OECD/OCDE

OECD GUIDELINE FOR THE TESTING OF CHEMICALS
DRAFT PROPOSAL FOR A NEW TEST GUIDELINE

ROS (Reactive Oxygen Species) assay for photosafety

INTRODUCTION

1. Phototoxicity is elicited after exposure of the skin and/or eye to topically or systemically administered chemicals in the presence of environmental light. Several classes of chemicals at non-toxic dose often cause phototoxic reactions, and phototoxicity can be categorized as photoirritation, photoallergy, and photogenotoxicity.

2. In 2002, regulatory agencies in the US (US Food and Drug Administration, FDA) and EU (European Medicines Agency, EMA) published guidelines for photosafety assessments of drug candidates (1)(2). In 2004, the Organisation for Economic Co-operation and Development (OECD) adopted Test Guideline 432: *In vitro* 3T3 Neutral Red Uptake (NRU) Phototoxicity Test as a validated methodology for evaluating the phototoxic potential of chemicals (3). The EMA also published a concept paper in 2008 (4), which proposes a testing strategy that merges the testing proposals recommended by FDA and EMA. Considering these documents, the International Council of Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) published ICH S10 guideline, "Photosafety Evaluation of Pharmaceuticals" in 2014 (5).

3. In these guidelines, chemicals or drug candidates, administered to the skin and/or eyes and distributed to the skin and/or eyes after administration, need to be examined for their phototoxic potential. The Grotthuss-Draper law (6), also known as the first law of photochemistry, states that light must be absorbed by a compound in order for photochemical reactions to take place. On the basis of this principle, the guidelines have suggested that the phototoxic potential of chemicals is related to the photochemical properties of compounds, especially light absorption properties within 290–700 nm, and they have described the need for measurement of the light absorption properties of chemicals as a first screening. The ICH S10 guideline recommends UV-visible light absorption spectral analysis as a criterion for evaluating the phototoxic potentials of drugs; however, UV-visible light absorption of chemicals would not always correlate directly with their phototoxic potential, so a combination of MEC with other appropriate screening systems might be advantageous in avoiding false predictions.

4. In addition to light absorption and distribution to light-exposed tissue, the generation of a reactive species from chemicals following absorption of UV-visible light is described as a key determinant of chemicals for causing phototoxic reactions in the guidance document (7). On the basis of the key characteristic, the Reactive Oxygen Species (ROS) assay (8) (9) has been also recommended by ICH S10 guideline as an *in vitro* tool for evaluating the photosafety of pharmaceuticals (5). Recent attention has been drawn to the strategic use of the ROS assay for photosafety assessment on cosmetic and food additives (9) (10) (11) (12), as well as pharmaceutical substances. The cosmetics industry has been directly affected by the 7th Amendment the Cosmetic Directive (13), which called for a ban on marketing of cosmetic products containing ingredients that have been tested in animals for toxicity as of March 2013. Therefore, a reliable and comprehensive non-animal photosafety screening strategy is urgently needed. Currently, the Personal Care Products

Council (PCPC) guidance "Evaluation of Photoirritation and Photoallergy Potential" recommends the use of the ROS assay for photosafety evaluation of cosmetic ingredients (14).

5. As an *in vitro* test method, the OECD TG432 (4) has recommended an *in vitro* 3T3 NRU Phototoxicity Test and set specific criteria for evaluating phototoxic risk. The assay was drafted as an alternative method for *in vivo* phototoxicity testing and submitted to the OECD by the European Centre for the Validation of Alternative Methods (ECVAM) and the European Cosmetics, Toiletry and Perfumery Association (COLIPA) (15). However, the *in vitro* 3T3 NRU Phototoxicity Test often provides false-positive results and the results from the assessments would not always reflect other types of *in vitro* phototoxic risk, including photogenotoxicity and photoallergy as well as *in vivo* phototoxicity. Thus, inclusive *in vitro* screening methodologies and strategies are also needed for more reliable photosafety evaluation induced by the excited chemical after exposure to environmental light. The 3T3 NRU Phototoxicity 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, following systemic application and distribution to the skin, or after topical application.

