

Proposed Reference Substances For Optimization And Validation Studies With *In Vitro* Ocular Test Methods

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Introduction

NICEATM evaluated four *in vitro* ocular test methods, the Bovine Corneal Opacity and Permeability (BCOP) assay, the Hen's Egg Test - Chorioallantoic Membrane (HET-CAM) assay, the Isolated Chicken Eye (ICE) assay, and the Isolated Rabbit Eye (IRE) assay, for their ability to identify substances that cause ocular corrosion or severe irritation. During these evaluations, a proposed list of reference substances for future optimization and validation studies of these and other alternative test methods intended to detect ocular corrosives/severe irritants was developed. Based on the ICCVAM Submission Guidelines (ICCVAM 2003; see <http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm>), substances included in this list are intended to:

- (1) represent the range of ocular responses (i.e., corrosive/severe irritant; nonsevere irritant/noncorrosive) that the test method is expected to be capable of predicting
- (2) represent the range of chemical/product classes and physicochemical properties (e.g., solid, liquid) that the test method is expected to be capable of testing
- (3) represent the range of known or anticipated mechanisms or modes of action for severe/irreversible ocular irritation or corrosion
- (4) have been generated by high-quality *in vivo* rabbit eye test studies following Organization for Economic Cooperation and Development (OECD) Test Guideline (TG) 405 (OECD [1987]) and preferably conducted in compliance with Good Laboratory Practices (GLP) guidelines (OECD [1998]; EPA [2004a, 2004b]; FDA [2004])
- (5) have a well-defined chemical composition
- (6) be tested at a defined concentration and at a defined purity
- (7) be readily available

Following completion of any optimization and validation studies for each test method, reference substances from this list could be selected for inclusion in performance standards and for proficiency testing. On November 1, 2004, draft Background Review Documents (BRDs) on IRE, ICE, BCOP, and HET-CAM, were released to the public for comment¹.

In addition to information on the validation status of these four *in vitro* test methods, the BRDs contained a list of 89 proposed reference substances. On January 11-12, 2005, ICCVAM convened an Expert Panel to independently evaluate the list of proposed reference substances². Based on Panel recommendations and on the availability of additional *in vivo* rabbit eye test data, the original list has been revised; this revised list is summarized here.

The complete list of candidate substances and proposed reference substances, organized by name, Chemical Abstracts Services Registry Number (CASRN), GHS ocular hazard category classification, chemical class, and product class are available in the Addendum to *In Vitro* Ocular Toxicity Draft Background Review Documents, on the NICEATM/ICCVAM website at <http://iccvam.niehs.nih.gov/methods/ocudocs/reanalysis.htm>. Also included in this Addendum for the proposed reference substances is information on physicochemical properties (e.g., chemical structure, molecular weight, pH, water solubility, color), commercial source, the concentration and purity tested, the physical form tested, the extent of damage induced (corneal, iridal, conjunctival scores), its classification according to other ocular hazard classification systems (e.g., US Environmental Protection Agency [EPA, 1996], European Union [EU 2001]), and selected summary information on effects in human and animals.

¹The draft BRDs can be obtained at http://iccvam.niehs.nih.gov/methods/ocudocs/ocu_brd.htm
²The Expert Panel Report can be obtained at <http://iccvam.niehs.nih.gov/methods/ocudocs/EPReport/ocureport.htm>

Proposed Reference Substances

Candidate Substances (Table 1)

210 candidate substances were identified including:

- 10 substances that produce severe ocular damage in humans
- 93 candidate GHS³ Category 1 substances (i.e., irreversible/serious effects on the eye)
- 17 candidate GHS Category 2A substances (i.e., irritating to eyes)
- 23 candidate GHS Category 2B substances (i.e., mildly irritating to eye)
- 77 candidate GHS Nonirritant substances

