human volunteers in the testing of potentially cutaneous irritant cosmetic ingredients or mixtures of ingredients (SCCNFP/0003/98 Final) and in skin compatibility testing of finished products (SCCNFP/0068/98 Final) in order to provide recommendations on the use of human volunteers in the safety evaluation of cosmetics, taking into account scientific and ethical aspects of the problem;

6. The SCCNFP will be monitoring on a regular basis, scientific progress in the development and validation of alternative methods, and it will also evaluate their applicability to the safety testing of cosmetics, as well as immediately report its opinion to the Commission.

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添付資料-3

IN VITRO PHOTOIRRITATION

DRAFT PROPOSAL

OECD GUIDELINE

OECD GUIDELINE FOR TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE

In Vitro 3T3 NRU Phototoxicity Test

INTRODUCTION

1. Phototoxicity (photoirritation) 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.

2. 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.

3. 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.

4. The *in vitro* 3T3 NRU phototoxicity test was developed and validated in a joint EU/COLIPA project from 1992-1997 (1-3), to establish a valid *in vitro* alternative to the various *in vivo* tests in use, none of which has been accepted by the OECD. In 1996 an OECD workshop recommended an *in vitro* tier testing approach for phototoxicity assessment (4).

5. 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*.

6. Definitions used in this Guideline are set out in Annex 1.

7. A sequential approach to phototoxicity testing of chemicals is set out in Annex 2.

INITIAL CONSIDERATION

8. Many types of chemicals have been reported to induce phototoxic effects (5-8). 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 according to OECD Test Guideline 101. If the molar extinction / absorption coefficient is less than 10 litre × mol^{-1} × 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 (see Annex 2).

PRINCIPLE OF THE TEST METHOD

9. Four mechanisms have been identified by which absorption of light by a (chemical) chromophore can result in a phototoxic response. 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.

10. 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/cm² 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. 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.

DESCRIPTION OF THE TEST METHOD

Preparations

Cells

11. 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.

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

13. 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.

Media and culture conditions

14. 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 assure a cell cycle time within the normal historical range of the cells or cell line used.

Preparation of cultures

15. 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.

16. 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.

17. 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).

Preparation of test chemicals

18. 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.

19. 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.

20. 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.

21. 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.

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

Preparation of UV irradiation

23. *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 photosensitization (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 photosensitizers. Furthermore, the wavelengths and doses employed should not be unduly deleterious to the test system, which includes the emission of heat (infra red region).

24. 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.

25. For the *in vitro* 3T3 NRU phototoxicity test an irradiance spectrum practically devoid of UVB should be used (UVA:UVB ~ 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).

26. *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/cm² (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/cm² within a time period of 50 min, irradiance has to be adjusted to 1.666 mW/cm². 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:

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

Test conditions

Test chemical concentrations

27. 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).

28. 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.

Controls

29. *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/cm², 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/cm² UVA is not less than 80% of the viability of dark controls. At the highest UVA dose of 9 J/cm², viability should not be less than 50% of that of dark controls. This check should be repeated about every 10 th passage of the cells.

30. *UVA sensitivity of the negative control cells, current test:* The test meets the quality criteria if negative controls (cells in EBSS with or without 1% DMSO or 1% 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).

31. *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.

32. *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 Inhibition Factor (PIF), i.e. the shift of EC_{50} should be at least 6.

33. 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 should be adequately defined as acceptance criteria for the test.

Test procedure [details of the validated 3T3 NRU PT test(1-3, 13)]:

1st day:

34. Prepare a cell suspension of $1x10^5$/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)**.

35. 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.

2nd day:

36. 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).

37. 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/cm² UVA (= 5 J/cm²). 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).

38. 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).

3rd day:

Microscopic evaluation

39. 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

40. 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.

41. 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.)

42. Add exactly 150 µL NR desorb solution (freshly prepared ethanol/acetic acid)

43. 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.

44. 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.

DATA AND REPORTING:

Quality and quantity of data

45. 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 a such 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.

46. 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.

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

48. 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.

Data analysis: EC_{50}

49. 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.

Evaluation of results: Prediction Model Version 1 (PIF)

50. 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:

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

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

51. If a chemical is only cytotoxic +UVA and is not cytotoxic when tested -UVA, the PIF cannot be calculated, although this is a result which 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":

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

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

52. 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

(3) $$PIF = *1 = \frac{C_{max}(-UV)}{C_{max}(-UV)}$$

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

53. In cases (2) and (3), concentrations achieved in the *in vitro* 3T3 NRU phototoxicity test should be carefully taken into consideration when predicting phototoxic potential.