6. Definitions used are provided in Annex 1.

INITIAL CONSIDERATION AND LIMITATIONS

7. Many types of chemicals have been reported to induce phototoxic events (16) (17) (18) (19). 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 photosafety assessments are considered, a UV-visible light absorption spectrum of the test chemical should be determined according to OECD Test Guideline 101. The OECD Test Guideline 432 has been suggested that the chemical is unlikely to be photoreactive if the MEC is less than 10 $L \cdot mol^{-1} \cdot cm^{-1}$ (4). The ICH S10 guideline has been suggested that no further photosafety testing is needed if the MEC of a chemical is less than 1,000 $L \cdot mol^{-1} \cdot cm^{-1}$ (5). Some phototoxic chemicals indicated the MEC less than 1,000 $L \cdot mol^{-1} \cdot cm^{-1}$ (11). Thus, the criterion for the UV-visible light absorption spectrum is in accordance with OECD TG 432 (4), and a chemical with the MEC of less than 10 $L \cdot mol^{-1} \cdot cm^{-1}$ may not need to be tested in the ROS assay or any other photosafety assessments (7) (16).

8. The multi-laboratory validation studies on the ROS assay using two different solar simulators, and the reliability and relevance of the ROS assay was recently evaluated (20) (21). The ROS assay on 2 standards and 42 coded chemicals (200 μM) provided no false negative predictions upon defined criteria as compared with the *in vitro/in vivo* phototoxicity. The ROS assay was designed for qualitative photoreactivity assessment of chemicals, the principle of which is monitoring of type I and II photochemical reactions in test chemicals exposed to simulated sunlight, possibly leading to photodegradation and various phototoxic reactions including photoirritation, photoallergy, and photogenotoxicity. The test has not been designed to address indirect mechanisms of phototoxicity, effects of metabolites of a test substance.

9. The applicability domain of the ROS assay is currently restricted to only those chemicals that meet the solubility criteria outlined in the protocol. As experience is gained from use of the ROS assay, the applicability domain could be more fully described in terms of physicochemical properties and/or chemical classes. This would contribute to increased efficiency by providing criteria that can

be used to identify whether a chemical may be satisfactorily tested in the ROS assay, or whether an alternate assay should be used initially. Insoluble chemicals in the reaction mixtures are not suitable for testing with the ROS assay and may be able to be tested by ROS assay with addition of solubilizing enhancers, such as Tween 20 and bovine serum albumin (BSA), in the reaction mixtures as follow-up assays (22) (23) (24). However, further characterization and standardization of procedures using these alternative vehicles should be performed before incorporation into routine use. In the ROS assay, superoxide anion (SA) can be measured upon the reduction of nitroblue tetrazolium, and the determination of singlet oxygen (SO) can be made on the basis of bleaching of *p*-nitrosodimethylaniline by oxidized imidazole (9). Test chemicals that interfere with these reactions may be considered outside of the applicability domain of the ROS assay. For example, ascorbic acid and other reducing substances reduce the tetrazolium salt to a formazan directly (25). Some skin-lightning cosmetics may also have potent reducing properties that interfere with ROS determinations. Ascorbic acid also accelerates the oxidation of imidazole derivatives (26).

10. The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the ROS assay to the testing of mono-constituent substances, multi-constituent substances and/or mixtures. On the basis of the data currently available, the ROS assay was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, phototoxic potency (as determined in in vivo studies) and physicochemical properties. Limited information is currently available on the applicability of the ROS assay to multi-constituent substances/mixtures (13). The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. However, before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Moreover, when testing multi-constituent substances or mixtures, consideration should be given to possible interference of cytotoxic constituents with the observed responses.

11. As the ROS assay can evaluate the photochemical properties of chemicals, possibly leading to phototoxic reactions and photodegradation, the risk for chemical phototoxicity including photoirritation, photoallergy, and photogenotoxicity could be evaluated by ROS assay. ROS generation from chemicals has been shown to be associated with clinical outcomes on phototoxicity (photoirritation) and photoallergy (9) (10) (27). In contrast, the relationship between ROS generation and photogenotoxicity has yet to be established. Therefore, the ROS assay would be applicable to evaluate chemicals for phototoxicity and photoallergy. Phototoxicity has been reported for the metabolites of some phototoxic chemicals, including amiodarone, chlorpromazine, and fenofibrate (28) (29) (30). Thus, the primary metabolites of phototoxic chemicals should also be evaluated for reliable photosafety assessment.

