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Validation Status of the Bovine Corneal Opacity and Permeability (BCOP) Test Method

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Abstract

Concerns about animal welfare, as well as interest in understanding eye irritation at the tissue and cellular levels, have led researchers to develop alternative *in vitro* test methods for the current rabbit eye test. NICEATM evaluated four *in vitro* ocular test methods for their ability to identify substances that cause irreversible or severe eye irritation or corrosion. One of these test methods, BCOP, is an organotypic model that provides short-term maintenance of normal physiological and biochemical function of the cornea in an isolated system. The ability of BCOP to correctly identify ocular corrosives and severe irritants using available BCOP and corresponding *in vivo* eye irritation data was evaluated according to current hazard classification schemes for the U.S. EPA (n=117), the European Union (n=157), and the UN Globally Harmonized System (n=120). Depending on the classification scheme used, BCOP had a false positive rate of 17-23%, and a false negative rate of 22.5-27%. In terms of reliability, the assay was determined to have acceptable intra- and inter-laboratory reproducibility. A proposed standardized test method protocol and a proposed recommended list of reference substances have been developed for future validation and testing studies to further assess the accuracy, reliability, and the applicability domain of BCOP for the detection of ocular corrosives/severe irritants. Consideration should be given to using BCOP prior to eye irritation testing in animals. BCOP may be useful in a tiered testing strategy where positive results can be used to classify and label a substance, while substances with negative results would undergo additional testing to identify false negative ocular corrosives/severe irritants and to identify those chemicals with reversible ocular effects. This approach would reduce the number of animals used for eye irritation testing and reduce the number of animals experiencing pain and distress. ILS staff supported by NIEHS contract N01-ES 35504.

Introduction

In the United States, many consumer products, pesticides, and materials used in the workplace are required to undergo testing to evaluate their acute eye irritation potential in humans. The accepted methods in the United States for evaluating eye irritation are based on the Draize eye test (Draize et al. 1944), in which a test substance is placed in the eye of a rabbit, and damage to the cornea, iris, and conjunctiva is assessed up to 21 days after exposure. In recent years, increased efforts have focused on the development of *in vitro* alternatives to this *in vivo* test.

Recently, the U.S. Environmental Protection Agency (EPA) formally nominated four *in vitro* test methods to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for evaluation of the methods' ability to identify potential ocular corrosives and severe irritants in a tiered testing strategy. One of the four test methods nominated for review is the Bovine Corneal Opacity and Permeability (BCOP) test method. NICEATM worked with the ICCVAM Ocular Toxicity Working Group (OTWG) and the European Centre for the Validation of Alternative Methods (ECVAM) to compile information and data to support the ICCVAM technical evaluation of the test method. Adequate validation of a test method, which is the process by which the reliability and relevance of a test method are established for a specific purpose, is a prerequisite for it to be considered for regulatory acceptance (ICCVAM 1997, 2003).

NICEATM, which administers ICCVAM and provides scientific support for ICCVAM activities, prepared a comprehensive background review document (BRD) reviewing the available data and information for the BCOP test method. The objectives of this BRD were to:

- Describe the current validation status of the BCOP test method, including what is known about its accuracy and reliability, the scope of the substances tested, and the availability of a standardized protocol for its use in a regulatory tiered testing strategy (e.g., the UN Globally Harmonized System; GHS [UN 2003])
- Identify the usefulness and limitations of the BCOP test method, based on existing data, for identifying ocular corrosives and severe irritants in a regulatory tiered testing strategy
- Propose a standardized BCOP test method protocol
- Propose additional test method protocol optimization studies that might enhance the accuracy and/or reliability of the BCOP test method and validation studies to further characterize its usefulness and limitations
- Propose reference substances for future optimization/validation studies of this and other alternative test methods intended to identify ocular corrosives and severe irritants

This BRD was based on published studies using the BCOP test method, and other data and information submitted in response to a 2004 *Federal Register* Notice (Vol. 69, No. 57, pp. 13859-13861; available at http://iccvam.niehs.nih.gov/methods/eyeirrit.htm). The BCOP test method was reviewed by an independent Expert Panel on January 11-12, 2005, and the panel report, including conclusions and recommendations, will be available in mid-March on the ICCVAM website. After considering the Expert Panel's report and any public comments, ICCVAM will prepare final recommendations on the BCOP test method, which will be published in a report that is expected to be available in August 2005.

