

11. It is important that UV sensitivity of the cells is checked regularly according to the quality control procedure described in this guideline. Because the UVA sensitivity of cells may increase with the number of passages, Balb/c 3T3 cells of the lowest obtainable passage number, preferably less than 100, should be used. (See paragraph 29and Annex 3).

Media and culture conditions

12. Appropriate culture media and incubation conditions should be used for routine cell passage and during the test procedure, e.g., for Balb/c 3T3 cells these are DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% new-born calf serum, 4 mM glutamine, penicillin (100 IU), and streptomycin (100 µg/mL), and humidified incubation at 37^0 C, 5-7.5% CO_2 depending on the buffer (see paragraph 17). It is particularly important that cell culture conditions assure a cell cycle time within the normal historical range of the cells or cell line used.

Preparation of cultures

13. 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.

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

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

Preparation of test substance

16. Test substances must be prepared fresh, immediately prior to use unless data demonstrate their stability in storage. It is recommended that all chemical handling and the initial treatment of cells be performed under light conditions that would avoid photoactivation or degradation of the test substance prior to irradiation.

17. Test chemicals shall be dissolved in buffered salt solutions, e.g. Earle's Balanced Salt Solution (EBSS), or other physiologically balanced buffer solutions, which must be free from protein components, light absorbing components (e.g., pH-indicator colours and vitamins) to avoid interference during irradiation.

18. Test chemicals of limited solubility in water should be dissolved in an appropriate solvent. If a solvent is used it must be present at a constant volume in all cultures, i.e. in the negative (solvent) controls as well as in all concentrations of

the test chemical, and be noncytotoxic at that concentration. Test chemical concentrations should be selected so as to avoid precipitate or cloudy solutions.

19. Dimethylsulphoxide (DMSO) and ethanol (ETOH) are the recommended solvents. Other solvents of low cytotoxicity may be appropriate. Prior to use, all solvents should be assessed for specific properties, e.g., reaction with the test chemical, quenching of the phototoxic effect, radical scavenging properties and/or chemical stability in the solvent.

20. Vortex mixing and/or sonication and/or warming to appropriate temperatures may be used to aid solubilisation unless this would affect the stability of the test chemical.

Irradiation Conditions

21. *Light source:* the choice of an appropriate light source and filters is a crucial factor in phototoxicity testing. Light of the UVA and visible regions is usually associated with phototoxic reactions *in vivo* (3)(11), whereas generally UVB is of less relevance but is highly cytotoxic; the cytotoxicity increases 1000-fold as the wavelength goes from 313 to 280 nm (12). Criteria for the choice of an appropriate light source must include the requirement that the light source emits wavelengths absorbed by the test chemical (absorption spectrum) and that the dose of light (achievable in a reasonable exposure time) should be sufficient for the detection of known photocytotoxic chemicals. Furthermore, the wavelengths and doses employed should not be unduly deleterious to the test system, e.g., the emission of heat (infrared region).

22. Simulation of sunlight with solar simulators is considered the optimal artificial light source. The irradiation power distribution of the filtered solar simulator should be close to that of outdoor daylight given in (13). Both, Xenon arcs and (doped) mercury-metal halide arcs are used as solar simulators (14). The latter have the advantage of emitting less heat and being cheaper, but the match to sunlight is less perfect compared to that of xenon arcs. Because all solar simulators emit significant quantities of UVB they should be suitably filtered to attenuate the highly cytotoxic UVB wavelengths. Because cell culture plastic materials contain UV stabilisers the spectrum should be measured through the same type of 96-well plate lid as will be used in the assay. 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 is given in (8). See also Annex 3 Figure 1.

23. *Dosimetry:* 'The intensity of light (irradiance) should be regularly checked before each phototoxicity test using a suitable broadband UV-meter. The intensity should be measured through the same type of 96-well plate lid as will be used in the assay. 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.