PRINCIPLE OF THE TEST

12. Chemical phototoxicity can be caused by topical and systemic application of chemicals in combination with exposure to environmental light. There are several classes of chemicals that are nontoxic by themselves but could become reactive in the skin or eyes when exposed to environmental light and thereby result in toxicity. The primary event in any phototoxic reaction is the absorption of photons of a wavelength that induces excitation of the chromophore. The excitation energy is often transferred to oxygen molecules, followed by generation of ROS, including SA through type I photochemical reactions and SO through type II photochemical reactions by photo-excited molecules. These appear to be the principal intermediate species in the phototoxic responses. Therefore, the determination of ROS generation from chemicals irradiated with simulated sunlight would be of value in recognizing their phototoxic potential.

13. In the ROS assay, SO generation was detected by spectrophotometric measurement of *p*-nitrosodimethyl aniline (RNO) bleaching, followed by decreased absorbance of RNO at 440 nm (31). Although SO does not react chemically with RNO, the RNO bleaching is a consequence of SO capture by the imidazole ring, which results in the formation of a trans-annular peroxide intermediate capable of inducing the bleaching of RNO, as follows:

SO + Imidazole → [Peroxide intermediate] → Oxidized imidazole

[Peroxide intermediate] + RNO → RNO + Products

SA generation was detected by the observing the reduction of nitroblue tetrazolium (NBT) as indicated below; NBT can be reduced by SA via a one-electron transfer reaction, yielding partially reduced (2 e^-) monoformazan (NBT^+) as a stable intermediate (32). Thus, SA can reduce NBT to NBT^+, the formation of which can be monitored spectrophotometrically at 560 nm.

$SA + NBT \rightarrow O_2 + NBT^+$

PROCEDURE

Solar simulator

14. An appropriate solar simulator is to be used for irradiation of UV and visible light. The irradiation power distribution is to be kept as close to that of outdoor daylight as possible by using an appropriate UVC cut filter. Recommended solar simulators and UVA intensity on the plate position measured by UVA detector #0037 (Dr. Hönle AG) are as follows:

Suntest CPS+ or CPS (Atlas) with UV cut filter (<290 nm)

- 1.8 to 2.2 mW/cm^2 (e.g. the indicator setting value of 250 W/m^2 for CPS+) for 1 hour,
- 6.5 to 7.9 J/cm^2 of UVA intensity (Annex 2).

SXL-2500V2 (Seric) with UV cut filter (<300 nm)

- 3.0 to 5.0 mW/cm^2 for 1 hour,
- 11 to 18 J/cm^2 of UVA intensity (Annex 2).

The solar simulator is to be equipped with an appropriate temperature control or fan to stabilize the temperature during irradiation, because ROS production is affected by temperature. Standard temperature for a solar simulator with temperature control is 25°C. The acceptable temperature range during irradiation is 20° to 29°C. If a solar simulator other than the two recommended models is used, the reference chemical set listed in Annex 3 is to be tested prior to performing the ROS assay to ensure that measured values of SO and SA are close to those mentioned in Annex 3.

Quartz reaction container

15. A quartz reaction container is used to avoid loss of UV due to passing through a plastic lid and vaporization of the reaction mixture (33). Specifications for the recommended container are provided in Annex 4. If a different container is used, a lid or seal with high UV transmittance should be used. In this case, a feasibility study using the reference chemicals is to be conducted to determine an appropriate level of exposure to UV and visible light.

Reagents

16. All reagents should be used within 1 month after preparation and should be sonicated immediately prior to use. Representative preparation methods are shown as follows;

20 mM sodium phosphate buffer (NaPB), pH 7.4

- Weigh 593 mg of $NaH_2PO_4 \cdot 2H_2O$ (CAS No. 13472-35-0) and 5.8 g of $Na_2HPO_4 \cdot 12H_2O$ (CAS No. 10039-32-4), add 900 mL of purified water, adjust with HCl to a pH of 7.4, dilute with purified water up to 1 L, and mix.
- Store in a refrigerator or at room temperature.

0.2 mM *p*-Nitrosodimethylaniline (RNO, CAS No. 138-89-6)

- Dissolve 3 mg of RNO in 100 mL of 20 mM NaPB.
- Store in a refrigerator and protect from light.

20 mM imidazole (CAS No. 288-32-4)

- Dissolve 13.6 mg of imidazole in 10 mL of 20 mM NaPB.
- Dilute the 2×10^{-2} M imidazole solution 100 times with 20 mM NaPB.
- Store in a refrigerator and protect from light.

