Performance of the OptiSafe Ocular Irritation Assay in a Three-Laboratory Validation Study

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Introduction

- OptiSafe assesses a test substance's potential to produce eye irritation by measuring damage to macromolecules.
- The test method allows for detection of substances with different mechanisms of ocular injury.
  - Denaturation of water-insoluble polymers in the membrane that model the phospholipid bilayer of cells predicts damage to the corneal epithelium and conjunctiva.
  - Indirect denaturation of molecules and fluid transfer across the membrane by osmotic effects predict damage to the cornea and conjunctiva.
  - Denaturation of macromolecules that model ordered collagen predicts damage to the cornea and conjunctiva.
- OptiSafe has a number of useful features:
  - Sold as a shelf-stable kit
  - Includes step-by-step instructions for easy implementation in a basic laboratory
  - Requires no specialized equipment
  - Can be conducted on a benchtop (no cell culture required)
  - Can be completed in less than 24 hours
  - Includes a pre-test that assesses the test substance's physical and chemical properties to identify the optimal procedure
- NICEATM coordinated a three-laboratory validation study of OptiSafe (**Table 1**) in order to:
  - Assess transferability to naïve laboratories
  - Characterize usefulness and limitations

Table 1. Validation Study Phases

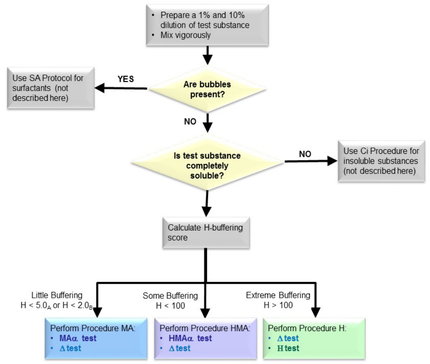
Phase	Activities
Pre-Study Phase	• Formation of VMT - composed of ICCVAM agency scientists and international representatives • Selection of naïve laboratories • Finalization of documents, reporting forms, and performance criteria
Phase I	• Qualification and training of naïve laboratories • Testing of all practice chemicals by lead and naïve laboratories
Phase II	• Testing of 30 chemicals by lead and naïve laboratories
Phase III	• Testing of 60 chemicals by lead laboratory
Reporting Phase	• Preparation of validation report

Chemical Selection

- NICEATM collaborated with the validation study management team (VMT) on chemical selection for all phases of the validation effort.
- Factors used in the chemical selection process included physicochemical properties, ocular irritancy responses, and use in other in vitro validation studies.
- Surfactants were included to assess accuracy of the pre-test procedure.

Test Method Protocol

Pre-test Flow Chart



Main Test Procedure Options

- Procedure Δ – assess water-insoluble denaturation and oxidative damage/excessive reactivity
- Procedure α – assess collagen denaturation and oxidative damage/excessive reactivity
  - MAα – non-surfactants with limited or little buffering capacity
  - HMAα – non-surfactants with moderate buffering capacity
  - Ci – insoluble compounds
- Procedure H – assess ocular pH shift

Option 1. Δ Test Flow Chart

Add test substance or standards to individual cells
↓
Add Δ reagent to each cell
↓
Incubate 18 hours at 30.6 °C
↓
Read optical density on specrophotometer
↓
Irritancy classification based on Δ decision criteria

Option 2. α Test Flow Chart

Aliquot active agent into 24-well plates
↓
Add ocular discs to wells
↓
Titrate test substance into wells, and add standards & quality controls to remaining wells
↓
Incubate 18 hours at 30.6 °C
↓
Discard ocular discs and read optical density of solution with spectrophotometer
↓
Irritancy classification based on decision criteria for specified α method protocol

Option 3. H Test Flow Chart

Add test substance to tubes (25-125 µL)
↓
Adjust pH of active agent to 7.4
↓
Add active agent to each of the test substance tubes
↓
Vortex and incubate for 10-15 mins at room temperature
↓
Measure pH
↓
Irritancy classification based on H decision criteria

Phase I Outcomes

- Intralaboratory reproducibility was greater than 90% (n = 15) for all testing laboratories. The VMT concluded Phase II should proceed.
- Minor protocol additions were made to further increase transferability and test method reproducibility in Phase II.
  - Procedures for when an incubation box should be discarded
  - Methods to ensure solids were fully in contact with the membrane
  - Additional quality control procedures

Phase II Outcomes

- Accuracy statistics for each laboratory under the U.S. Environmental Protection Agency (EPA) and United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS) classification systems are shown in **Table 2** and **Table 3.**
- Interlaboratory accuracy statistics for the EPA and GHS classification systems are shown in **Table 4.**
- Intralaboratory reproducibility for the EPA and GHS classification systems was greater than 93% for non-surfactants.
- Interlaboratory reproducibility was 91% for both classification systems.

Table 2. Phase II Test Method Accuracy for Individual Labs: EPA Classification

	Lead Laboratory	Naïve Laboratory 1	Naïve Laboratory 2
Accuracy	88% (22/25[a])	81.5% (22/27[b])	85.2% (23/27[b])
Sensitivity	100% (13/13)	100% (14/14)	92.9% (13/14)
Specificity	75% (9/12)	61.5% (8/13)	76.9% (10/13)
False Positive	25% (3/12)	38.5% (5/13)	23.1% (3/13)
False Negative	0% (0/13)	0% (0/14)	7.1% (1/14)
Negative Predictivity	100% (9/9)	100% (8/8)	90.9% (10/11)
Positive Predictivity	81.3% (13/16)	73.7% (14/19)	81.3% (13/16)

[a] Five chemicals were excluded from the analysis: one chemical outside of the applicability domain, one chemical tested using a non-standard test method, and three chemicals identified as surfactants.