24. A dose of 5 J/cm^2 (as measured in the UVA range) was determined in the validation study to be non-cytotoxic to Balb/c 3T3 cells and sufficiently potent to excite chemicals to elicit phototoxic reactions, e.g. to achieve 5 J/cm^2 within a time period of 50 min, irradiance was adjusted to 1.7 mW/cm^2. See Annex 3 Figure 2. If another cell line or a different light source are used, the irradiation dose may have to be calibrated so that a dose regimen can be selected that is not deleterious to the cells but sufficient to excite standard phototoxins. The time of light exposure is calculated in the following way:

$$t\,(\text{min}) = \frac{\text{irradiation dose}\,(\text{J/cm}^2) \times 1000}{\text{irradiance}\,(\text{mW/cm}^2) \times 60} \qquad (1\,\text{J} = 1\,\text{Wsec})$$

Test conditions

Test substance concentrations

25. The ranges of concentrations of a chemical tested in the presence (+Irr) and in the absence (-Irr) of light should be adequately determined in dose range-finding experiments. It may be useful to assess solubility initially and at 60 min (or whatever treatment time is to be used), as solubility can change during time or during the course of exposure. To avoid toxicity induced by improper culture conditions or by highly acidic or alkaline chemicals, the pH of the cell cultures with added test chemical should be in the range 6.5 - 7.8.

26. The highest concentration of the test substance should be within physiological test conditions, e.g. osmotic and pH stress should be avoided. Depending on the test chemical, it may be necessary to consider other physico-chemical properties as factors limiting the highest test concentration. For relatively insoluble substances that are not toxic at concentrations up to the saturation point the highest achievable concentration should be tested. In general, precipitation of the test chemical at any of the test concentrations should be avoided. The maximum concentration of a test substance should not exceed 1000 µg/mL; osmolality should not exceed 10 mmolar. A geometric dilution series of 8 test substance concentrations with a constant dilution factor should be used (see paragraph 47).

27. If there is information (from a range finding experiment) that the test chemical is not cytotoxic up to the limit concentration in the dark experiment (-Irr), but is highly cytotoxic when irradiated (+Irr), the concentration ranges to be selected for the (+Irr) experiment may differ from those selected for the (–Irr) experiment to fulfill the requirement of adequate data quality.

Controls

28. *Radiation sensitivity of the cells, establishing of historical data:* Cells should be checked regularly for sensitivity to the light source by exposure to increasing doses of irradiation. These doses are easiest quantitated by measurements of UV parts of the light source. Cells are seeded at the density used in the *in vitro* 3T3 NRU phototoxicity test irradiated the next day. Cell viability is then determined one day later using Neutral Red uptake. It should be demonstrated that the resulting highest non-cytotoxic dose (e.g. in the validation study: 5 J/cm^2 [UVA]) was sufficient to classify the reference chemicals (Table 1) correctly.

29. *Radiation sensitivity, check of current test:* The test meets the quality criteria if the irradiated negative/solvent controls show a viability of more than 80% when compared with non-irradiated negative/solvent control.

30. *Viability of solvent controls:* The absolute optical density ($OD_{540\ NRU}$) of the Neutral Red extracted from the solvent controls indicates whether the $1x10^4$ cells seeded per well have grown with a normal doubling time during the two days of the assay. A test meets the acceptance criteria if the mean $OD_{540\ NRU}$ of the untreated controls is $\geq$ 0.4 (i.e. approximately twenty times the background solvent absorbance).

31. *Positive control:* A known phototoxic chemical shall be tested concurrently with each *in vitro* 3T3 NRU phototoxicity test. Chlorpromazine (CPZ) is 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 (+Irr): IC_{50} = 0.1 to 2.0 µg/ml, CPZ non-irradiated (-Irr): IC_{50} = 7.0 to 90.0 µg/mL. The Photo Irritation Factor (PIF), should be > 6. The historical performance of the positive control should be monitored.

32. Other phototoxic chemicals, suitable for the chemical class or solubility characteristics of the chemical being evaluated, may be used as the concurrent positive controls in place of chlorpromazine.