Test Method Overview

The BCOP assay is an *in vitro* organotypic model that provides short-term maintenance of normal physiological and biochemical function of the cornea in an isolated system. The test method was developed by Gautheron et al. (1992), who focused on a cornea-based assay since the cornea is one of the main targets during accidental eye exposures, and damage to it can result in visual impairment or loss.

In the BCOP assay, ***opacity*** and ***permeability*** are the most commonly used endpoints to evaluate the extent of damage to the cornea following exposure to a test substance:

- ***Opacity*** is quantitatively measured, most often with an opacitometer, by the amount of light transmission through the cornea
- ***Permeability*** is quantitatively measured by UV/VIS spectrophotometry (OD_{490}) as the amount of the small molecule, sodium fluorescein (NaF), that penetrates all corneal cell layers

During a BCOP study, corneas are isolated from the eyes of cattle, which have been processed for human consumption. As described in **Figure 1**, the corneas are inspected for defects and handled carefully to avoid damage. Corneas are then mounted in corneal holders, which consist of anterior and posterior chambers that interface with the epithelial and endothelial sides of the cornea, respectively. After an equilibration period, the corneas are treated. Two treatment protocols are used, one for liquids and surfactants, and one for solids. Test substances are applied to the epithelial surface of the cornea by addition to the anterior chamber of the corneal holder. Historically, negative control corneas have been used to correct opacity and permeability values of treated corneas.

Mean corrected opacity and mean corrected permeability values are calculated for each treatment group. An *In Vitro* Irritancy Score is calculated using the following empirically-derived formula (Sina et al. 1995):

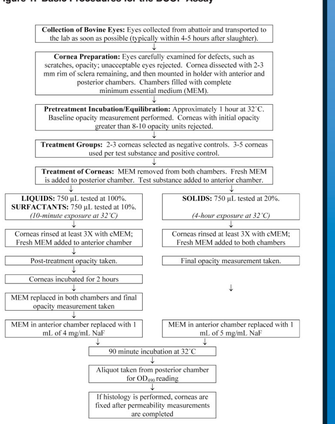
- *In Vitro* Irritancy Score = Opacity value + (15 x OD_{490} value)
- *In Vitro* Irritancy Score ≥ 55.1 is considered a severe eye irritant.

Some substances, such as anionic and nonionic surfactants, produce permeability without significant opacity; thus, only permeability values are used for certain chemical classes.

- Permeability (OD_{490}) > 0.600 is considered a severe irritant.

In addition, histopathological evaluation of the treated cornea is used on a case-by-case basis to identify damage that does not produce opacity or permeability in the cornea (Curren et al. 2000). Histology procedures are conducted after permeability data have been collected.

Figure 1. Basic Procedures for the BCOP Assay



Test Method Database

A total of 166 substances in eight studies were available and used to evaluate the accuracy of the BCOP test method for detecting ocular corrosives and severe irritants (**Table 1**). BCOP data for 65 substances were available and used to evaluate intralaboratory repeatability, data for 41 substances were used to evaluate intralaboratory reproducibility, and data for 127 substances were used to evaluate interlaboratory reproducibility.