Evaluation of results: Prediction Model Version 2 (MPE)

54. 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, D), which can be obtained free of charge.

Interpretation of data

55. 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.

56. 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* model).

Test Report

57. 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 (1): *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 (2): *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 (3); *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 (4); *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 of 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 vs. 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 Inhibition Factor (PIF), or by calculation of the Mean Photo Effect (MPE)
- classification of phototoxic potential
- test acceptance criteria (1), *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 (2), *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

Conclusion

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ANNEX 1

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 (315-400nm), UVB (280-315nm) and UVC (100-280nm). Other designations are also used: the division between UVB and UVA is often placed at 320nm, and the UVA may be divided into UV-A1 and UV-A2 with a division made at about 340nm.

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 non-cytotoxic 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 with topically applied chemicals.

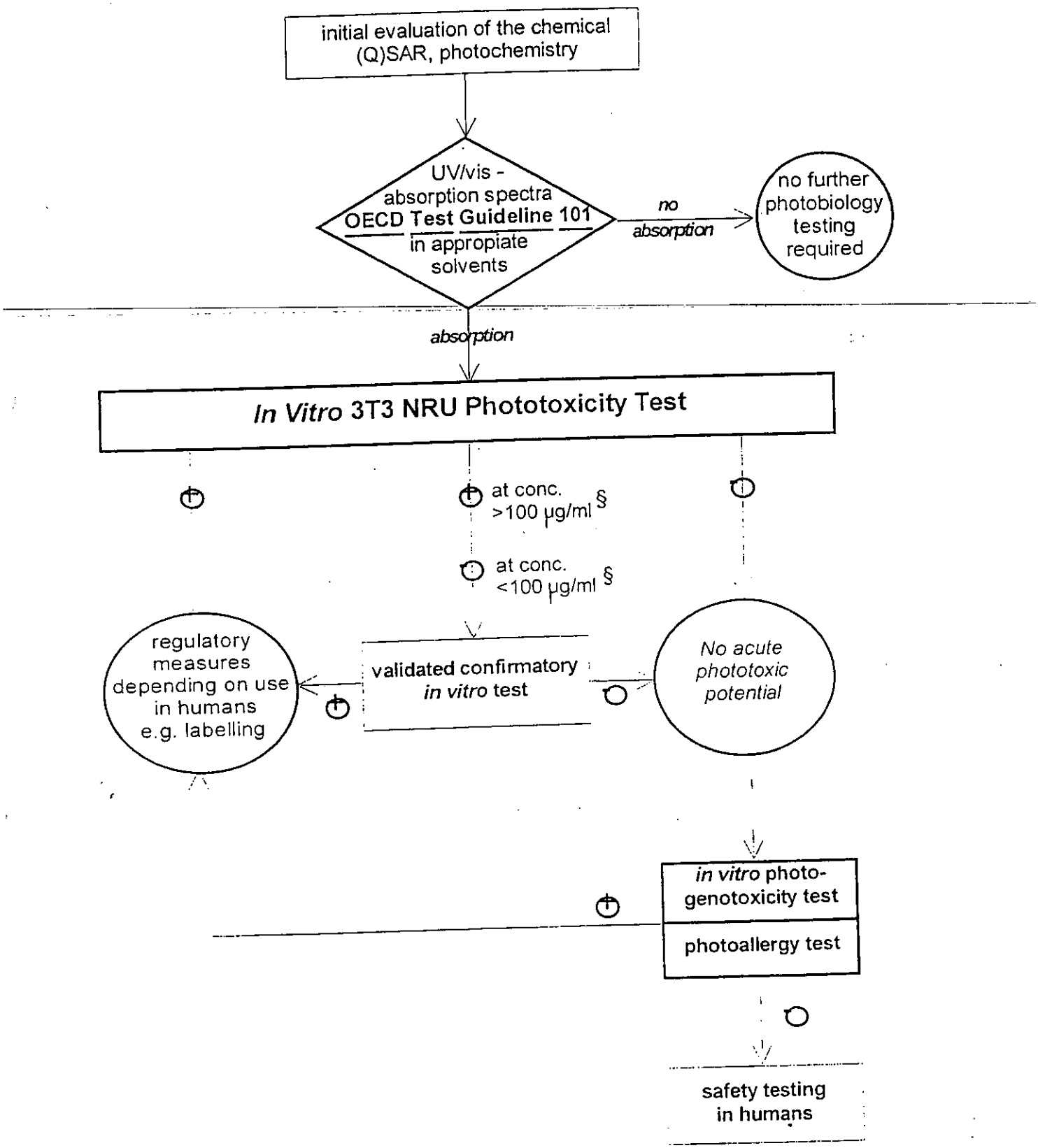
Photoallergy. an acquired immunological reactivity, which does not occur on first treatment with chemical and light, and needs an induction period of one or two weeks before skin reactivity can be demonstrated.