Test procedure (6)(7)(8)(15)(16):

1st day:

33. Dispense 100 µL culture medium 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 $1x10^5$ cells/mL in culture medium (= $1x10^4$ cells/well). Two plates should be prepared for each series of individual test substance concentrations, and for the solvent and positive controls.

34. Incubate cells for 24 h (see paragraph 13) until they form a half confluent monolayer. This incubation period allows for cell recovery, adherence, and exponential growth.

2nd day:

35. After incubation, decant culture medium from the cells and wash carefully with 150 µL of the buffered solution used for incubation. Add 100 µL of the buffer containing the appropriate concentration of test chemical or solvent (solvent control). Apply 8 different concentrations of the test chemical. Incubate cells with the test substance in the dark for 60 minutes (see paragraph 12).

36. From the two plates prepared for each series of test substance concentrations and the controls, one is selected, generally at random, for the determination of cytotoxicity (-Irr) (i.e., the control plate), and one (the treatment plate) for the determination of photocytotoxicity (+Irr).

37. To perform the +Irr exposure, irradiate the cells at room temperature for 50 minutes through the lid of the 96-well plate with the highest dose of radiation that is non-cytotoxic (see also Annex 3). Keep non-irradiated plates (-Irr) at room temperature in a dark box for 50 min (= light exposure time).

38. Decant test solution and carefully wash twice with 150 µL of the buffered solution used for incubation. Replace the buffer with culture medium and incubate (see paragraph 12) overnight (18-22 h).

3rd day:

Microscopic evaluation

39. Cells should be examined for growth, morphology, and integrity of the monolayer using a phase contrast microscope. Changes in cell morphology and effects on cell growth should be recorded.

Neutral Red Uptake test

40. Wash the cells with 150 µL of the warmbuffering solution used for incubation. Remove the washing solution by gentle tapping. Add 100 µL of a 50 µg/mL Neutral Red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, CAS number 553-24-2; C.I. 50040) in medium without serum (15) and incubate as described in paragraph 12, for 3 h.

41. After incubation, remove the NR medium, and wash cells with 150 µL of the buffer. Decant and remove excess buffer by blotting or centrifugation.

42. Add exactly 150 µL NR desorb solution (freshly prepared 49 parts water + 50 parts ethanol + 1 part acetic acid).

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

44. Measure the optical density of the NR extract at 540 nm in a spectrophotometer, using blanks as a reference. Save data in an appropriate electronic file format for subsequent analysis.

DATA AND REPORTING:

Quality and quantity of data

45. The test data should allow a meaningful analysis of the concentration-response obtained in the presence and in the absence of irradiation. If cytotoxicity is found, both the concentration range and the intercept of individual concentrations shall be set in a way to allow the fit of a curve to the experimental data.

46. For both clearly positive and clearly negative results (see paragraph 53), the primary experiment, supported by one or more preliminary dose range-finding experiment(s), may be sufficient.

47. Equivocal, borderline, or unclear results should be clarified by further testing (see also paragraph 56). In such cases, modification of experimental conditions should be considered. Experimental conditions that might be modified include the concentration range or spacing, the pre-incubation time, and the irradiation-exposure time. A shorter exposure time may be appropriate for water-unstable chemicals.

Evaluation of results

48. To enable evaluation of the data, a Photo-Irritation-Factor (PIF) or Mean Photo Effect (MPE) may be calculated.

49. For the calculation of the measures of photocytotoxicity (see below) the set of discrete dose-response values has to be approximated by an appropriate continuous dose-response curve (model). Fitting of the curve to the data is commonly performed by a non-linear regression method (17). To assess the influence of data variability on the fitted curve a bootstrap procedure is recommended.

50. A Photo-Irritation-Factor (PIF) is calculated using the following formula:

$$PIF = \frac{IC_{50}\,(-Irr)}{IC_{50}(+Irr)}$$

If an IC_{50} in the presence or absence of light cannot be calculated, a PIF cannot be determined for the test material.