Twenty-seven different chemical classes were represented among these substances with the most commonly represented being:

- Surfactant-containing formulations
- Alcohols
- Ethanol-containing formulations
- Cationic surfactants

Twenty different product classes were represented with the most commonly represented being:

- Solvents
- Chemical/synthetic intermediates
- Drugs/pharmaceuticals/therapeutic agents
- Petroleum products
- Cleaners (e.g., household)
- Personal care cleansers

For the accuracy analysis, detailed *in vivo* data were necessary, including cornea, iris and conjunctiva scores for each test animal at 24, 48, and 72 hours and/or assessment of the presence or absence of lesions at 7, 14, and 21 days. These data were used to determine the appropriate EPA (1996), European Union (EU 2001), and GHS (UN 2003) ocular irritancy hazard classifications.

Table 1. Primary BCOP Data Sources

Study	Accuracy (S/NS)			Intrlab	Interlab	
	GHS (UN 2003)	EPA (1996)	EU (2001)	CVs	CVs	GHS class (S/NS)
Gautheron et al. (1994)	7/6	6/6	8/43	-	52	7/6
Balls et al. (1995)	22/35	20/35	21/38	-	59	22/35
Swanson et al. (1995)	6/3	6/3	5/4	18[a]	-	-
Gettings et al. (1996)	8/17	10/15	6/19	25[b]	-	-
Casterton et al. (1996)	26/30	26/29	24/36	-	-	-
Southee (1998)	6/8	6/8	5/10	16[c]	16	6/8
Swanson and Harbell (2000)	1/8	4/5	1/8	-	-	-
Balls et al. (2006)	3/13	3/13	3/13	-	-	-
Dr. Joseph Sina's submission[d]	-	-	-	29[e]	-	-

Abbreviations: class, = classification; CV = coefficient of variation; EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = United Nations Globally Harmonized System for Classification and Labeling of Chemicals; NS = nonsevere irritants or nonirritants; S = severe or corrosive irritants.
[a]Intralaboratory repeatability was evaluated (n=3 replicate experiments).
[b]Intralaboratory reproducibility was evaluated (n=3 replicate experiments).
[c]Intralaboratory reproducibility and reproducibility were evaluated.
[d]Intralaboratory reproducibility was evaluated (n=4 corneas).
[e]*In vivo* data not available for this study.

This table summarizes the number of substances used from each study for the accuracy analysis, the quantitative intralaboratory analysis, and the quantitative and qualitative interlaboratory analyses conducted for the BCOP BRD. The first value in each cell represents the number of severe irritants that were used, while the second number represents the number of nonsevere/nonirritants irritants used, as defined by the particular hazard classification system.

Test Method Protocols

The BCOP test method protocols used in the reviewed studies were similar to each other, but not identical. Examples of test method components that differed among the protocols include:

- Number of corneas used (n=3-5)
- Storage conditions of bovine eyes during transport (e.g., use of antibiotics; at ambient temperature or over ice)
- Negative controls always used but some labs used saline, while others used MEM or sterile deionized water
- Some labs used positive controls, others didn't
- Most labs used an opacitometer to measure opacity; one used a UV/VIS spectrophotometer

Test Method Accuracy Analysis

The ability of the BCOP test method to correctly identify ocular corrosives and severe irritants, as defined by the GHS (UN 2003), EPA (1996), and EU (2001) classification systems, was evaluated. For the purposes of this analysis, an ocular corrosive or severe irritant was defined as a substance that would be classified as Category 1 according to the GHS classification system (UN 2003), as Category I according to the EPA classification system (EPA 1996), or as R41 according to the EU classification system (EU 2001). Accuracy statistics were calculated for each BCOP test method study with appropriate *in vivo* and *in vitro* data and, where appropriate:

- Classifications were pooled into one classification per substance (i.e., majority call among studies used)
- Using individual studies, where a balanced design existed (multiple substances in multiple labs as in Balls et al. 1995)

The overall BCOP test method accuracy with regard to each of the three classification systems ranged from 77% to 80%, while the false positive and false negative rates ranged from 17% to 23% and 22.5% to 27%, respectively (**Table 2**).