0.4 mM nitroblue tetrazolium chloride (NBT, CAS No. 298-83-9)

- Dissolve 32.7 mg of NBT in 100 mL of 20 mM NaPB.
- Store in a refrigerator and protect from light.

Test chemicals

17. Test chemicals are to be stored as recommended by manufacturers until termination of the study and their stability during the test period is to be confirmed. One concentration level, 200 µM (final concentration), is to be used. A 20-µM concentration can be used if precipitation before light exposure, coloration, or other interference is observed in the reaction mixture at 200 µM.

18. The test chemical solutions are to be prepared using a solvent just before use. Each test chemical is to be weighed in a tube, and solvent added to a concentration 10 mM. The tube is to be mixed with a vortex mixer and sonicated for 5 to 10 minutes under UV-cut illumination or shade. All preparations are to be protected from light. The final concentration in a reaction mixture is to be 200 µM. When precipitation before light exposure, coloration, or other interference is observed in the reaction mixture at 200 µM, a 1-mM solution (20 µM as the final concentration) is to be prepared using the solvent. For chemicals that are not soluble in DMSO, 20 µL of DMSO is to be contained in the reaction mixture.

Positive and negative controls

19. Stock solutions of quinine hydrochloride (a positive control, CAS No. 6119-47-7) and sulisobenzone (a negative control, CAS No. 4065-45-6) are to be prepared at 10 mM each in DMSO (final concentration of 200 µM) according to the above procedure, divided into tubes, and stored in a freezer (generally below -20˚C) for up to 1 month. The stock solution is to be thawed just before the experiment and used within the day.

Solvents

20. Use analytical grade DMSO at first. For chemicals that are not soluble in DMSO, 20 mM of NaPB is to be used. When a test chemical is insoluble in either DMSO or 20 mM NaPB, try BSA or Tween 20 (22) (23) (24). Prior to use of BSA or Tween 20, however, perform a feasibility study (see Annex 3) using the reference chemicals to determine appropriate test conditions. The results of ROS assays using BSA or Tween 20, however, are not suitable for regulatory purposes until these solvents have been properly evaluated.

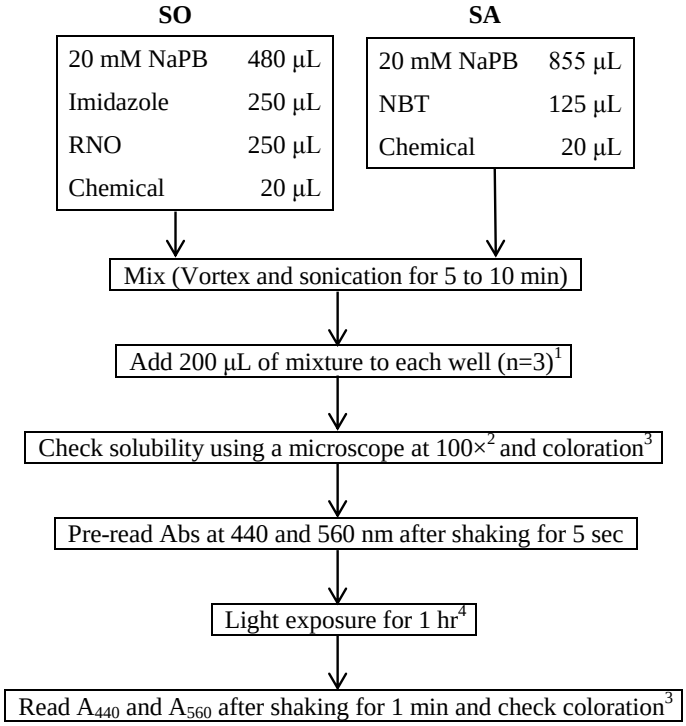
Test procedure

21. A typical 96-well plate configuration is as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A					Singlet oxygen							
B		B	P	N	T1	T2	T3	T4	T5	T6	T7	
C		B	P	N	T1	T2	T3	T4	T5	T6	T7	
D		B	P	N	T1	T2	T3	T4	T5	T6	T7	
E		B	P	N	T1	T2	T3	T4	T5	T6	T7	
F		B	P	N	T1	T2	T3	T4	T5	T6	T7	
G		B	P	N	T1	T2	T3	T4	T5	T6	T7	
H					Superoxide anion							

B: Blank
P: Positive control (Quinine)
N: Negative control (Sulisobenzone)
T1-T7: Test chemical No. 1-7

22. A tube (e.g. 1.5 mL micro tube) and a plastic clear flat bottomed 96-well microplate are to be used. The reaction mixture is to be prepared by vortex mixing and/or sonication under UV-cut illumination or shade. The same volume of DMSO, 20 µL, is to be added in a blank instead of test chemical solution.