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.

ANNEX 2

Role of the 3T3 NRU PT in a Sequential Approach to Phototoxicity Testing of Chemicals



§ = based on (13)

In Vitro 3T3 NRU Phototoxicity Test に対するコメント

試験法の問題点等

1. p.8. 吸光度による適用範囲の制限：

分子吸光係数 ε =10 (litre/mol/cm)以下は、光毒性なしと評価

…・・分子吸光係数 ε が求められない混合物等の原料については適用できない

2. p.29 細胞の品質基準：以下のチェックは 10 継代ごとに行うべき

…・・現実的には厳しいのでは？

3. p.30. 陰性対照細胞の UVA 感受性：

…・・照射量の記述が無い、(恐らく 5 J/cm2)

4. p.31. 陰性対照の生存率：

合格基準：平均 OD540 NRU≧0.2

…・・(これを生存率 100%とするには) 異常に低いのでは？

5. p.32. 陽性対照：

Photo Inhibition Factor (PIF)= {EC50(-UV) } ／ {EC50(+UV) }：6 以上

…・・ Photo irritation Factor では？

6. p.37. 試験手順：

…・・加える培地の量の記述が無い

7. p.40. 試験手順：

100 uL の NR 培地を加え、7.5%CO2, 37℃で 3 時間培養

…・・ NR 培地の調製方法及び NR 濃度の記述が無い

8. p.42. 試験手順：

ETOH/酢酸は新鮮に調製

…・・ ETOH/酢酸の濃度・調製法の記述が無い

9. p.50. 結果の評価：予測モデル Version 1(PIF)

PIF＝＊1＝ {Cmax(-UV) } ／ {Cmax(-UV)} …… (3)

…・・ PIF＝＊1＝ {Cmax(-UV) } ／ {Cmax(+UV)} の誤り

10. p.57. 試験報告：

UV/vis 照射下及び非照射下における被験物質濃度選択の理論的根拠

UV/vis 照射時間

…・・試験のプロトコール等には vis(可視部)の記述が無いのに、試験結果の報告の項目で唐突に vis のことが出現している。

vis でも試験を実施する必要があるのか？

OECDガイドライン *in vitro* 光毒性試験に対するコメント

Session 9	光毒性反応の4作用機構を説明してほしい。また、それらの文献を引用すべきと考える。
Session 10	培養細胞を用いる光毒性試験はUVAを照射するのか？ UVA/vis lightを照射するのか？例として、(+UV)の表記法は、Session 17の表記法(+UVA/vis)との統一性が必要である。
Session 14	通常、インキュベーターのCO2濃度は5％である。7.5%でなければいけないのか？ 培養液中の血清はnew-born calf serum 10%ではなく、人工血清Serum Plus 10%にnew-born calf serum 2%配合でもよいか。人工血清を用いることにより、陰性対照のデータ管理がしやすい。この細胞はもっとも血清のロット差の影響を受けやすいはずである。
Session 18	red lightではなく、yellow lightでもよいか？一般的にはyellow lightを光関連実験には使用すると考える。
Session 23	UVAとvisible regionが光感作に関係していることはわかったが、光毒性との関係はどうか？説明と文献を引用してほしい。
Session 26	ここまでの内容から、「Balb/c 3T3細胞を用いる光毒性試験はUVAを光の代表として扱う。よって、以後はすべて（+UVA/vis）を（UVA）とする」という記載がどこかで必要である。
Session 28	推奨する被験物質の希釈倍率を記載してほしい。例えば、公比2又はlog10など。
Session 32	positive control試験は被験物質毎に、必ず実施するのか？
Session 34	周囲のwellをブランクとすることから、使用well数は60で良いか？陰性対照の位置などその構成をできれば推奨いただきたい。
Session 36	96wellを用いる細胞培養試験にしては洗浄回数が多すぎる。洗い方によっては、細胞が剥がれるので、注意が必要との記載がどこかに必要である。
Session 40	なぜNR mediumのNR濃度を記載していなのか。NR溶液の調整法も含め記載すべきである。
Session 42	NR抽出溶媒のエタノール／酢酸濃度を記載すべきである。
Session 49	x-scale:log及びy-scale:probitの解析は妥当か？文献を示してほしい。日本の細胞毒性試験バリデーションの検討では別の式を推奨している（Ohno, T, et al., AATEX 5,1&2,1-38）。
Session 55	human in vitro skin modelの結果は信頼できるバリデーションがなされているのか？
Session 57	Cells ：必要ならばcell cycle timeの記載 Test conditions（I）： 血清のメーカー名、ロットが必要 結果は予備試験結果も不要、再現性も確認する必要なく、1回限りの記載で良いのか？仮に再現性が必要とされるならば、同一日に、同一細胞、同一被験物質調製による試験結果を用いても良いのか？
Annex 1	光毒性の光(photo)の定義を明確にしてほしい。

