OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Short Time Exposure *In Vitro* Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

INTRODUCTION

1.   The Short Time Exposure (STE) test method is an *in vitro* method that can be used under certain circumstances and with specific limitations for hazard classification and labeling of chemicals (substances and mixtures) that induce serious eye damage as well as those that do not require classification for either serious eye damage or eye irritation, as defined by the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (1).

2.   For many years, the eye hazard potential of chemicals has been evaluated primarily using an *in vivo* rabbit eye test (TG 405). It is generally accepted that, in the foreseeable future, no single *in vitro* alternative test will be able to fully replace the *in vivo* rabbit eye test to predict across the full range of serious eye damage/eye irritation responses for different chemical classes. However, strategic combinations of alternative test methods used in a (tiered) testing strategy may well be able to fully replace the rabbit eye test (2). The top-down approach is designed for the testing of chemicals that can be expected, based on existing information, to have a high irritancy potential or induce serious eye damage. Conversely, the bottom-up approach is designed for the testing of chemicals that can be expected, based on existing information, not to cause sufficient eye irritation to require a classification. While the STE test method is not considered to be a complete replacement for the *in vivo* rabbit eye test, it is suitable for use as part of a tiered testing strategy for regulatory classification and labeling, such as the top-down/bottom-up approach, to identify without further testing (i) chemicals inducing serious eye damage (UN GHS Category 1) and (ii) chemicals (excluding highly volatile substances and all solid chemicals other than surfactants) that do not require classification for eye irritation or serious eye damage (UN GHS No Category) (1) (2). However, a chemical that is neither predicted to cause serious eye damage (UN GHS Category 1) nor UN GHS No Category (does not induce either serious eye damage or eye irritation) by the STE test method would require additional testing to establish a definitive classification. Furthermore, the appropriate regulatory authorities should be consulted before using the STE in a bottom-up approach under classification schemes other than the UN GHS. The choice of the most appropriate test method and the use of this Test Guideline should be seen in the context of the OECD Guidance Document on an Integrated Approaches on Testing and Assessment for Serious Eye Damage and Eye irritation (14).

3.   The purpose of this test guideline (TG) is to describe the procedures used to evaluate the eye hazard potential of a test chemical based on its ability to induce cytotoxicity in the Short Time Exposure Test method. The cytotoxic effect of chemicals on corneal epithelial cells is an important mode of action (MOA) leading to corneal epithelium damage and eye irritation. Cell viability in the STE test method is

This Guideline was adopted by the OECD Council by written procedure on 9 October 2017 [C(2017)97].

assessed by the quantitative measurement, after extraction from cells, of blue formazan salt produced by the living cells by enzymatic conversion of the vital dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), also known as Thiazolyl Blue Tetrazolium Bromide (3). The obtained cell viability is compared to the solvent control (relative viability) and used to estimate the potential eye hazard of the test chemical. A test chemical is classified as UN GHS Category 1 when both the 5% and 0.05% concentrations result in a cell viability smaller than or equal to ($\leq$) 70%. Conversely, a chemical is predicted as UN GHS No Category when both 5% and 0.05% concentrations result in a cell viability higher than (>) 70%.

4.   The term "test chemical" is used in this Test Guideline to refer to what is tested and is not related to the applicability of the STE test method to the testing of substances and/or mixtures. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS AND LIMITATIONS

5.   This Test Guideline is based on a protocol developed by Kao Corporation (4), which was the subject of two different validation studies: one by the Validation Committee of the Japanese Society for Alternative to Animal Experiments (JSAAE) (5) and another by the Japanese Center for the Validation of Alternative Methods (JaCVAM) (6). A peer review was conducted by NICEATM/ICCVAM based on the validation study reports and background review documents on the test method (7).