DATA AND REPORTING

Data analysis

23. Data from three wells for each chemical concentration is used to calculate mean and standard deviation.

SO

Decrease of A_{440} ×1000 = [A_{440} (–) – A_{440} (+) – (a – b)] ×1000
A_{440} (–): Absorbance before light exposure at 440 nm
A_{440} (+): Absorbance after light exposure at 440 nm

a: Blank before light exposure (mean)
b: Blank after exposure (mean)

SA

Increase of A_{560} ×1000 = [A_{560} (+) – A_{560} (–) – (b – a)] ×1000
A_{560} (–): Absorbance before light exposure at 560 nm
A_{560} (+): Absorbance after light exposure at 560 nm
a: Blank before light exposure (mean)
b: Blank after exposure (mean)

[1] Avoid using peripheral wells. More than one test chemical can be tested on a plate.
[2] Some chemicals might precipitate in the reaction mixture. It is therefore important to check solubility prior to irradiation. Solubility of each reaction mixture in its well is to be observed with a microscope prior to irradiation. Test chemical concentrations are to be selected so as to avoid precipitation or cloudy solutions.
[3] The reaction mixture is to be checked for coloration with the naked eye.
[4] The 96-well plate is to be placed in the quartz reaction container. A quartz cover is to be set on the plate and fastened with bolts. Ensure that temperature and other ambient conditions are stable when using the solar simulator. Measure UVA intensity and temperature at the plate position using a UVA detector and thermometer both before and after irradiation.

Criteria for data acceptance

24. The following criteria are to be satisfied in each experiment.

- No precipitation of test chemical in the reaction mixture before light exposure.
- No coloration of test chemical in the reaction mixture before or after light exposure.
- No technical problems, including temperature range (20–29°C), when collecting data set.
- The ranges of raw A_{440} and A_{560} values: 0.02 to 1.5.
- Historical positive and negative control values are to be developed by each laboratory based on a mean +/-2 SD. The following range was defined based on the 95% confidence interval (mean +/- 1.96SD) obtained from the validation data. When a solar simulator other than a recommended model is used, establish modified criteria based on 95% confidence interval.

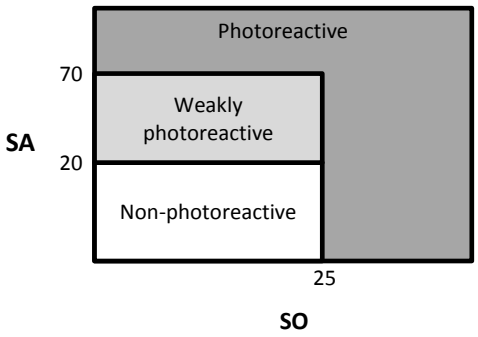
  Positive control value at 200 μM (mean of 3 wells)
  SO: 319 to 583
  SA: 193 to 385

  Negative control value at 200 μM (mean of 3 wells)
  SO: -9 to 11
  SA: -20 to 2

Criteria for judgment

25. Each test chemical is to be judged as follows:

Judgment[1, 2]	Concentration[3]	SO (mean of 3 wells)		SA (mean of 3 wells)
Photoreactive	200 μM	≥25	and	≥70
		<25 and/or I[4]	and	≥70
		≥25	and	<70 and/or I[4]
Weakly photoreactive	200 μM	<25	and	≥20, <70
Non-photoreactive	200 μM	<25	and	<20
Inconclusive	The results do not meet the above-mentioned criterion.			



[1] A single experiment is sufficient for judging results, because the ROS assay shows good intra- and inter-laboratory reproducibility in the validation studies.

[2] If precipitation, coloration, or other interference is observed at both 20 and 200 μM, the chemical is considered incompatible with the ROS assay and judged as inconclusive.