以上

In Vitro Photoirritation ガイドライン案について

コメント

1）推奨している溶媒の DMSO はラジカルスカベンジャーとしての作用をがある。しかし実際には 1%以下では影響はみられずに、当社での実験でも活性酸素種の反応が検出できた。だから厳密なラジカル検出には不向きであるが、スクリーニングでは問題ないと考える。資生堂の杉山さんたちはアセトンを使用しているが、水不溶性物質が多い光毒性物質の溶媒としては DMSO の方が優れている。特に溶解性条件が結果のばらつきに影響するとしていることから DMSO の利用は止むを得ない。

2）PIF 値５（UV 照射での細胞毒性が UV 未照射での 1/5 の濃度で見られるもの）というカットオフポイントは、化学物質全体のバリデーション結果から得られたものであるが、この妥当性について検討する必要がある。確かに５以上が毒性を示すことは理解できるが、１<PIF<５の物質を negative と断定するのには問題がある。実際に香料（ファントライド 3.2 やガラクソライド 1.6:当社結果）や殺菌剤（TCSA：資生堂の結果）などは in vivo では光毒性を示しているが、この評価法では negative になってしまう。false negative を防ぐために、スキームの中にこれらを反映させるプロトコールに変更する必要がある。

3）試験の繰り返しは、結果が明確であれば必要としないとしているが、一方で、試験のばらつきは試験条件、特にｻﾝﾌﾟﾙの溶解性に左右されるとしている。確かに同様に調製した試料の試験の再現性が良好なことは当社でも確認しているが、UV 照射と未照射の両方で毒性が見られる場合や溶解性に問題がある場合などは一度の試験で評価すると結果がばらつくことがある。従って、疑わしい範囲（PIF 値で示す）の結果の場合は試験の繰り返しが必要という設定が必要である。

4）in vivo 動物試験による確認過程が今回提示のスキームから削除されていることの説明がなされていないので、この点についての OECD の見解を知りたい。

分担研究報告書

In vitro経皮吸収試験法ガイドラインについて
メチルパラベンおよびエチルパラベン経皮吸収試験

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要　約

1996年6月、OECDは、In vitro経皮吸収試験法の第2次ガイドライン（案）を作成し、各国に公表した。我が国では、国立医薬品食品衛生研究所の大野薬理部長を中心として意見を取りまとめ、同年10月にOECDに意見を提出した。この第2次OECD ガイドライン（案）に対する回答がOECDから提出されていないが、予め、「In vitro試験法を用いた化粧品の安全性評価法およびその国際的ハーモナゼーションによる研究」の一環として,前年に引き続きIn vitro経皮吸収試験法を防腐剤の一種であるメチルパラベンおよびエチルパラベンの経皮吸収試験に実施した。縦型のFranz型拡散セルを用いて検討し、それら試験物質のdonor側のvehicleへの溶解性を考慮することが、In vitro経皮吸収試験を実施する場合の大きな要因であることが示唆された。