6.   When used to identify chemicals (substances and mixtures) inducing serious eye damage (UN GHS Category 1 (1), data obtained with the STE test method on 125 chemicals (including both substances and mixtures), showed an overall accuracy of 83% (104/125), a false positive rate of 1% (1/86), and a false negative rate of 51% (20/39) as compared to the *in vivo* rabbit eye test (7). The false negative rate obtained is not critical in the present context, since all test chemicals that induce a cell viability of $\leq$ 70% at a 5% concentration and > 70% at 0.05% concentration would be subsequently tested with other adequately validated *in vitro* test methods or, as a last option, in the *in vivo* rabbit eye test, depending on regulatory requirements and in accordance with the sequential testing strategy and weight-of-evidence approaches currently recommended (1) (8). Mainly mono-constituent substances were tested, although a limited amount of data also exist on the testing of mixtures. 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. Such considerations are not needed when there is a regulatory requirement for testing of the mixture. The STE test method showed no other specific shortcomings when used to identify test chemicals as UN GHS Category 1. Investigators could consider using this test method on test chemicals, whereby cell viability $\leq$ 70% at both 5% and 0.05% concentration should be accepted as indicative of a response inducing serious eye damage that should be classified as UN GHS Category 1 without further testing.

7.   When used to identify chemicals (substances and mixtures) not requiring classification for eye irritation and serious eye damage (i.e. UN GHS No Category), data obtained with the STE test method on 130 chemicals (including both substances and mixtures), showed an overall accuracy of 85% (110/130), a false negative rate of 12% (9/73), and a false positive rate of 19% (11/57) as compared to the *in vivo* rabbit eye test (7). If highly volatile substances and solid substances other than surfactants are excluded from the dataset, the overall accuracy improves to 90% (92/102), the false negative rate to 2% (1/54), and the false positive to 19% (9/48) (7). As a consequence, the potential shortcomings of the STE test method when used to identify test chemicals not requiring classification for eye irritation and serious eye damage (UN GHS No Category) are a high false negative rate for i) highly volatile substances with a vapor pressure over 6 kPa and ii) Solid chemicals (substances and mixtures) other than surfactants and mixtures composed only of surfactants. Such chemicals are excluded from the applicability domain of the STE test method (7).

8.   In addition to the chemicals mentioned in paragraphs 6 and 7, the STE test method generated dataset also contains in-house data on 40 mixtures, which when compared to the *in vivo* Draize eye test, showed an accuracy of 88% (35/40), a false positive rate of 50% (5/10), and a false negative rate of 0% (0/30) for predicting mixtures that do not require classification under the UN GHS classification system (9). The STE test method can therefore be applied to identify mixtures as UN GHS No Category in a bottom-up approach with the exception of solid mixtures other than those composed only of surfactants as an extension of its limitation to solid substances. Furthermore, mixtures containing substances with vapour pressure higher than 6kPa should be evaluated with care to avoid potential under-predictions, and should be justified on a case-by-case basis.

9.   The STE test method cannot be used for the identification of test chemicals as UN GHS Category 2, Category 2A (eye irritation) or UN GHS Category 2B (mild eye irritation), due to the considerable number of UN GHS Category 1 chemicals under-predicted as UN GHS Category 2, 2A, or 2B and UN GHS No Category chemicals over-predicted as UN GHS Category 2, 2A, or 2B (7). For this purpose, further testing with another suitable method may be required.

10.   The STE test method is suitable for test chemicals that are dissolved or uniformly suspended for at least 5 minutes in physiological saline, 5% dimethyl sulfoxide (DMSO) in saline, or mineral oil. The STE test method is not suitable for test chemicals that are insoluble or cannot be uniformly suspended for at least 5 minutes in physiological saline, 5% DMSO in saline, or mineral oil. The use of mineral oil in the STE test method is possible because of the short-time exposure. Therefore, the STE test method is suitable for predicting the eye hazard potential of water-insoluble test chemicals (*e.g.*, long-chain fatty alcohols or ketones) provided that they are miscible in at least one of the three above proposed solvents (4).

