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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE 4XX

The Cytosensor Microphysiometer Test Method: An *In Vitro* Method for Identifying Chemicals Not Classified as Irritant, as well as Ocular Corrosive and Severe Irritant Chemicals

INTRODUCTION

1. The Cytosensor Microphysiometer (CM) test method is an *in vitro* test method that can be used, in conjunction with other test methods, as part of a tiered testing strategy for hazard classification and labelling of chemicals for eye corrosion/irritation, according to the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS), the European Union (EU) Regulation on Classification, Labelling and Packaging of Substances and Mixtures (CLP), and the U.S. Environmental Protection Agency (EPA) (1)(2)(3). While the CM test method is not considered valid as a complete replacement for the *in vivo* rabbit eye test, the CM is recommended for use, under certain circumstances and with specific limitations, for regulatory classification and labelling, as an initial step within a Top-Down approach to identify ocular corrosives and severe irritants (UN GHS Category 1; EU CLP Category 1; U.S. EPA Category I) from all other toxicity classes, for water soluble chemicals (substances and mixtures). Furthermore, the test method can be used an initial step within a Bottom-Up approach to identify chemicals not classified as irritant (UN GHS No Category; EU CLP No Category; U.S. EPA Category IV) from all other classes, water soluble surfactants and surfactant-containing mixtures (4).

2. It is currently generally accepted that, in the foreseeable future, no single *in vitro* eye irritation test will be able to replace the *in vivo* Draize eye test to predict across the full range of irritation for different chemical classes. However, strategic combinations of several alternative test methods within a (tiered) testing strategy may be able to replace the Draize eye test (5). The Top-Down approach (5) is designed to be used when, based on existing information, a chemical is expected to have high irritancy potential. Test chemicals (substances and mixtures) that produce severe effects in the CM test method can be classified as ocular corrosives or severe irritants (UN GHS Category 1; EU CLP Category 1; U.S. EPA Category I) without any further testing. The Bottom-Up approach (5) is designed to be used when, based on existing information, a chemical is expected not to cause sufficient eye irritation to require a classification. Test chemicals (substances and mixtures) that are negative in the CM test method can be considered as not classified (UN GHS No Category; EU CLP No Category; U.S. EPA Category IV) without any further testing. A chemical that tests as mild or moderate irritant would need to be tested in another test method. The CM method is so far the only validated *in vitro* test method that can be used to identify chemicals not classified as eye irritants.

3. The purpose of this Test Guideline is to describe the procedures used to evaluate the potential for ocular corrosivity or irritancy of a test chemical as measured by its ability to induce changes in cellular metabolism which occur after chemical exposure. The CM test method estimates the decrease in metabolic rate (glucose utilization rate) of the cells exposed to the test chemical by measuring the rate of change in pH of the medium (acidity) per unit time as compared to the basal metabolic state. The reduction of the metabolic rate of the exposed cells can be used to estimate the ocular toxicity potential of a test chemical.

4. This Test Guideline also includes a set of Performance Standards (PS) (Annex II – *still need to be developed*) for the assessment of similar and modified CM test methods in accordance with the principles of Guidance Document No. 34 (6). Before a proposed similar or modified *in vitro* CM test method can be

used for regulatory purposes, its reliability, relevance (accuracy), and limitations for its proposed use should be determined in order to ensure that it can be regarded as similar to that of the validated test method, in accordance with the requirements of the PS set out in this Test Guideline (Annex II).

5. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS AND LIMITATIONS

6. This Test Guideline is based on the modified version of the current INVITTOX protocol No. 102[1] (7) that has been evaluated in an international validation study by the European Centre for the Validation of Alternative Methods (ECVAM) (8), in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (9) and the Japanese Center for the Validation of Alternative Methods (JaCVAM).

7. The CM test method is not recommended for the identification of mild/moderate irritant chemicals (substances and mixtures), as demonstrated by the validation study (4, 8).

8. The test method is only applicable to water soluble chemicals (substances and mixtures) (8). When used to initiate a Top-Down approach, the test method is considered suitable for all water soluble chemicals (substances and mixtures), while when used to initiate a Bottom-Up approach, it is considered suitable only for water soluble surfactants and surfactant containing mixtures.