[3] 20 μM can be used for judgment when precipitation or coloration is observed at 200 μM. For regulatory purposes, the stability of the test chemical in the reaction mixture both before and after light exposure is to be confirmed when results at 20 μM are used for judgment as a non-photoreactive chemical for which no further phototoxicity testing is necessary.

[4] Interference such as precipitation or coloration.

Data quality

26. Studies for regulatory purposes are to be conducted to the highest of quality standards, with data collection records readily available, in compliance with GLP/GMP regulations whenever possible, and all documents checked by the Quality Assurance Unit of the laboratory.

Test report

27. The test report should include the following information:

Test substance:
- identification data, common generic names and IUPAC and CAS number, if known;
- physical nature and purity;
- physicochemical properties relevant to conduct of the study;
- UV/vis absorption spectrum;
- stability and photostability, if known.

Control chemicals:
- name, manufacturer, and lot No.;
- physical nature and purity;
- storage condition;
- preparation of control chemical solutions;
- final concentrations tested.

Solvent:
- name, manufacturer, and lot No.;
- justification for choice of solvent;
- solubility of the test chemical in solvent.

Irradiation condition:
- manufacturer and type of the solar simulator used;
- rationale for selection of the solar simulator used;
- UVA detector used;
- UVA irradiance, expressed in mW/cm^2 UVA dose, expressed in J/cm^2;
- temperature before and after irradiation.

ROS assay procedure.

Acceptance and decision criteria.

Results.

Discussion.

Conclusions.

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OECD/OCDE
ANNEX 1

DEFINITIONS

3T3 NRU Phototoxicity Test: *In vitro* 3T3 neutral red uptake phototoxicity test.

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

Dose of light: The quantity [= intensity × time (seconds)] of UV or visible light incident on a surface, expressed in J/m^2 or J/cm^2.

MEC: Molar Extinction Coefficient (also called molar absorptivity) is a constant for any given molecule under a specific set of conditions (e.g., solvent, temperature, and wavelength) and reflects the efficiency with which a molecule can absorb a photon (typically expressed as $L\ mol^{-1}\ cm^{-1}$).

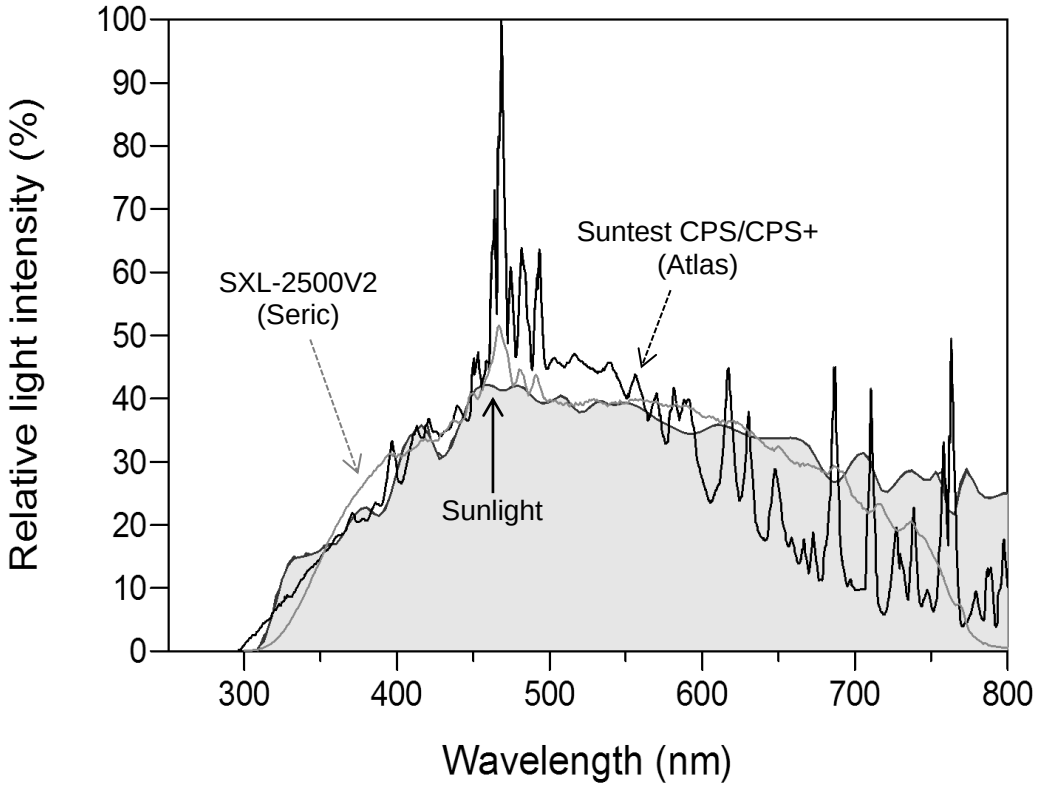
Photoreactivity: The property of chemicals that react with another molecule as a consequence of absorption of photons.