11.   The term "test chemical" is used in this Test Guideline to refer to what is being tested[11] and is not related to the applicability of the STE test method to the testing of substances and/or mixtures.

PRINCIPLE OF THE TEST

12.   The STE test method is a cytotoxicity-based *in vitro* assay that is performed on a confluent monolayer of Statens Seruminstitut Rabbit Cornea (SIRC) cells, cultured on a 96-well polycarbonate microplate (4). After five-minute exposure to a test chemical, the cytotoxicity is quantitatively measured as the relative viability of SIRC cells using the MTT assay (4). Decreased cell viability is used to predict potential adverse effects leading to ocular damage.

13.   It has been reported that 80% of a solution dropped into the eye of a rabbit is excreted through the conjunctival sac within three to four minutes, while greater than 80% of a solution dropped into the human eye is excreted within one to two minutes (10). The STE test method attempts to approximate these exposure times and makes use of cytotoxicity as an endpoint to assess the extent of damage to SIRC cells following a five-minute exposure to the test chemical.

DEMONSTRATION OF PROFICIENCY

14.   Prior to routine use of the STE test method described in this test guideline, laboratories should demonstrate technical proficiency by correctly classifying the eleven substances recommended in Table 1. These substances were selected to represent the full range of responses for serious eye damage or eye irritation based on results of *in vivo* rabbit eye tests (TG 405) and the UN GHS classification system (1). Other selection criteria included that the substances should be commercially available, that high-quality *in*

---

[1]   In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term "test chemical" describing what is being tested should now be applied in new and updated Test Guidelines.

vivo reference data should be available, and that high-quality *in vitro* data from the STE test method should be available (3). In situations where a listed substance is unavailable or where justifiable, another substance for which adequate *in vivo* and *in vitro* reference data are available could be used provided that the same criteria as described here are used.

Table 1: List of Proficiency Substances

Substance	CASRN	Chemical class[1]	Physical state	*In Vivo UN GHS Cat.*[2]	Solvent in STE test	STE UN GHS Cat.
Benzalkonium chloride (10%, aqueous)	8001-54-5	Onium compound	Liquid	Category 1	Saline	Category 1
Triton X-100 (100%)	9002-93-1	Ether	Liquid	Category 1	Saline	Category 1
Acid Red 92	18472-87-2	Heterocyclic compound; Bromine compound; Chlorine compound	Solid	Category 1	Saline	Category 1
Sodium hydroxide	1310-73-2	Alkali; Inorganic chemical	Solid	Category 1[3]	Saline	Category 1
Butyrolactone	96-48-0	Lactone; Heterocyclic compound	Liquid	Category 2A	Saline	No prediction can be made
1-Octanol	111-87-5	Alcohol	Liquid	Category 2A/B[4]	Mineral Oil	No prediction can be made
Cyclopentanol	96-41-3	Alcohol; Hydrocarbon, cyclic	Liquid	Category 2A/B[5]	Saline	No prediction can be made
2-Ethoxyethyl acetate	111-15-9	Alcohol; Ether	Liquid	No Category	Saline	No Category
Dodecane	112-40-3	Hydrocarbon, acyclic	Liquid	No Category	Mineral Oil	No Category
Methyl isobutyl ketone	108-10-1	Ketone	Liquid	No Category	Mineral Oil	No Category
n,n-Dimethylguanidine sulfate	598-65-2	Amidine; Sulfur compound	Solid	No Category	Saline	No Category

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

[1]Chemical classes were assigned using information obtained from previous NICEATM publications and if not available, using the National Library of Medicine's Medical Subject Headings (MeSH®) (via ChemIDplus® [National Library of Medicine], available at http://chem.sis.nlm.nih.gov/chemidplus/) and structure determinations made by NICEATM.

[2]Based on results from the *in vivo* rabbit eye test (OECD TG 405) and using the UN GHS (1).