Selection of the Proposed List of 122 Reference Substances

- Based purely on a statistical evaluation, several hundred substances could potentially be required to evaluate accuracy with a reasonably high level of confidence
- For the detection of ocular corrosives and severe irritants, the list of reference substances needs to include substances that:
 - induce very severe responses within a relatively short time period, as well as those where the toxic response is delayed
 - adversely affect the cornea, iris, and/or conjunctiva
 - induce persistent versus non-persistent lesions (when assessed at 21 days post treatment)
 - represent diverse chemical classes and physicochemical properties

To meet these needs, the total number of substances in the list was increased from 89 substances in the original *in vitro* ocular BRDs to 122. The proposed list of reference substance includes:

- 79 GHS Category 1 substances (10 of which were classified as severe irritants based on human data only),
- 28 GHS Category 2 substances (14 Category 2A substances, 13 GHS Category 2B substances, and one substance [Triton X-100] that induced a GHS Category 2A response in one study and a 2B response in another study when tested at a 5% concentration)
- 15 nonirritants
- These substances cover 34 chemical and 24 product classes, with 79 substances tested in liquid form and 43 tested as solids.

³GHS = the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS). New York & Geneva: United Nations Publications, 2003 Available: <http://www.unece.org/trans/danger/publi/ghs/officialtext.html>.

Table 1. Distribution of Substances in the *In Vitro* Rabbit Eye Test Database and Molecular Weight Ranges of the Proposed Reference Substances, by GHS³ Ocular Hazard Classification

Classification (GHS)	Number of Entries in the <i>In Vitro</i> Rabbit Eye Test Database ¹ with a GHS Classification		Number of Candidate Substances (i.e., GHS-Classified Substances ² Determined to be Commercially Available)		Number of Proposed Reference Substances	Additional Substances Identified as Causing Severe Ocular Damage in Humans	Final Number of Proposed Reference Substances		Molecular Weight Range for Proposed Reference Substances
	Revised/Original	Revised/Original ³	Revised/Original	Revised/Original			Revised/Original	Revised/Original	
Category 1	220/123	93/48	69/48	10	79/48	10	30.0 - 546.8		
Category 2A	62/24	17/11	15/11	5	15/11	5	58.1 - 384.4		
Category 2B	51/68	23/27	13/15	-	13/15	-	80.0 - 265.3		
Nonirritant	497/277	77/111	15/15	-	15/15	-	86.2 - 1227.5		
Total	830/492	210/197	112/89	10	122/89	10	30.0 - 1227.5		

¹GHS = Globally Harmonized System (UN [2003]).
²The complete database includes multiple entries for some substances, as well as formulations, coded substances, and substances that could not be classified according to the GHS ocular hazard classification system.
³"Substance" is defined as a unique entry (i.e., a single substance tested at a single concentration). The substances identified as causing severe ocular effects in humans are substances for which individual rabbit eye test results were not located. One substance (Triton X-100), when tested at 5%, induced a GHS Category 2A response in one study and a Category 2B response in another study; for purposes of classification in this table, Triton X-100 is classified as a Category 2A substance.
⁴The number of entries decreased for some GHS classification categories due to (1) the reclassification of some substances as GHS Category 1 irritants, based on the persistence of any lesion to day 21 post-treatment; (2) a reassessment of current commercial availability; and (3) collapsing multiple studies with the same substance tested at the same concentration into a single entry.
⁵NI = nonirritant.