Phototoxicity: 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 systemic administration of a chemical.

ROS: Reactive Oxygen Species, including superoxide anion (SA) and singlet oxygen (SO).

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.

Spectrum of solar simulators used in the validation studies



Proficiency substances

Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency by correctly obtaining the expected ROS prediction for the 9 proficiency substances recommended in Table 1 and 2. These proficiency substances were selected to represent the range of responses for phototoxic potential. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the ROS assay are available, and that they were used in the JaCVAM-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study.

Table 1 The expected ROS prediction for 9 proficiency substances using solar simulators of Suntest CPS/CPS+ (Atlas) or SXL-2500V2 (Seric) and the acceptable range

No.	Chemical	CAS No.	SO	SA	Solvent	Concentration
11	Doxycycline hydrochloride	10592-13-9	115 to 429	230 to 468	DMSO	200 µM
12	Norfloxacin	70458-96-7	131 to 271	57 to 161	DMSO	200 µM
13	8-Methoxy psoralen	298-81-7	31 to 137	0 to 126	DMSO	200 µM
14	Fenofibrate	49562-28-9	77 to 203	-31 to 11	DMSO	20 µM
15	p-Aminobenzoic acid	150-13-0	-8 to 12	-11 to 7	DMSO	200 µM
16	Benzocaine	94-09-7	-7 to 9	-7 to 17	DMSO	200 µM
17	Erythromycin	114-07-8	-15 to 11	-9 to 21	DMSO	200 µM
18	Octyl salicylate	118-60-5	-5 to 11	-8 to 20	DMSO	20 µM
19	L-Histidine	71-00-1	-8 to 12	8 to 120	NaPB	200 µM

The values were calculated as mean +/- 1.96 SD from the validation data.

Table 2 The expected ROS prediction for 17 proficiency substances using the other solar simulators and the acceptable range

No.	Chemical	CAS No.	SO	SA	Solvent	Concentration
21	Acridine	260-94-6	182 to 328	121 to 243	DMSO	200 µM
22	Chlorpromazine hydrochloride	69-09-0	-56 to 70	66 to 106	DMSO	200 µM
23	Diclofenac	15307-79-6	34 to 416	47 to 437	DMSO	200 µM
24	Doxycycline hydrochloride	10592-13-9	115 to 429	230 to 468	DMSO	200 µM
25	Furosemide	54-31-9	31 to 225	-7 to 109	DMSO	200 µM
26	Ketoprofen	22071-15-4	120 to 346	77 to 151	DMSO	200 µM
27	8-Methoxy psoralen	298-81-7	31 to 137	0 to 126	DMSO	200 µM
28	Nalidixic acid	389-08-2	54 to 246	88 to 470	DMSO	200 µM
29	Norfloxacin	70458-96-7	131 to 271	57 to 161	DMSO	200 µM
30	Omeprazole	73590-58-6	-221 to 103	30 to 216	DMSO	200 µM
31	Promethazine hydrochloride	58-33-3	20 to 168	-3 to 77	DMSO	200 µM
32	Fenofibrate	49562-28-9	77 to 203	-31 to 11	DMSO	20 µM
33	p-Aminobenzoic acid	150-13-0	-8 to 12	-11 to 7	DMSO	200 µM
34	Benzocaine	94-09-7	-7 to 9	-7 to 17	DMSO	200 µM
35	Erythromycin	114-07-8	-15 to 11	-9 to 21	DMSO	200 µM
36	Octyl salicylate	118-60-5	-5 to 11	-8 to 20	DMSO	20 µM
37	L-Histidine	71-00-1	-8 to 12	8 to 120	NaPB	200 µM

The values were calculated as mean +/- 1.96 SD from the validation data.

OECD/OCDE
ANNEX 4

Quartz reaction container used in the validation studies