[3]Classification as Cat.1 is based on skin corrosive potential of 100% sodium hydroxide (listed as a proficiency chemical with skin corrosive potential in OECD TG 435) and the criterion for UN GHS category 1 (1).

[4]Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these

two categories, i.e., 2 out of 6 vs 4 out of 6 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* dataset included 2 studies with 3 animals each. In one study two out of three animals showed effects at day 7 warranting a Cat. 2A classification (11), whereas in the second study all endpoints in all three animals recovered to a score of zero by day 7 warranting a Cat. 2B classification (12).

[5]Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, i.e., 1 out of 3 vs 2 out of 3 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* study included 3 animals. All endpoints apart from corneal opacity and conjunctivae redness in one animal recovered to a score of zero by day 7 or earlier. The one animal that did not fully recover by day 7 had a corneal opacity score of 1 and a conjunctivae redness of 1 (at day 7) that fully recovered at day 14 (11).

PROCEDURE

Preparation of the Cellular Monolayer

15.   The rabbit cornea cell line, SIRC should be used for performing the STE test method. It is recommended that SIRC cells are obtained from a well-qualified cell bank, such as American Type Culture Collection CCL60.

16.   SIRC cells are cultured at 37°C under 5% CO_2 and humidified atmosphere in a culture flask containing a culture medium comprising Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50–100 units/mL penicillin and 50–100 µg/mL streptomycin. Cells that have become confluent in the culture flask should be separated using trypsin-ethylenediaminetetraacetic acid solution, with or without the use of a cell scraper. Cells are propagated (e.g. 2 to 3 passages) in a culture flask before being employed for routine testing, and should undergo no more than 25 passages from thawing.

17.   Cells ready to be used for the STE test are then prepared at the appropriate density and seeded into 96-well plates. The recommended cell seeding density is 6.0×10^3 cells per well when cells are used four days after seeding, or 3.0×10^3 cells per well when cells are used five days after seeding, at a culture volume of 200 µL. Cells used for the STE test that are seeded in a culture medium at the appropriate density will reach a confluence of more than 80% at the time of testing, *i.e.*, four or five days after seeding.

Application of the Test Chemicals and Control Substances

18.   The first choice of solvent for dissolving or suspending test chemicals is physiological saline. If the test chemical demonstrates low solubility or cannot be dissolved or suspended uniformly for at least five minutes in saline, 5% DMSO (CAS#67-68-5) in saline is used as a second choice solvent. For test chemicals that cannot be dissolved or suspended uniformly for at least five minutes in either saline or 5% DMSO in saline, mineral oil (CAS#8042-47-5) is used as a third choice solvent.

19.   Test chemicals are dissolved or suspended uniformly in the selected solvent at 5% (w/w) concentration and further diluted by serial 10-fold dilution to 0.5% and 0.05% concentration. Each test chemical is to be tested at both 5% and 0.05% concentrations. Cells cultured in the 96-well plate are exposed to 200 µL/well of either a 5% or a 0.05% concentration of the test chemical solution (or suspension), for five minutes at room temperature. Test chemicals (mono-constituent substances or multi-constituent substances or mixtures) are considered as neat substances and diluted or suspended according to the method, regardless of their purity.

20.   The culture medium described in paragraph 15 is used as a medium control in each plate of each repetition. Furthermore, cells are to be exposed also to solvent control samples in each plate of each

repetition. The solvents listed in paragraph 17 have been confirmed to have no adverse effects on the viability of SIRC cells.

21.   In the STE test method, 0.01% Sodium lauryl sulfate (SLS) in saline is to be used as a positive control in each plate of each repetition. In order to calculate cell viability of the positive control, each plate of each repetition has to also include a saline solvent control.

22.   A blank is necessary to determine compensation for optical density and should be performed on wells containing only phosphate buffered saline, but no calcium and magnesium (PBS-) or cells.