Table 2. BCOP Test Method Accuracy*

Statistic	GHS (n=120)		EPA (n=117)		EU (n=157)**	
	%	n	%	n	%	n
Accuracy	79	95/120	80	93/117	77	121/157
Sensitivity	76	32/42	73	33/45	77.5	31/40
Specificity	81	63/78	83	60/72	77	90/117
False Positive Rate	19	15/78	17	12/72	23	27/117
False Negative Rate	24	10/42	27	12/45	22.5	9/40

Abbreviations: EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = United Nations Globally Harmonized System for Classification and Labeling of Chemicals.

*BCOP data from the following studies were pooled for this analysis: Gautheron et al. (1994), Balls et al. (1995), Swanson et al. (1995), Gettings et al. (1996), Southee (1998), Swanson & Harbell (2000), Balley et al. (2006).
**Additional 37 chemicals available for EU analysis only (individual animal data not available for GHS or EPA classification at time of analysis).

Table 3 lists the false negative and false positive rates of the BCOP test method for identifying ocular corrosives/severe irritants, as defined by the GHS (UN 2003) hazard classification system, based on chemical class and physicochemical properties. This table highlights the fact that, among this limited dataset, alcohols appear to be frequently overpredicted (44% [4/9] false positive rate), as do alcohol-containing formulations (29% [2/7] false positive rate) and ketones (67% [2/3] false positive rate) when using the 10-minute exposure protocol for liquids. Regarding physical properties, solids were commonly underpredicted (33% [4/12] false negative rate).

Table 3. BCOP Test Method Accuracy According to Chemical Class and Physical Property (GHS System)

Category	n	False Negative Rate %	False Positive Rate %	
		No.	No.	
EYE-IRRITANT	74	10/42	15	11/74
Formulation, surfactant containing	33	1/1	30	10/33
Alcohol	10	1/1	44	4/9
Formulation, ethanol containing	8	0	29	2/7
Surfactant, cationic	7	0	0/6	0/1
Acetate	6	-	0	0/6
Formulation, petrochemical	6	1/1	0	0/3
Acid	5	0	50	1/2
Heterocyclic compound	5	2/5	1/4	0/1
Surfactant, anionic	4	-	50	2/4
Aromatic hydrocarbon	3	-	0	0/3
Inorganic chemical	3	1/2	0	0/1
Ketone	3	-	67	2/3
Alkali	2	0	100	1/1
Amine	2	-	0	0/2
Cyclic hydrocarbon	2	-	0	0/2
Oil	2	-	0	0/2
Petrochemical, cutting fluid	2	-	0	0/2
Polycyclic aromatic hydrocarbon	2	0	0/2	-
Surfactant, anionic	2	1/1	0	0/1
Acyl halide	1	-	0	0/1
Aldehyde	1	-	0	0/1
Amide	1	-	0	0/1
Amidine	1	0	0/1	-
Chlorinated hydrocarbon	1	-	0	0/1
Diol	1	1/1	-	-
Lactone	1	-	0	0/1
Organometallic	1	-	0	0/1
Organophosphate	1	-	100	1/1
Quaternary ammonium surfactant	1	1/1	-	-
Terpene	1	-	0	0/1
Thiophthalimide	1	1/1	-	-
Tin cn	1	-	0	0/1
Physical Properties				
Solids	19	4/12	29	2/7
Liquids	94	5/28	21	14/66

False Negative Rate = the proportion of all positive substances that are falsely identified as negative in vitro; False Positive Rate = the proportion of all negative substances that are falsely identified as positive in vitro; n = number of substances

Limitations of the Accuracy Analysis

There were notable limitations of the BCOP test method accuracy analysis. For the majority of the substances included in this analysis, *in vivo* data were available from only a single Draize rabbit eye test per test substance, and it was assumed that the positive or negative results from this single *in vivo* study was "correct". For the few substances (e.g., ethanol, 1% benzalkonium chloride) that were evaluated in more than one Draize rabbit eye test, the more severe hazard classification was used. Thus, this accuracy analysis does not account for variability among rabbit eye studies. In addition, a lack of appropriate individual rabbit test data prevented an accuracy evaluation for all 166 substances using the GHS and EPA classification schemes. Finally, the small number of substances representing most chemical classes allows for only limited conclusions with respect to the accuracy of BCOP by chemical class or physicochemical property. However, it appears that alcohols and ketones tend to be overpredicted, while solids tend to be underpredicted in the BCOP test method.