Table 2. NICEATM-Defined Subcategories for the Proposed GHS³ Category 1 Reference Substances

Subcategory	Criteria for Classification as a GHS Category 1	# of Substances Revised/Original
0 ²	Not Classifiable	12/0
1	Positive response based on a persistent lesion involving the cornea, iris, and/or conjunctiva through to day 21 in at least one of three rabbits and not on severity	9/18
2	Positive response based on mean for first 3 days (CO ³ score >3 and <4 or IR ⁴ score >1.5) in at least two of three rabbits but lesions do not persist through day 21	4/4
3	Positive response based on mean for first 3 days (CO score >3 and <4 or IR score >1.5) in at least two of three rabbits and a persistent (>21 days) lesion in at least one rabbit	4/2
4	CO score = 4 at any time in at least one of three rabbits	50/24
	Total	79/48

¹GHS = United Nations Globally Harmonized System (UN [2003]).
²Included are two GHS Category 1 substances that could not be subclassified because classification was based on an extreme response shortly after treatment in the only animal tested and 10 substances classified as GHS Category 1 irritants because they induced a severe ocular response in accidentally-exposed humans, and appropriate *in vivo* rabbit ocular irritation test data was not located for these 10 substances.
³CO = corneal opacity.
⁴IR = iritis.

Table 3. Chemical Classes and Properties of Interest Represented Among the Proposed Reference Substances, According to GHS³ Ocular Hazard Classification Category

Category	Number of Candidate Substances	Number of Proposed Reference Substances	GHS Category 1		GHS Category		
			Based on Human Data	Based on Rabbit Data	2A	2B	NI ²
Chemical Class^{3,4,5}							
Acid (inorganic)	2	2	2(2)	5	-	-	-
Acid (organic)	20	17	-	13(14)	2(2)	1(3)	1(1)
Acyl Halide	3	3	-	2(2)	1(1)	-	-
Alcohol	30	22	-	11(17)	4(6)	4(4)	3(10)
Aldehyde	6	4	-	2(2)	1(1)	1(2)	0(1)
Alkali	3	3	2(2)	1(1)	-	-	-
Amide	2	2	-	1(1)	-	1(1)	-
Amidine	6	5	-	4(5)	-	-	1(1)
Amine	23	17	-	14(18)	-	2(2)	1(3)
Amino Acid	1	1	-	1(1)	-	-	-
Boron Compound	1	1	-	1(1)	-	-	-
Ester	30	15	-	8(9)	3(3)	2(5)	2(15)
Ether	22	11	-	8(12) ⁶	1(1) ⁷	2(2) ⁷	3(14)
Heterocyclic Compound	13	13	-	9(13) ⁸	2(2) ⁸	2(2)	1(3)
Hydrocarbon (acyclic)	7	1	1(1)	-	-	-	0(6)
Hydrocarbon (cyclic)	11	2	-	-	1(1)	0(1)	1(9)
Hydrocarbon, Halogenated	13	2	-	-	-	-	2(13)
Isocyanate	2	2	-	2(2)	-	-	-
Ketone	8	5	-	-	2(2)	2(4)	1(2)
Lactone	1	1	-	-	1(1)	-	-
Nitrate	2	2	1(1)	-	-	1(1)	-
Nitrile	3	3	-	1(1)	1(1)	1(1)	-
Nitro Compound	5	2	-	2(2)	-	-	-
Onium Compound	6	6	-	5(6) ⁹	1(1) ⁹	1(1)	-
Organophosphorus Compound	3	2	-	1(1)	1(1)	-	0(1)
Organosilicon Compound	5	4	-	3(3)	-	1(1)	-
Phenol	6	6	-	5(7)	-	-	1(1)
Polycyclic Compound	4	3	-	2(3)	1(1)	-	-
Quinone	1	1	-	1(1)	-	-	-
Salt (inorganic)	12	12	7(7)	4(4)	-	-	1(1)
Salt (organic)	13	12	-	6(7)	-	2(4)	3(3)
Sulfur Compound (inorganic)	1	1	1(1)	-	-	-	-
Sulfur Compound (organic)	15	9	-	7(8)	-	-	2(8)
Urea Compound	1	1	-	-	-	1(1)	-
Total⁹	281	193	14(14)	116(143)	22(24)	24(37)	23(95)
Properties of Interest							
Liquid	163	79	6(6)	40(61)	14(16) ¹⁰	9(13)	10(67)
Solid	53	43	4(4)	29(31)	1(1)	4(8)	5(9)