23.   Each sample (test chemical at 5% and 0.05%, medium control, solvent control, and positive control) should be tested in triplicate in each repetition by exposing the cells to 200 μL of the appropriate test or control chemical for five minutes at room temperature.

24.   Benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative irritancy potential of an ocular irritant within a specific range of irritant responses.

Cell Viability Measurement

25.   After exposure, cells are washed twice with 200 μL of PBS and 200 μL of MTT solution (0.5 mg MTT/mL of culture medium) is added. After a two-hour reaction time in an incubator (37˚C, 5% CO_2), the MTT solution is decanted, MTT formazan is extracted by adding 200 μL of 0.04 N hydrochloric acid-isopropanol for 60 minutes in the dark at room temperature, and the absorbance of the MTT formazan solution is measured at 570 nm with a plate reader. Interference of test chemicals with the MTT assay (by colorants or direct MTT reducers) only occurs if significant amount of test chemical is retained in the test system following rinsing after exposure which is the case for 3D Reconstructed human cornea or Reconstructed human epidermis tissues but is not relevant for the 2D cell cultures used for the STE test method.

Interpretation of Results and Prediction Model

26.   The optical density (OD) values obtained for each test chemical are then used to calculate cell viability relative to the solvent control, which is set at 100%. The relative cell viability is expressed as a percentage and obtained by dividing the OD of test chemical by the OD of the solvent control after subtracting the OD of blank from both values.

$$\text{Cell viability (\%)} = \frac{(\text{OD}_{570}\text{ of test chemical}) - (\text{OD}_{570}\text{ of blank})}{(\text{OD}_{570}\text{ of solvent control}) - (\text{OD}_{570}\text{ of blank})} \times 100$$

Similarly, the relative cell viability of each solvent control is expressed as a percentage and obtained by dividing the OD of each solvent control by the OD of the medium control after subtracting the OD of blank from both values.

27.   Three independent repetitions, each containing three replicate wells (*i.e.*, n=9), should be performed. The arithmetic mean of the three wells for each test chemical and solvent control in each independent repetition is used to calculate the arithmetic mean of relative cell viability. The final arithmetic mean of the cell viability is calculated from the three independent repetitions.

28.   The cell viability cut-off values for identifying test chemicals inducing serious eye damage (UN GHS Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) are given hereafter.

6

Table 2: Prediction model of the STE test method

Cell viability		UN GHS Classification	Applicability
At 5%	At 0.05%		
> 70%	> 70%	No Category	Substances and mixtures, with the exception of: i) highly volatile substances with a vapor pressure over 6 kPa[1] and ii) Solid chemicals (substances and mixtures) other than surfactants and mixtures composed only of surfactants
≤ 70%	> 70%	No prediction can be made	Not applicable
≤ 70%	≤ 70%	Category 1	Substances and mixtures [2]

[1] Mixtures containing substances with vapour pressure higher than 6kPa should be evaluated with care to avoid potential under-predictions, and should be justified on a case-by-case basis.

[2] Based on results obtained mainly with mono-constituent substances, although a limited amount of data also exist on the testing of mixtures. The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. 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. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

Acceptance Criteria

29.   Test results are judged to be acceptable when the following criteria are all satisfied:

a) Optical density of the medium control (exposed to culture medium) should be 0.3 or higher after subtraction of blank optical density.

b) Viability of the solvent control should be 80% or higher relative to the medium control. If multiple solvent controls are used in each repetition, each of those controls should show cell viability greater than 80% to qualify the test chemicals tested with those solvents.

c) The cell viability obtained with the positive control (0.01% SLS) should be within two standard deviations of the historical mean. The upper and lower acceptance boundaries for the positive control should be frequently updated i.e., every three months, or each time an acceptable test is conducted in laboratories where tests are conducted infrequently (i.e., less than once a month). Where a laboratory does not complete a sufficient number of experiments to establish a statistically robust positive control distribution, it is acceptable that the upper and lower acceptance boundaries established by the method developer are used, i.e., between 21.1% and 62.3% according to its laboratory historical data, while an internal distribution is built during the first routine tests.

d) Standard deviation of the final cell viability derived from three independent repetitions should be

7

less than 15% for both 5% and 0.05% concentrations of the test chemical.

