The Cytosensor Microphysiometer (CM) Test Method – Validation Status and Appropriate Use

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Supporting NICEATM


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Bethesda, MD
NICEATM-ICCVAM Evaluation of CM

- Reviewed available data and information regarding the usefulness and limitations for assessing the ocular hazard potential of chemicals and products

- Determined validation status
  - Accuracy: sensitivity and specificity
  - Reproducibility for identifying ocular corrosives/severe irritants vs. all other hazard categories
  - Scope of substances tested
  - Availability of a standardized test method protocol

- Independent international scientific peer review panel
Overview of CM

- L929 mouse fibroblast cells are treated with the test substance
  - Seven concentrations (predetermined in the dose range-finding assay)
  - Diluted in low-buffered treatment medium
  - At least two independent runs

- L929 cells are treated with the positive control in each run
  - 10% (w/v) sodium lauryl sulfate (SLS)

- Time of exposure: 13 minutes 30 seconds
- Endpoint measured: Rate of pH change
Validation Database

- **53 water-soluble surfactants** (32 surfactant-containing formulations and 21 surfactant substances tested across seven different laboratories)
  - Most of the 32 formulations, which are limited to cosmetic and personal care products, contain one or more surfactants at a final concentration of greater than five percent
  - No pesticide formulations included

- **29 water-soluble nonsurfactants** (27 nonsurfactant chemicals and 2 nonsurfactant formulations tested in seven laboratories)
  - For example, acids, alcohols, alkalis, and ketones

- Reproducibility data from two validation studies
  - Balls et al. (1995): 4 laboratories
  - Brantom et al. (1997): 2 laboratories
## Decision Criteria Proposed to Classify CM Data

<table>
<thead>
<tr>
<th>MRD&lt;sub&gt;50&lt;/sub&gt; (mg/mL)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>EPA</th>
<th>GHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;80</td>
<td>Category IV</td>
<td>NA</td>
</tr>
<tr>
<td>&gt;2; ≤80</td>
<td>No prediction can be made</td>
<td>NA</td>
</tr>
<tr>
<td>&gt;10</td>
<td>NA</td>
<td>No Category</td>
</tr>
<tr>
<td>&gt;2; ≤10</td>
<td>NA</td>
<td>No prediction can be made</td>
</tr>
<tr>
<td>≤2</td>
<td>Category I</td>
<td>Category 1</td>
</tr>
</tbody>
</table>

<sup>1</sup>**MRD<sub>50</sub>: Metabolic rate decrement of 50%. The concentration of test substance (weight/volume) required to cause 50% inhibition of the basal acidification (metabolic) rate.**
### CM Test Method Accuracy: Ocular Corrosives and Severe Irritants

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>False Positive Rate</th>
<th>False Negative Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td><strong>EPA</strong></td>
<td>52</td>
<td>85</td>
<td>44/52</td>
<td>78</td>
<td>18/23</td>
<td>90</td>
</tr>
<tr>
<td><strong>GHS</strong></td>
<td>53</td>
<td>94</td>
<td>50/53</td>
<td>91</td>
<td>21/23</td>
<td>97</td>
</tr>
</tbody>
</table>

1. EPA = Cat I vs. Cat II/III/IV, GHS = Cat 1 vs. Cat2A/2B/NC
2. The three false positives when using the EPA classification system are classified as Category II (n=2) or III (n=1) based on *in vivo* data. The one false positive when using the GHS classification system is Not Classified based on *in vivo* data.
3. The false negative substances were classified as mild or moderate irritants *in vitro* based on the EPA and GHS classification systems (i.e., EPA Category II/III; GHS Category 2A/2B).
<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>False Positive Rate</th>
<th>False Negative Rate²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>EPA</td>
<td>25</td>
<td>92</td>
<td>23/25</td>
<td>71</td>
<td>5/7</td>
<td>100</td>
</tr>
<tr>
<td>GHS</td>
<td>29</td>
<td>83</td>
<td>24/29</td>
<td>55</td>
<td>6/11</td>
<td>100</td>
</tr>
</tbody>
</table>

¹EPA = Cat I vs. Cat II/III/IV, GHS = Cat 1 vs. Cat2A/2B/NC
²Two substances were false negatives when using the EPA classification system and were classified in vitro as either Category II/III (n = 1) or IV (n = 1). Five substances were false negatives using the GHS classification system and were classified in vitro as either Category 2A/2B (n = 4) or Not Classified (n = 1).
<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>False Positive Rate$^2$</th>
<th>False Negative Rate$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>EPA</td>
<td>52</td>
<td>92</td>
<td>48/52</td>
<td>98</td>
<td>45/46</td>
<td>50</td>
</tr>
<tr>
<td>GHS</td>
<td>53</td>
<td>68</td>
<td>36/53</td>
<td>100</td>
<td>28/28</td>
<td>32</td>
</tr>
</tbody>
</table>

$^1$EPA = Cat IV vs. Cat I/II/III; GHS = NC vs. Cat 1/2A/2B

$^2$Three substances were false positive when using the EPA classification system and were classified *in vitro* as Category II/III. Seventeen substances were false positive when using the GHS classification system and were classified *in vitro* as Category 2A/2B (n=16) or Category 1 (n=1).