A. 研究目的

医薬品、化粧品あるいは添加物等のin vitro経皮吸収試験法は、適切な実験原理に基づき、適切な解析を行い、in vitro経皮吸収試験法の結果を正確に記述できるデータを提示する必要がある。1996年6月、OECDは、the European Cosmetic Toiletry and Perfumery AssociationおよびEuropean Center for the Validation of Alternative Methodsのガイドラインを基に第2次OECDガイドライン（案）を作成し、各国に公表した。このガイドライン（案）に関して、我が国では、国立医薬品食品衛生研究所の大野薬理部長を中心として意見の取りまとめを行い、同年10月に意見をOECDに提出したところである。この第2次OECDガイドライン（案）に対する回答がOECDから報告されていないが、予め、「in vitro試験法を用いた化粧品の安全性評価法およびその国際的ハーモナゼーションによる研究」の一環として、前年度に引き続き、防腐剤の一種であるメチルパラベン（MP）およびエチルパラベン（EP）の経皮吸収的な評価をするため、in vitro経皮吸収試験を縦型のFranz型拡散セルを用いて検討した。また、それら化合物を化粧水に添加したときのin vitro経皮吸収試験を実施し、vehicle の経皮吸収試験に及ぼす影響を検討したので報告する。

B．研究方法

1）試薬および試液

MP、EP および液体クロマトグラフ（HPLC）用アセトニトリルは和光純薬工業株式会社より購入した。HPLC 用カラムは CAPCELL PAK C_{18} カラム（粒径 5 μ m、内径 4.6mm，長さ 25cm）を資生堂から購入した。化粧水は市販品 2 種類を用いた。その他の試薬は，試薬特級品を用いた。

MP あるいは EP 原液：MP あるいは EP 約 0.025 g を精密に量り，20%プロピレングリコール（PG）溶液に溶かし、正確に 10mL とした（0.25w/v%W/V%）。

MP あるいは EP 溶液：MP あるいは EP 原液 1.0 mL に水 4.0mL を加えて調製した(0.05w/v%)。

MP 含有化粧水：市販化粧水は防腐剤として、0.125w/v%の MP を含有していた。化粧水 2.0mL に PG1.0mL および水 2.0mL を加えて調製した。

EP 含有化粧水：化粧水 2.0mL に EP 原液 1.0mL および水 2.0mL を加えて調製した。

20%PG 溶液：PG20mL に水を加えて 100mL とした。

0.1M リン酸試液：リン酸 9.8g に水を加えて 1000mL とした。

0.1M リン酸二ナトリウム試液：リン酸二ナトリウム（無水）14.1g を水に溶かし、1000mL とした。

50mM リン酸緩衝液（PH4）：0.1M リン酸試液に 0.1M リン酸二ナトリウム試液を加えて PH4.0 に調整した後、水にて 2 倍に希釈した。

2)剥離皮膚の調製法

モルモット（ハートレー系、オス、250 － 300g）をエーテル麻酔後，エーテルでの深麻酔および呼吸停止により処理した。腹部の毛を電気バリカンで除いた後，腹部の皮膚を剥離した。皮下脂肪を除き，凍結保存し，必要なときに解凍して用いた。

3)ＭＰおよびＥＰの皮膚透過実験

モルモット剥離皮膚を縦型の Franz 型拡散セル（static セル系、有効透過面積 0.246cm^2）に表皮を donor 側に向けて装着した。donor 側に MP あるいは EP 溶液 1.0mL を加え、receptor 側に 20%PG 溶液を加え、32 ℃で 2 ～ 8 時間後に receptor 側の溶液 0.2ml を分取した。この液 20 μ l を用いて HPLC 法にて測定を行った。あらかじめ作成した検量線を用いて透過した MP あるいは EP 量を求めた。なお，receptor 側に 20%PG 溶液 0.2ml を加え，receptor 側の容量を一定とした。

（HPLC 条件）

検出器：紫外吸光光度計（測定波長：255nm）

カラム：資生堂製 CAPCELL PAK C_{18} カラムを用いた。

カラム温度：35 ℃付近の一定温度

移動相：50mM リン酸塩緩衝液（pH4.0）／アセトニトリル混液（3:2）

流量：1 mL/min

C．研究結果

1）MP および EP の検量線および再現性

MP あるいは EP0.05 ～ 0.8 μ g/mL の溶液 20 μ L を用いて HPLC 条件にて検量線の作成を行った。その結果を Fig.1 に示した。

濃度と Peak area の間には原点を通る良好な直線関係を示した。また、MP0.05 μ g/mL および 0.8 μ g/mL の 20 μ L を用いたときの再現性は、それぞれ、4806 μ V × sec（相対標準偏差 2.21 %）と 78 931 μ V × sec（相対標準偏差 0.41%）であった。同様に、EP0.05 μ g/mL および 0.8 μ g/mL の場合、それぞれ、4399 μ V × sec（相対標準偏差 4.08%）