[b] Three chemicals were excluded from the analysis because they were surfactants.

Table 3. Phase II Test Method Accuracy for Individual Labs: GHS Classification

	Lead Laboratory	Naïve Laboratory 1	Naïve Laboratory 2
Accuracy	88% (23/25[a])	81.5% (22/27[b])	77.8% (21/27[b])
Sensitivity	100% (12/12)	100% (13/13)	84.6% (11/13)
Specificity	76.9% (10/13)	64.3% (9/14)	71.4% (10/14)
False Positive	23.1% (3/13)	35.7% (5/14)	28.6% (4/14)
False Negative	0% (0/12)	0% (0/13)	15.4% (2/13)
Negative Predictivity	100% (10/10)	100% (9/9)	83.3% (10/12)
Positive Predictivity	80% (12/15)	72.2% (13/18)	73.3% (11/15)

[a] Five chemicals were excluded from the analysis: one chemical outside of the applicability domain, one chemical tested using a non-standard test method, and three chemicals identified as surfactants.

[b] Three chemicals were excluded from the analysis because they were surfactants.

Table 4. Phase II Test Method Accuracy (Summary of All Labs): EPA and GHS Classification

	EPA	GHS
Accuracy	88.5% (23/26[a])	88.5% (23/26[a])
Sensitivity	100% (14/14)	100% (13/13)
Specificity	75% (9/12)	76.9% (10/13)
False Positive	25% (3/12)	23.1% (3/13)
False Negative	0% (0/14)	0% (0/13)
Negative Predictivity	100% (9/9)	100% (10/10)
Positive Predictivity	82.4% (14/17)	81.3% (13/16)

[a] Four chemicals were excluded from the analysis: three chemicals were identified as surfactants, and no overall call could be made for the fourth chemical.

Phase III Outcomes

- To evaluate the applicability domain of OptiSafe, the misclassification rate of structural fragments present in the tested chemicals was evaluated (**Table 5**).
- The Organic Functional Group profiler in OECD Toolbox (v. 4.1), a quantitative structure activity relationship program developed by the Organisation for Economic Co-operation and Development, was used to identify structural fragments in the tested chemicals.
- There were no GHS underpredictions.
- Two chemicals classified as EPA Category III ("mild" irritants) based on in vivo studies (dodecane and 1,4-dibromobutane) were underpredicted as Category IV by OptiSafe
  - The Category III classifications are based on mild reactions in only one of three or six animals.
  - Considering the recognized variability of the in vivo test, if tested again these chemicals could be classified as Category IV.

Table 5. Misclassification of Phase III Chemicals by Organic Functional Groups

Structural Feature	EPA Classification						GHS Classification					
	-	+	False +	False -	False + Rate (%)	False - Rate (%)	-	+	False +	False -	False + Rate (%)	False - Rate (%)
Acetoxy	0	2	0	0	N/A	0	1	1	1	0	100	0
Alcohol	3	10	1	0	33	0	5	8	3	0	60	0
Aldehyde	2	2	1	0	50	0	2	2	1	0	50	0
Alkane, branched with secondary carbon	3	2	1	0	33	0	3	2	0	0	0	0
Alkane, branched with tertiary carbon	2	3	1	0	50	0	2	3	1	0	50	0
Alkene	2	3	1	0	50	0	4	1	2	0	50	0
Alkenyl (hetero)arenes	0	1	0	0	N/A	0	1	0	1	0	100	N/A
Alkyl	1	1	0	1	0	100	2	0	0	0	0	N/A
Alkyl (hetero)arenes	0	4	0	0	N/A	0	1	3	0	0	0	0
Alkyl halide	1	2	1	1	100	50	2	1	0	0	0	0
Alkyl-, alkenyl- and alkynyl (hetero)arenes	0	5	0	0	N/A	0	2	3	1	0	50	0
Allyl	2	2	1	0	50	0	3	1	1	0	33	0
Aryl	4	9	2	0	50	0	7	6	4	0	57	0
Benzyl	0	1	0	0	N/A	0	1	0	1	0	100	N/A
Carboxylic acid ester	3	6	2	0	67	0	4	5	3	0	75	0
Diketone	0	1	0	0	N/A	0	1	0	1	0	100	N/A
Dihydroxyl derivatives	2	1	1	0	50	0	2	1	1	0	50	0
Ether	4	2	3	0	75	0	6	0	5	0	83	N/A
Guanidine	0	1	0	0	N/A	0	1	0	1	0	100	N/A
Isopropyl	2	2	2	0	100	0	2	2	1	0	50	0
Ketone	0	4	0	0	N/A	0	1	3	1	0	100	0
Methacrylate	1	0	1	0	100	N/A	1	0	1	0	100	N/A
No functional group found	0	2	0	0	N/A	0	1	1	1	0	100	N/A
Phosphite ester	1	0	1	0	100	N/A	1	0	1	0	100	N/A
Sulfide	1	0	1	0	100	N/A	1	0	1	0	100	N/A
Thiol	2	1	1	0	50	0	2	1	1	0	50	0

Conclusions and Future Directions

- OptiSafe may represent a new tool for in vitro assessment of the ocular toxicity potential of chemicals in a tiered-testing system.
- OptiSafe exhibited high transferability and interlaboratory reproducibility in this study.
- High false positive rates for a limited number of substances in certain chemical classes are being further investigated by additional testing.

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