9. The applicability domain of the CM test method is influenced by some physical constrains imposed by the CM instrument itself. The instrument requires exposing the cells by pumping test chemical through a small diameter tube, and then washing the cells by pumping fresh media across the cells and out the chamber through another small diameter tube. Thus non-water-soluble solids, suspensions or viscous chemical can not be used, as they would tend to clog the machine or not be washed out once they have reached the exposure chamber.

10. The current applicability domain might be increased in some cases, but only after analyzing an expanded data set of studied test chemicals, preferably acquired through testing (4). This Test Guideline will be updated accordingly as new information and data are considered.

PRINCIPLE OF THE TEST

11. The CM test method is a cytotoxicity and cell-function based *in vitro* assay that is performed on a sub-confluent monolayer of adherent cells (mouse L929 fibroblasts) cultured on a transwell polycarbonate insert with a porous membrane, which functions as electrode, and a light-addressable potentiometric sensor detecting changes in pH (acidity). Mechanistically, the CM test method is intended to model the cytotoxic action of an irritant chemical on the cell membranes of the corneal and conjunctival epithelium where the test chemical would reside in an *in vivo* exposure (10).

12. The CM estimates the metabolic rate (glucose utilization rate) of a population of cells maintained in low volume flow-through chambers by measuring the rate of excretion of acid by-products and the resulting decrease in pH of the surrounding medium. The metabolic rate is determined indirectly by the number of protons excreted into the low buffer medium (change in pH) per unit time. The light-

[1] The INVITTOX protocol 102 is currently under revision at ECVAM.

addressable potentiometer forms the bottom of the flow through chamber and serves as a very sensitive and stable pH meter.

13. During the course of an experiment, test samples, prepared as dilution series of a test chemical, are introduced in order of increasing concentration to flow-through chambers containing the cells. Therefore, in the CM test method the same cell population is exposed progressively to increasing concentrations of the test chemical. The cells cultured in the chamber are exposed to the test chemical for a short period of time, followed by a rinse step with medium to remove the test chemical. Finally the flow is stopped and the change in pH is measured. All rate of acidification measurements are made on washed cells. These three steps are repeated with increasing concentrations of the test chemical until the highest testable concentration has been used or the metabolic rate has declined to effectively zero.

14. The rate of change in pH per unit time becomes the metabolic rate of the population of cells. If a test chemical causes cytotoxicity to this population of cells it is assumed that the metabolic rate will fall. A transient up-regulation of glucose metabolism can occur if the cells need energy to maintain their integrity in the face of a mild biochemical insult, but it soon falls below the basal level if exposure to the cytotoxic chemical is prolonged or intensified (higher concentration). The concentration of test chemical that leads to a 50% decline in the basal metabolic rate of the population (MRD_{50}) is the parameter used to measure the cytotoxic effect of the test chemical on the test system (L929 mouse fibroblast cells). The MRD_{50} value (mg/mL) for each test chemical is calculated from a concentration response curve (see paragraph 29), and is used to provide a measure of the ocular irritancy potential of the test chemical.

15. Recovery is an important part of a test chemical's toxicity profile that is also assessed by the *in vivo* ocular irritation test. The CM test method is non-invasive, thus it could be also used for the determination of recovery of the cells from toxic insult. Additional data, preferably acquired by further testing, would be required to confirm this usefulness (8). This Test Guideline will be updated accordingly as new information and data are considered.

PROCEDURE

Preparation of the cells grown on capsule cups

16. The L929 mouse fibroblasts, grown routinely in cell culture flasks, are trypsinised, centrifuged and an appropriate cell suspension is prepared in Dulbecco's Modified Eagle's Medium (DMEM). The cells are then seeded in the capsule cups at a density of ~6 x 10^5 cells/cup, and subsequently incubated for 16-32 hours under normal growth conditions before use in the CM test method. At the time of use the cells should be <80 % confluent. Use of a fully confluent monolayer may interfere with communication between the upper and lower surfaces of the Transwell porous membrane of the chamber, causing inaccurate pH readings.