If one or several of these criteria is not met, the results should be discarded and another three independent repetitions should be conducted.

DATA AND REPORTING

Data

30.   Data for each individual well (e.g., cell viability values) of each repetition as well as overall mean, SD, and classification are to be reported.

Test Report

31.   The test report should include the following information:

Test Chemical and Control Substances

- Mono-constituent substance : chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers;

- Multi-constituent substance, UVCB and mixture: Characterization as far as possible by e.g., chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;

- Physical state, volatility, pH, LogP, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available;

- Purity, chemical identity of impurities as appropriate and practically feasible, etc;

- Treatment prior to testing, if applicable (e.g., warming, grinding);

- Storage conditions and stability to the extent available.

Test Method Conditions and Procedures

- Name and address of the sponsor, test facility and study director;

- Description of the test method used;

- Cell line used, its source, passage number and confluence of cells used for testing;

- Details of test procedure used;

- Number of repetitions and replicates used;

- Test chemical concentrations used (if different than the ones recommended);

- Justification for choice of solvent for each test chemical;

- Duration of exposure to the test chemical (if different than the one recommended);

- Description of any modifications of the test procedure;

- Description of evaluation and decision criteria used;

- Reference to historical positive control mean and Standard Deviation (SD):

- Demonstration of proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or demonstration of reproducible performance of the test method over time.

Results

- For each test chemical and control substance, and each tested concentration, tabulation should be given for the individual OD values per replicate well, the arithmetic mean OD values for each independent repetition, the % cell viability for each independent repetition, and the final arithmetic mean % cell viability and SD over the three repetitions;

- Results for the medium, solvent and positive control demonstrating suitable study acceptance criteria;

- Description of other effects observed;

- The overall derived classification with reference to the prediction model/decision criteria used.

Discussion of the Results

Conclusions

LITERATURE

1) United Nations UN (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Fifth revised edition. New York & Geneva: United Nations Publications. ISBN: 978-92-1-117006-1. Available at: [http://www.unece.org/trans/danger/publi/ghs/ghs_rev05/05files_e.html].

2) Scott L, et al. (2010). A proposed Eye Irritation Testing Strategy to Reduce and Replace *in vivo* Studies Using Bottom-Up and Top-Down Approaches. *Toxicol. In Vitro* 24, 1-9.

3) Mosmann T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to 7 Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* 65, 55-63.

4) Takahashi Y, et al. (2008). Development of the Short Time Exposure (STE) Test: an *In Vitro* Eye Irritation Test Using SIRC Cells. *Toxicol. In Vitro* 22,760-770.

5) Sakaguchi H, et al. (2011). Validation Study of the Short Time Exposure (STE) Test to Assess the Eye Irritation Potential of Chemicals. *Toxicol. In Vitro* 25,796-809.

6) Kojima H, et al. (2013). Second-Phase Validation of Short Time Exposure Tests for Assessment of Eye Irritation Potency of Chemicals. *Toxicol. In Vitro* 27, pp.1855-1869.

7) ICCVAM (2013). Short Time Exposure (STE) Test Method Summary Review Document, NIH. Available at: [http://www.ntp.niehs.nih.gov/iccvam/docs/ocutox_docs/STE-SRD-NICEATM-508.pdf].

8) OECD (2012). Test Guideline for Testing of Chemicals (No.405): Acute Eye Irritation/Corrosion.Organisation for Economic Cooperation and Development, Paris.

9) Saito K, et al. (2015). Predictive Performance of the Short Time Exposure Test for Identifying Eye Irritation Potential of Chemical Mixtures.