$^3$The one false negative was EPA Category III based on *in vivo* data. For this substance, six test animals were included in the *in vivo* test. One test animal had no observable effects, three test animals had conjunctival redness (score = 1), and two test animals had corneal opacity (score = 1) that cleared after one day.
CM Test Method Accuracy: Substances Not Labeled as Irritants¹: Nonsurfactant Substances

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>False Positive Rate</th>
<th>False Negative Rate²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>EPA</td>
<td>29</td>
<td>66</td>
<td>19/29</td>
<td>67</td>
<td>16/24</td>
<td>60</td>
</tr>
<tr>
<td>GHS</td>
<td>25</td>
<td>64</td>
<td>16/25</td>
<td>62</td>
<td>13/21</td>
<td>75</td>
</tr>
</tbody>
</table>

¹EPA = Cat IV vs. Cat I/II/III; GHS = NC vs. Cat 1/2A/2B
²Eight substances were false negative when using the EPA and GHS classification systems. In the EPA system, they were classified \textit{in vivo} as Category 1 (n = 1) and Category II (n = 3) and Category III (n = 4). In the GHS system, they were classified \textit{in vivo} as Category 1 (n = 1) and Category 2A (n = 7).
## CM Interlaboratory Reproducibility

<table>
<thead>
<tr>
<th>Material Type</th>
<th>Number of Laboratories</th>
<th>Agreement Among Laboratories</th>
<th>Percentage (# correct/total)</th>
<th>Maximum Mean CV</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surfactants</td>
<td>4</td>
<td>100%</td>
<td>55% (6/11)</td>
<td>37%</td>
<td>EC/HO – Balls et al. (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75%</td>
<td>27% (3/11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50%</td>
<td>18% (2/11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsurfactants</td>
<td></td>
<td>100%</td>
<td>48% (11/23)</td>
<td>51%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>75%</td>
<td>22% (5/23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>67%</td>
<td>4% (1/23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50%</td>
<td>13% (3/23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surfactants</td>
<td>2</td>
<td>100%</td>
<td>90% (9/10)</td>
<td>23%</td>
<td>COLIPA – Brantom et al. (1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0%</td>
<td>10% (1/10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surfactant-based formulations</td>
<td>2</td>
<td>100%</td>
<td>100% (7/7)</td>
<td>16%</td>
<td></td>
</tr>
<tr>
<td>and mixtures</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsurfactants</td>
<td></td>
<td>100%</td>
<td>78% (7/9)</td>
<td>51%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0%</td>
<td>22% (2/9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ICCVAM Recommendations for CM\textsuperscript{1}: Usefulness and Limitations – Ocular Corrosives and Severe Irritants

**Usefulness**

- Can be used for identification of ocular corrosives and severe irritants (EPA Category I, GHS Category 1) in appropriate circumstances and with certain limitations

**Limitations**

- Limited to water-soluble substances (i.e., water-soluble surfactants, surfactant-containing formulations, and nonsurfactants)

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ICCVAM Recommendations for CM\textsuperscript{1}: Usefulness and Limitations – Substances Not Labeled as Irritants

**Usefulness**

- Can be used for identification of substances not labeled as irritants (EPA Category IV) in appropriate circumstances and with certain limitations

**Limitations**

- Restricted to water-soluble surfactant chemicals and certain types of surfactant-containing formulations (e.g., cosmetics and personal care product formulations, but not pesticide formulations), but **not** nonsurfactants

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ICCVAM-Recommended CM Protocol

Prepare Instrument and Cells
1. Prepare instrument and check background rates
2. Seed each capsule cup with L929 cells in seeding medium
3. Incubate L929 cells at 37±1°C in 5±1% CO₂ for 16-32 hrs in seeding medium

Prepare Test Substance
1. Evaluate test substance suitability:
   - Must be water-soluble
   - May not be viscous or a suspension
   - Other considerations depending on test objective
2. Prepare seven 3-fold serial dilutions of test substance from 10 mg/mL in low-buffered treatment medium and a positive control to be run concurrently

Perform Range-finding Test
1. Expose cells to test substance starting with lowest concentration:
   - Exposure time 13 min 30 sec (flow rate 100 uL/min for first minute, then 20 uL/min)
   - Washout time 6 minutes (100 uL/min)
   - Measure acidification rate with no flow for 25 sec
2. Repeat test until highest concentration is reached or the concentration that leads to a 50% decline in the metabolic rate of the population (the MRD₅₀) has been surpassed
3. Using Cytosoft software, calculate the MRD₅₀

Perform Definitive Test
1. Repeat test as above at least two more times using a range of concentrations that includes three each at >50%, 50%, and <50% survival rates:
   - Use 10% sodium lauryl sulfate in water as positive control
   - Include benchmarks if appropriate
2. Derive MRD₅₀ value from two definitive assays and the dose range finding data when appropriate
3. Assign hazard classification using criteria in Table 1

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1 Available at http://iccvam.niehs.nih.gov/docs/protocols/IVOcular-CM.pdf
ICCVAM Recommendations: Future Studies

- Additional studies to expand the applicability domain of CM for ocular corrosives and severe irritants and for substances not labeled as irritants
  - Use ICCVAM-recommended reference substances\(^1\) or a reference set from this list

- Optimization studies to increase performance of CM for identifying all categories of ocular irritancy hazard classification

- ICCVAM encourages users to provide all data from future studies to further evaluate the usefulness and limitations of CM

\( ^1\) Available at http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_tmer.htm
Draft OECD Test Guideline Currently Under Consideration

- Draft OECD Guideline for the Testing of Chemicals

  *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*

  - Based on international validation study by ECVAM, in collaboration with ICCVAM and JaCVAM

1Available at http://www.oecd.org/document/55/0,3343,en_2649_34377_2349687_1_1_1_1,00.html
2010 ICCVAM Evaluation of CM

- In 2010, ICCVAM also evaluated CM for identifying nonsevere irritants
- ICCVAM concluded that CM is **not** recommended to identify moderate and mild ocular irritants as defined by the EPA and GHS classification systems
ACKNOWLEDGEMENTS

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