17. The L929 cell cultures should be kept in incubators in a humidified atmosphere, at 5 ± 1% CO_2 and 37 ± 1ºC. The cells should be free of contamination by bacteria, viruses, mycoplasma and fungi. The capsule cups containing cells are placed in the Cytosensor chambers at the time the assay is performed.

Application of the Test and Control Chemicals

18. A fresh stock solution of test chemical should be prepared for each experimental run and used within 30 minutes of preparation. Test chemicals should be prepared in the low-buffered treatment medium (serum-free, $NaCO_3$-free DMEM with additional NaCl and supplemented with 2 mM L-

glutamine and 50 µg/mL gentamicin). The data provided by the Cytosensor machine are based on time-dependent changes in pH which occur as a result of cellular metabolism. Use of fully buffered medium would essentially eliminate the ability to detect the necessary level of pH changes.

19. A dose range finding assay is performed to establish an appropriate test chemical dose range for the definitive toxicity test. Solutions at different concentrations are prepared by serial three-fold dilutions in treatment medium that has been left to equilibrate to room temperature overnight. The concentrations to be tested in the dose-range finding assay are as follows: 10 mg/mL; 3.33 mg/mL; 1.11 mg/mL; 0.370 mg/mL; 0.123 mg/mL; 0.0412 mg/mL and 0.0137 mg/mL. If possible, the test chemical concentration that results in the reduction of the metabolic rate to 50% of its basal rate (MRD_{50} value) should be calculated from the dose-finding assay.

20. In the definitive assays seven concentrations are tested. Generally, three concentrations are chosen below the expected MRD_{50} value, one at approximately the MRD_{50} value, and three above the expected MRD_{50}. If the test chemical fails to cause 50% toxicity in the dose range finding assay, the maximum concentration used will be generally 270 mg/mL or less, based on the ability of the test chemical to form single phase solution/suspension in the treatment buffer. If the test chemical cannot form a single phase solution/suspension at the concentration of 1.11 mg/mL, it could not be tested in the CM test method using standard techniques.

21. Once a concentration range which includes the MRD_{50} value has been found, the same range of concentrations should be tested twice more, meaning that the mean MRD_{50} value is derived from three independent runs. If the MRD_{50} results could be determined from the results of the dose-range finding assay, these data could be also included as the results of the definitive assay.

22. A concurrent positive control should be used in each experimental run. A solvent control is recommended when a solvent other than low-buffered treatment medium is used. The suggested positive control chemical is a 10% (w/v) Sodium lauryl sulphate (CAS No. 151-21-3) stock solution in DI H_2O. This stock solution is considered the "neat" test chemical and should be diluted in low-buffered treatment medium for testing. A dose range finding assay should be performed once on the positive control to set the appropriate ranges for the subsequent definitive trials. Historical data for the positive control must be established in each user laboratory to ensure that the Cytosensor machine provides similar readings from day-to-day, and to enable comparing data for different test chemicals tested on different days. The negative control to obtain the basal metabolic rate is low-buffered treatment medium alone.

Determination of the change in metabolic rate

23. Prior to the start of the assay, the medium in capsule cups containing the cultured L929 cells is changed to low-buffered treatment medium. The capsules are then placed in the Cytosensor chambers. The medium flow in the machine is adjusted and obvious bubbles are cleared. While medium is flowing through the chamber, the pH is stable and governed by the medium. When the flow of medium is stopped, the pH begins to drop in a linear fashion over time. The actual change in pH during this measurement is generally less than 0.2 pH units.

24. It must always be ascertained that the Cytosensor machine and cells are stable before the experiment can begin, since all subsequent data points are interpreted based on the baseline rate. Thus, at the beginning of each assay, at least four to five measurements are taken to assess the basal acidification rate, which is used as the negative control for each cell culture. Baseline rates are expected to fall between 50 and 200 µV/s after a stabilization period of approximately 1 hour. If a capsule with cells in a chamber fails to achieve these ranges it should be discarded and replaced with another capsule.

25. After the baseline data points have been taken, the cells contained in the chamber undergo cycles of exposure to the test chemical consisting of three phases (exposure, wash-out and measurement). The cycles start from the lowest concentration tested and are repeated for the increasing concentrations of the test chemical in the same cell population. Each cycle takes 20 minutes. For standard safety assays the exposure time should be 810 seconds in order to match the experimental conditions for which the main prediction model was established. Longer or shorter exposure times will change the calculated MRD_{50} since toxicity is a function of exposure time.