¹GHS = Globally Harmonized System (UN [2003]).
²NI = nonirritant.
³Chemical Class—Based on the MeSH Medical Subject Heading. Available <http://www.nlm.nih.gov/mesh/>; substances may be assigned into one or more chemical classes.
⁴Numbers in parenthesis indicate the number of candidate substances for that GHS category.
⁵Substances were assigned into one or more chemical classes.
⁶- indicates that there are no substances in this category.
⁷Triton X-100 classified as GHS Category 1, 2A/2B and NI, depending on the concentration tested
⁸Cetylpyridinium bromide classified as GHS Category 1 and 2A, depending on the concentration tested
⁹The total number is greater than the total number of proposed reference substances because some substances were assigned to more than one chemical class.
¹⁰Triton X-100, when tested at 5%, induced a GHS Category 2A response in one study and a Category 2B response in another study; for purposes of classification in this table, Triton X-100 is classified as a Category 2A substance.

Table 4. Product Classes Represented Among the Proposed Reference Substances, According to GHS³ Ocular Hazard Classification Category

Product Class	Total ²	GHS Category 1		GHS Category ³ 2A	GHS Category 2B	GHS NI ⁴
		Based on Human Data	Based on Rabbit Data			
Adjuvant, Solubilizer, Wetting Agent	1	1 ⁴	1	-	-	-
Anesthetic	2	1	1	-	-	-
Anti-Fungal	6	1	4	1	-	-
Anti-Infective	12	3	7 ⁵	3 ³	-	-
Battery Acid	1	1	-	-	-	-
Building Material	2	1	-	-	-	1
Caustic Agent	2	-	2	-	-	-
Chemical Intermediate	43	8	23	4	3	5
Cleaner or Cleaning Agent	15	6	6	1	1	1
Cosmetic Ingredients, & Perfumes	11	-	8	-	2	1
Fertilizers	4	4	-	-	-	-
Flame Retardant	3	2	1	-	-	-
Food Additives	9	2	4	1	1	1
Herbicides	5	2	2	1	-	-
Industrial Chemicals & Dyes	46	11	28	2	2	3
Laboratory Chemicals	28	3	16 ⁶	3 ³	3	4
Pesticide & Pesticide Intermediates	17	1	11	1	1	3
Pharmaceuticals & Pharmaceutical Intermediates	29	5	15	1	4	4
Polish	1	-	1	-	-	-
Preservative	4	2	1	1	-	-
Refrigerant	1	1	-	-	-	-
Solvent	21	1	8	8	3	1
Surfactants:	7	-	5 ³	2 ³	2 ³	2 ³
Anionic	3	-	1	-	1	1
Cationic	2	-	2 ³	1 ³	-	-
Nonionic	2	-	2 ³	1 ³	1 ³	1 ³
Veterinary Agent	6	2	4	-	-	-

¹GHS = United Nations Globally Harmonized System (UN [2003]).
²All substances were assigned into one or more product classes by referencing the National Library of Medicine Hazards Substances Database (HSDB); other information was obtained from Material Safety Data Sheets (MSDS) obtained from the commercial supplier; therefore, the total number is greater than the total number of proposed reference substances.
³Some substances, when tested at different concentrations, were assigned a different GHS ocular hazard classification. For this table, these substances (Triton X-100 and cetylpyridinium bromide) appear in more than one GHS category column; thus the total numbers in these columns do not add up to the numbers of substances in the total column.
⁴- indicates that there are no substances in this category.

Conclusions

This proposed list of reference substances is intended to represent the minimum number of substances that should be used to evaluate the accuracy and reliability of an *in vitro* ocular test method proposed for the detection of ocular corrosives and severe irritants. Testing the complete list of all reference substances will facilitate future validation efforts and comparison of performance among different test methods and protocols.

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