51. The mean photo effect (MPE) is based on comparison of the complete concentration response curves (18). It is defined as the weighted average across a representative set of photo effect values

$$MPE = \frac{\sum_{i=1}^{n} w_i \, PE_{c_i}}{\sum_{i=1}^{n} w_i}$$

The photo effect (PE_c) at any concentration (C) is defined as the product of the response effect (RE_c) and the dose effect (DE_c) i.e. $PE_c = RE_c \times DE_c$. The response effect (RE_c) is the difference between the responses observed in the absence and presence of light, i.e. $RE_c = R_c\,(-Irr) - R_c\,(+Irr)$. The dose-effect is given by

$$DE_c = \left| \frac{C/C^{*}-1}{C/C^{*}+1} \right|$$

where C* represents the equivalence concentration, i.e. the concentration at which the +Irr response equals the –Irr response at concentration C. If C* cannot be determined because the response values of the +Irr curve are systematically higher or lower than RE_c the dose effect is set to 1. The weighting factors i are given by the highest response value, i.e. w_i = MAX {Ri (+Irr), Ri (-Irr) }. The concentration grid Ci is chosen such that the same number of points falls into each of the concentration intervals defined by the concentration values used in the experiment. The calculation of MPE is restricted to the maximum concentration value at which at least one of the two curves still exhibits a response value of at least 10%. If this maximum concentration is higher than the highest concentration used in the +Irr experiment the residual part of the +Irr curve is set to the response value "0". Depending on whether the MPE value is larger than a properly chosen cut-off value (MPE_c= or not, the chemical is classified as phototoxic).

52. A software package for the calculation of the PIF and MPE is available from

the Secretariat (19).

Interpretation of Results

53. Based on the validation study (8), a test substance with a PIF < 2 or an MPE < 0.1 predicts: "no phototoxicity". A PIF >2 and < 5 or an MPE > 0.1 and < 0.15 predicts: "probable phototoxicity" and a PIF > 5 or an MPE > 0.15 predicts: "phototoxicity".

54. For any laboratory initially establishing this assay, the reference materials listed in Table 1 should be tested prior to the testing of test substances for phototoxic assessment. PIF or MPE values should be close to the values mentioned in Table 1.

TABLE 1

Chemical and CAS No	PIF	MPE	Absorption Peak	Solvent[1]
Amiodarone HCL [19774-82-4]	>3.25	0.27-0.54	242 nm 300 nm (shoulder)	ethanol
Chloropromazine HCL [69-09-0]	>14.4	0.33-0.63	309 nm	ethanol
Norfloxacin [70458-96-7]	>71.6	0.34-0.90	316 nm	acetonitrile
Anthracene [120-12-7]	>18.5	0.19-0.81	356 nm	acetonitrile
Protoporphyrin IX, Disodium [50865-01-5]	>45.3	0.54-0.74	402 nm	ethanol
L – Histidine [7006-35-1]	no PIF	0.05-0.10	211 nm	water
Hexachlorophene [70-30-4]	1.1-1.7	0.00-0.05	299 nm 317 nm (shoulder)	ethanol
Sodium lauryl sulfate [151-21-3]	1.0-1.9	0.00-0.05	no absorption	--

Interpretation of data

55. If a positive result is obtained only at the highest test concentration, (especially for water soluble test chemicals) additional considerations may be necessary for assessment of hazard. These may include data on skin absorption, and accumulation of the chemical in the skin and / or data from other tests, e.g., testing of the chemical in *in vitro* animal or human skin, or skin models.

56. If no toxicity is demonstrated (+Irr and -Irr), and if poor solubility limited the concentrations that could be tested, then the compatibility of the test substance with the assay may be questioned and confirmatory testing should be considered (using, e.g., *in vitro* skin, *ex vivo* skin, or *in vivo* models).

Test Report

[1] Solvent used for measuring absorption.