26. In the first phase of an exposure cycle, the test chemical is introduced into the sensor for 810 seconds. The nominal medium flow rate is 100 µL/min for the first minute and 20 µL/min for the remaining 12 minutes and 30 seconds. During the second phase, which lasts 6 minutes (at a flow rate of 100 µL/min) , the test chemical is washed out from the sensor chamber using the low-buffered treatment medium not containing the test chemical. In the third phase the flow is stopped after 25 seconds and the pH is measured. These cycles (exposure, wash-out and measurement phases) are repeated with increasing concentrations of the test chemical until the highest concentration is reached.

Interpretation of results and Prediction model

27. The acidification rates that occur after exposure to each test chemical concentration are calculated and compared to the mean basal acidification rate of the same cells prior to exposure to the test chemical. The percent of control acidification rate is determined by comparing the dose response acidification rate to the basal acidification rate.

The following equation for the calculation of % control acidification rate should be applied:

$$\% \text{ of control acidification rate} = \frac{\text{acidification rate after exposure to test chemical}}{\text{basal acidification rate}} \times 100$$

The percent of control acidification rates for each concentration are then plotted against the test chemical concentrations. The concentration of the test chemical that results in a 50% reduction in acidification rate is interpolated from the obtained curve and referred to as the MRD_{50}.

28. The cut-off values of MRD_{50} for distinguishing chemicals not classified as irritant, mild/moderate irritants and strong irritants are given below:

MRD_{50} (mg/mL)	**UN GHS C&L**	**EU CLP C&L**	**U.S. EPA C&L**
> 80 mg/mL	N/A	N/A	Category IV
> 2; ≤ 80 mg/mL	N/A	N/A	Category II or III
> 10 mg/mL	No Category	No Category	N/A
> 2; ≤ 10 mg/mL	Category 2A or 2B	Category 2	N/A
≤ 2 mg/mL	Category 1	Category 1	Category I

N/A: Not applicable for the particular classification and labelling system

It has to be noted that the CM test method is recommended only for the identification of ocular corrosives and severe irritants (UN GHS Category 1, EU CLP Category 1, U.S. EPA Category I) and for the identification of chemicals not classified as irritant (UN GHS No Category; EU CLP No Category; U.S. EPA Category IV), within a previously stated applicability domain (see paragraphs 1 and 8). In this context, the MRD_{50} cut-off value < 2 mg/mL is recommended for identification of ocular corrosives and severe irritants (for UN GHS, EU CLP and U.S. EPA C&L) for water soluble substances and mixtures (5, 7, 8), while the MRD_{50} cut-off value > 10 mg/mL (for UN GHS and EU CLP C&L) or > 80 mg/mL (for U.S. EPA C&L) are recommended for identification of chemicals not classified as irritant for water soluble surfactants and water soluble surfactant-containing mixtures (4, 7, 8).

Acceptance of results

29. Acceptance criteria are normally based on the performance of the positive control. The test is considered acceptable if the MRD_{50} of the positive control falls within 2 standard deviations of the historical range.

DATA AND REPORTING

Data

30. For each run, data from individual replicate measurements (e.g. basal acidification rate, acidification rate after exposure to a concentration of the test chemical and calculated % of control acidification rate) should be reported in tabular form. In addition means ± SD of individual replicate measurements in each run should be reported.

Test Report

31. The test report should include the following information:

Test and Control Chemicals

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known;
- Chemical CAS number, if known;
- Purity and composition of the chemical or mixture (in percentage(s) by weight), to the extent this information is available;
- Physical-chemical properties relevant to the conduct of the study (e.g. physical state, volatility, pH, stability, water solubility, chemical class);
- Treatment of the test/control chemical prior to testing, if applicable (*e.g.*, warming, grinding);
- Storage conditions;

Justification of the Test Method and Protocol Used

Test Conditions

- Description of cell system used;

- Details of test procedure used;
- Test chemical concentration(s) used;
- Duration of exposure to the test chemical;
- Description of any modifications of the test procedure;
- Description of evaluation criteria used;
- Reference to historical data of the model (*e.g.*, negative and positive controls, solvent control, benchmark chemicals, if applicable);

Results

- Tabulation of data from individual test chemicals for each run and each replicate measurement;
- Description of other effects observed.