Test Method Reliability Analysis

Intralaboratory Repeatability

Intralaboratory repeatability was evaluated for three studies (**Table 4**) by performing a quantitative analysis using a coefficient of variation (CV) calculation to compare variability in the *In Vitro* Irritancy Score obtained for replicate corneas.

Table 4. BCOP Intralaboratory Repeatability – % CV Values for *In Vitro* Irritancy Scores from Replicate Corneas

	%CV	Dr. Sina's Study[1] (n=4 corneas)	Swanson et al. 1995[1] (n=5)	Southee 1998 Lab 1[1] (n=3)	Southee 1998 Lab 2[1] (n=3)	Southee 1998 Lab 3[1] (n=3)
All Data	Mean	71	10.9	48.3	39.2	30.5
	Median	35	9.4	14.2	11.8	12.4
	Range	1.1 - 479	1.9 - 32.5	0.1 - >500[3]	2.1 - >500[3]	4.3 - >500[3]
Severe *In Vitro*	Mean	8.2	8.9	8.4	8.4	11.1
	Median	8.1	7.9	7.1	8.1	9.3
	Range	1.1 - 13	1.9 - 25.7	0.1 - 22	2.1 - 21.7	5.1 - 30.3

[1]In this study, 29 substances were evaluated using a protocol that incubated the corneas at 32°C; of these, 5 were classified as severe *in vitro*.
[2]Only 18 of the 20 test substances evaluated in this study were used for this analysis, since two of the substances produced negative *In Vitro* Irritancy Scores, which resulted in negative CV% values. Of these 18 substances, 16 were classified as severe *in vitro*.
[3]In this study, 16 substances were evaluated in 3 laboratories multiple times (2-5 experiments). Each laboratory performed a total of 41 tests.
[4]Sodium oxalate, which was tested twice in this laboratory, produced a mean *in vitro* score <1 and %CV> 500. Results around the baseline of the assay tended to produce higher %CVs.
[5]In this laboratory, one trial of Tween 20 produced a mean *in vitro* score <1 and a %CV >500. Results around the baseline of the assay tended to produce higher %CVs.

Intralaboratory Reproducibility

Intralaboratory reproducibility was evaluated for two studies by performing a quantitative analysis using a CV calculation to compare variability in the *In Vitro* Irritancy Score (IVIS) obtained for different experiments or trials performed by the same laboratory.

- Gettings et al. 1996: 25 substances, 3 trials, 1 lab
  - Mean %CV and Median %CV for permeability value were 33.4 and 29.0, respectively
  - Substances spanned a range of irritancy
  - Surfactant-based personal care cleaning formulations
- Southee 1998: 16 substances, ≥ 2 trials, 3 labs
  - Mean %CVs for IVIS ranged from 12.6 to 14.8 for the 3 labs
  - Median %CVs for IVIS ranged from 6.7 to 12.4 for the 3 labs
  - Substances spanned a range of irritancy and chemical classes

Interlaboratory Reproducibility

Interlaboratory reproducibility was evaluated using data from three studies (Gautheron et al. 1994; Balls et al. 1995; Southee 1998). Two separate analyses were conducted:

- A qualitative analysis, in which the extent of agreement between testing laboratories when identifying ocular corrosives and severe irritants was compared
- A quantitative analysis using a CV calculation to compare variability in measurements of the *In Vitro* Irritancy Score

Table 5 summarizes the results of the qualitative analysis for each of the three regulatory classification systems.