10) Mikkelson TJ, Chrai SS and Robinson JR. (1973). Altered Bioavailability of Drugs in the Eye Due to Drug-Protein Interaction. *J. Pharm*. Sci.1648-1653.

11) ECETOC. (1998). Eye Irritation Reference Chemicals Data Bank. Technical Report (No. 48. (2)), Brussels, Belgium.

12) Gautheron P, et al. (1992). Bovine Corneal Opacity and Permeability Test: an *In Vitro* Assay of Ocular Irritancy. Fundam Appl *Toxicol.* 18, 442–449.

13) OECD (2005), Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Environmental, Health and Safety Publications,Series on Testing and Assessment (No. 34.). Organisation for Economic Cooperation and Development, Paris.

14) OECD (2017). Guidance Document on an Integrated Approaches on Testing and Assessment for Serious Eye Damage and Eye irritation. Series on Testing and Assessment No.263. ENV Publications, Organisation for Economic Cooperation and Development, Paris.

OECD/OCDE   491

ANNEX I

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with concordance to mean the proportion of correct outcomes of a test method (13).

Benchmark substance: A substance used as a standard for comparison to a test chemical. A benchmark substance should have the following properties; (i) a consistent and reliable source(s); (ii) structural and functional similarity to the class of substances being tested; (iii) known physical/chemical characteristics; (iv) supporting data on known effects, and (v) known potency in the range of the desired response.

Bottom-Up Approach: A step-wise approach used for a test chemical suspected of not requiring classification for eye irritation or serious eye damage, which starts with the determination of chemicals not requiring classification (negative outcome) from other chemicals (positive outcome)

Chemical: means a substance or mixture.

Eye irritation: Production of change in the eye following the application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Interchangeable with "reversible effects on the eye" and with UN GHS Category 2 (1)

False negative rate: The proportion of all positive chemicals falsely identified by a test method as negative. It is one indicator of test method performance.

False positive rate: The proportion of all negative chemicals that are falsely identified by a test method as positive. It is one indicator of test method performance.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

Medium control: An untreated replicate containing all components of a test system. This sample is processed with test chemical-treated samples and other control samples to determine whether the solvent interacts with the test system.

Mixture: A mixture or a solution composed of two or more substances in which they do not react (1).

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide.

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration $\geq$ 10% (w/w) and < 80% (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

OD: Optical Density.

Positive control: A replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (10).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (13).

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (10).

Serious eye damage: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test chemical to the anterior surface of the eye, which is not fully reversible within 21 days of application. Interchangeable with "irreversible effects on the eye" and with UN GHS Category 1 (1).

Solvent/vehicle control: An untreated sample containing all components of a test system, including the solvent or vehicle that is processed with the test chemical-treated and other control samples to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent or vehicle. When tested with a concurrent medium control, this sample also demonstrates whether the solvent or vehicle interacts with the test system.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (13).

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, inducing any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing it composition (1).

Surfactant: Also called surface-active agent, this is a chemical such as a detergent, that can reduce the surface tension of a liquid and thus allow it to foam or penetrate solids; it is also known as a wetting agent.

Test chemical: The term "test chemical" is used to refer to what is being tested.

Tiered testing strategy: A stepwise testing strategy where all existing information on a test chemical is reviewed, in a specified order, using a weight of evidence process at each tier to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier. If the irritancy potential of a test chemical can be assigned based on the existing information, no additional testing is required. If the irritancy potential of a test chemical cannot be assigned based on the existing information, a step-wise sequential animal testing procedure is performed until an unequivocal

classification can be made.

Top-Down Approach: step-wise approach used for a test chemical suspected of causing serious eye damage, which starts with the determination of chemicals inducing serious eye damage (positive outcome) from other chemicals (negative outcome).

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

UN GHS Category 1: See "Serious eye damage".

UN GHS Category 2: See "Eye irritation".

UN GHS No Category: Chemicals that are not classified as UN GHS Category 1 or 2 (2A or 2B).

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.