Discussion of the Results

Conclusions

LITERATURE

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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.

EPA Category 1: Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days (1).

EU CLP (European Commission Regulation on the Classification, Labelling and Packaging of Substances and Mixtures): Implements in the European Union (EU) the UN GHS system for the classification of chemicals (substances and mixtures)(2).

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.

GHS (Globally Harmonized System of Classification and Labelling of Chemicals by the United Nation (UN)): 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).

GHS Category 1: 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 (1).

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.

Me-too test: A colloquial expression for a test method that is structurally and functionally similar to a validated and accepted reference test method. Such a test method would be a candidate for catch-up validation. Interchangeably used with similar test method.

Mixture: Used in the context of the UN GHS (1) as a mixture or solution composed of two or more substances in which they do not react.

MRD_{20}: The concentration of test chemical as weight/volume %, required to reduce the acidification rate to 50%.

Negative control: An untreated replicate containing all components of a test system. This sample is processed before exposure to the test chemical, to assess the basal acidification rate.

Not-classified: Chemicals that are not classified as UN GHS Categories 1, 2A, or 2B; EU CLP Categories 1 or 2; or U.S. EPA Categories I, II, or III ocular irritants (1)(2)(3).

Ocular corrosive: (a) A chemical that causes irreversible tissue damage to the eye. (b) Chemicals that are classified as UN GHS Category 1; EU CLP Category 1; or U.S. EPA Category I ocular irritants (1)(2)(3).

Ocular irritant: (a) A chemical that produces a reversible change in the eye following application to the anterior surface of the eye; (b) Chemicals that are classified as UN GHS Categories 2A, or 2B; EU CLP Category 2; or U.S. EPA Categories II or III ocular irritants (1)(2)(3).

Ocular severe irritant: (a) A chemical that causes tissue damage in the eye following application to the anterior surface of the eye that is not reversible within 21 days of application or causes serious physical decay of vision. (b) Chemicals that are classified as UN GHS Category 1; EU CLP Category 1; or U.S. EPA Category I ocular irritants (1)(2)(3).

Performance standards (PS): Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are; (i) essential test method components; (ii) a minimum list of Reference Chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals.

Positive control: A replicate containing all components of a test system and treated with a chemical 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.

Reference chemicals: Chemicals selected for use in the validation process, for which responses in the *in vitro* or *in vivo* reference test system or the species of interest are already known. These chemicals should be representative of the classes of chemicals for which the test method is expected to be used, and should represent the full range of responses that may be expected from the chemicals for which it may be used, from strong, to weak, to negative. Different sets of reference chemicals may be required for the different stages of the validation process, and for different test methods and test uses (6).

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 (6).

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.

Replacement test: A test which is designed to substitute for a test that is in routine use and accepted for hazard identification and/or risk assessment, and which has been determined to provide equivalent or improved protection of human or animal health or the environment, as applicable, compared to the accepted test, for all possible testing situations and chemicals.

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 (6).

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 negative 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.

Substance: Used in the context of the UN GHS as chemical elements and their compounds in the natural state or obtained by any production process, including 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 its composition.

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.

Validated test method: A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose (6).

Weight-of-evidence: The process of considering the strengths and weaknesses of various pieces of information in reaching and supporting a conclusion concerning the hazard potential of a chemical.

ANNEX II

PERFORMANCE STANDARDS

Performance standards for the CM test method are not yet available and need to be developed.

Note: The validated CM test method uses an instrument that is no longer commercially available. However, several testing laboratories still have a functional instrument being used on a routine basis for the assessment of eye irritation potential of chemicals, and the necessary consumables to secure the operation of the existing instruments are still commercially available. Moreover, some companies produce and commercialise a second generation Cytosensor Microphysiometer instrument, which measure the exact same endpoint as the original validated Cytosensor Microphysiometer instrument and may therefore substitute it in the CM test method, However, these would need to be validated according to the performance standards.