Table 5. BCOP Interlaboratory Reproducibility – Classification Agreement Among Laboratories

% Interlab Agreement	Balls et al. 1995 (5 labs): GHS		Southee 1998 (3 labs): GHS		Gautheron et al. 1994 (11 or 12 labs): GHS	
	%	N	%	N	%	N
100% (all)	68	41/60	94	15/16	71	36/51
≥80% (all)	85	51/60	94	15/16	91	46/51
100% (severe *in vivo* and *in vitro*)	82	14/17	100	3/3	60	3/5
≥80% (severe *in vivo* and *in vitro*)	94	16/17	-	-	80	4/5

Abbreviations: GHS = United Nations Globally Harmonized System for Classification and Labeling of Chemicals; N = number.

Table 6 summarizes the quantitative analysis using a CV calculation. Mean and median %CV values were calculated to provide an assessment of overall variability.

Table 6. BCOP Interlaboratory %CV Values for *In Vitro* Irritancy Scores

	%CV	Gautheron et al. 1994 (11 or 12 labs)	Balls et al. 1995 (5 labs)	Southee 1998 (3 labs)
All Data	Mean	168 (n=52)	50 (n=50)	32 (n=16)
	Median	46.9 (n=52)	26 (n=50)	23 (n=16)
	Range	16.5 - 1325 (n=52)	7.6 - 712 (n=50)	7.5 - 109 (n=16)
Substances Predicted as Severe	Mean	36 (n=17)	25 (n=32)	11.1 (n=5)
	Median	17 (n=17)	22 (n=32)	8.6 (n=5)
	Range	16.5 - 55.7 (n=17)	7.6 - 89.4 (n=32)	7.5 - 21.6 (n=5)

Draft BRD Proposals

Based on this evaluation of BCOP test method performance, a proposed BCOP test method protocol, which evaluates corneal opacity and permeability, and recommends histology on a case-by-case basis, is proposed in the BRD. The proposed standardized protocol is based on the method of the Institute for *In Vitro* Sciences (IIVS). The only major difference between the two protocols is that the proposed protocol lacks the detailed histology procedures used by IIVS for the BCOP assay.

Additional optimization studies are suggested that may enhance the performance of the BCOP test method for identifying ocular corrosives and severe irritants. These studies include:

- A retrospective analysis of decision criteria used to classify substances as corrosives and severe irritants
- An evaluation of possible increased interlaboratory variability for specific chemical classes appearing more variable (e.g., alcohols)
- An evaluation of reduced exposure times for alcohols, and possibly other volatile solvents
- Determining the utility of histopathology and when it should be included

Once these studies have been completed, additional validation studies are recommended to further assess the accuracy and reliability of BCOP, so that the test method applicability domain is better defined and data gaps are filled in.

Proposed Reference Substances for Optimization and Validation Studies

A common list of reference substances proposed for future optimization and validation studies of BCOP and other alternative test methods intended to detect ocular corrosives/severe irritants has been developed (see BCOP BRD http://iccvam.niehs.nih.gov/methods/ocudocs/ocu_brd.htm#bcop). Following completion of the proposed validation studies, reference substances from this list can be selected for inclusion in performance standards and for proficiency testing. Substances included in this list are intended to:

- Represent the range of responses (i.e., corrosive/severe irritant; nonsevere irritant/noncorrosive) that the test method is expected to be capable of measuring or predicting
- Represent the range of chemical/product classes and physicochemical properties that the test method is expected to be capable of testing
- Represent the range of known or anticipated mechanisms or modes of action for severe/irreversible ocular irritation or corrosion
- Have been generated by high-quality *in vivo* studies following Organisation for Economic Co-operation and Development (OECD) Test Guideline 405 and preferably conducted in compliance with Good laboratory Practice guidelines
- Have well-defined chemical composition, with defined purity, be readily available, and not associated with excessive hazard or prohibitive disposal costs

This list of 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.

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