APPENDIX D
ICCVAM RECOMMENDED BCOP TEST METHOD PROTOCOL

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ICCVAM Recommended Protocol for Future Studies Using the Bovine Corneal Opacity and Permeability (BCOP) Test Method

PREFACE

This proposed protocol for measuring corneal damage is based primarily on information obtained from 1) Dr. John Harbell of the Institute for *In Vitro* Sciences (IIVS), a nonprofit foundation that has performed the BCOP assay since 1997 in a Good Laboratory Practice (GLP) compliant testing facility; and 2) INVITTOX Protocol 124 (1999), which represents the protocol used for the European Community sponsored prevalidation study of the BCOP assay conducted in 1997-1998. Both of these protocols are based on the BCOP assay methodology first reported by Gautheron et al. (1992). Future studies using the BCOP test method could include further characterization of the usefulness or limitations of the BCOP in a weight of evidence approach for regulatory decision making. Users should be aware that the proposed test method protocol could be revised based on any additional optimization and/or validation studies that are conducted in the future. ICCVAM recommends that test method users consult the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov/) to ensure use of the most current test method protocol.

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the potential ocular irritancy of a test substance as measured by its ability to induce opacity and increase permeability in an isolated bovine cornea. Effects are measured by: 1) decreased light transmission through the cornea (opacity); 2) increased passage of sodium fluorescein dye through the cornea (permeability); and 3) evaluation of fixed and sectioned tissue at the light microscopic level, if applicable. The opacity and permeability assessments of the cornea following exposure to a test substance are considered individually and also combined to derive an *In Vitro* Irritancy Score, which is used to classify the irritancy level of the test substance. Histological evaluation of the corneas can be useful for identifying damage in tissue layers that does not produce significant opacity or permeability.

The focus of this protocol is on the use of the BCOP test method for the detection of ocular corrosives and severe irritants, as defined by the U.S. Environmental Protection Agency (EPA; EPA 1996), the European Union (EU; EU 2001), and in the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN 2003). Substances other than ocular corrosives and severe irritants (e.g., nonirritants and mild/moderate ocular irritants) have been tested using this protocol; however, the accuracy and reliability of the BCOP test method have not yet been formally evaluated for the other classes of ocular irritancy defined by the EPA (1996), the EU (2001), and the GHS (UN 2003).

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures with bovine eyes and bovine corneas should follow the institution's applicable regulations and procedures for handling human or animal substances, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions are recommended, including the use of laboratory coats, eye protection, and gloves. If available, additional precautions required for specific study substances should be identified in the Material Safety Data Sheet for that substance.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Source of Bovine Eyes

Eyes from young adult cattle are obtained from a cattle slaughterhouse located within close proximity of the testing facility. The cattle type (breed not specified) can be cows, heifers, steers, or bulls. Because cattle have a wide range of weights, depending on breed, age, and sex, there is no recommended weight for the animal at the time of slaughter.

Eyes from very old cattle are not recommended because the corneas tend to have a greater horizontal corneal diameter and vertical corneal thickness that could affect assay performance (Doughty et al. 1995; Harbell J, personal communication). Additionally, eyes from calves are not recommended since their corneal thickness and corneal diameter are considerably less than that of eyes from adult cattle.

3.2 Equipment and Supplies

- Corneal holders[1]
- Dissection equipment (scissors, scalpels, forceps)
- Electric screwdriver
- Falcon tubes (50 mL)
- Incubator or water bath
- Liquinox (or equivalent)
- Microplate reader or UV/VIS spectrophotometer
- Micropipettors and pipette tips
- Opacitometer
- Petri dishes
- Plastic containers for collection and transport of eyes
- Sample tubes (5 mL, glass) for permeability determination
- Spatula
- Specialized window-locking ring screwdriver
- Standard tissue culture and laboratory equipment
- Sterile deionized water
- Syringes (10 mL) and blunt tip needles (19 Gauge)
- Vacuum pump
- Volumetric flasks
- 96 well plates (polystyrene) or cuvettes of an appropriate size for UV/VIS spectrophotometer

3.3 Chemicals

- Ethanol (200 proof, absolute, anhydrous, ACS/USP grade)
- Imidazole
- Penicillin
- Sodium chloride
- Sodium fluorescein
- Streptomycin

3.4 Solutions

Follow the manufacturer's recommendations with regard to storage temperature and shelf life of stock solutions. Prepare assay solutions volumetrically.

- 0.9% (w/v) NaCl in sterile deionized water (saline).
- 1X Hanks' Balanced Salt Solution with Ca^{++} and Mg^{++} (HBSS) containing 100 IU/mL penicillin and 100 µg/mL streptomycin.

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[1]Users should be aware of a proposed corneal holder developed by Ubels et al. (2002). The ICCVAM Test Method Evaluation Report recommends, "Studies should be conducted to evaluate the impact of using a corneal holder that maintains normal curvature (e.g., the corneal mounting system designed by Ubels et al. 2002) on accuracy and/or reliability of the BCOP test method."

- Dulbecco's Phosphate Buffered Saline (DPBS).
- Eagle's Minimum Essential Medium without phenol red containing 1% (v/v) Fetal Bovine Serum (complete MEM), warmed to 32˚C.
- Eagle's Minimum Essential Medium with phenol red containing 1% Fetal Bovine Serum (complete MEM with phenol red, used only for rinsing test substances), warmed to 32˚C.
- Sodium fluorescein (Na-fluorescein) diluted in DPBS to 4 mg/mL for liquid test articles or 5 mg/mL for solid test articles.

4.0 TEST SUBSTANCE PREPARATION

All test substance solutions should be prepared fresh on the day of use.

4.1 Nonsurfactant Liquid Test Substances

Liquid test substances are usually tested undiluted. However, if prescribed, dilutions of aqueous soluble test substances should be prepared in 0.9% sodium chloride.

4.2 Nonsurfactant Solid Test Substances

Nonsurfactant solid test substances should be prepared as 20% (w/v) solutions or suspensions in 0.9% sodium chloride.

4.3 Surfactants

Solid and concentrated liquid surfactants should be prepared and tested as a 10% (w/v, v/v) dilution or suspension in 0.9% sodium chloride.

4.4 Surfactant Preparations

Surfactant-based preparations (e.g., product formulations) are usually tested neat, or can be diluted in 0.9% sodium chloride, with justification of the selected dilution.

5.0 CONTROLS

5.1 Negative Control

A negative control (e.g., 0.9% sodium chloride) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.2 Solvent/Vehicle Control

Untreated controls are recommended when solvents/vehicles other than 0.9% sodium chloride or distilled water are used to dissolve test substances, in order to demonstrate that the solvent/vehicle is not interfering with the test system.

5.3 Positive Control

A known ocular irritant is included in each experiment to verify that an appropriate response is induced. If the BCOP assay is being used only to identify corrosive or severe irritants, then the positive control should be a reference substance that induces a severe response *in vivo* as well as in the BCOP assay. However, to ensure that variability in the positive control response across time can be assessed, the magnitude of the severe response should not be excessive. The selection of positive control test substances should be based on the availability of high quality *in vivo* data.

a. Historically, the most commonly used positive control for liquid test substances has been 100% ethanol. In the *in vivo* rabbit eye test, 100% ethanol is classified as EPA Category III or I (EPA 1996), GHS Category 2B or 2A (GHS; UN 2003), and EU nonirritant (EU 2001) based on an ECETOC (1998) study and a S.C. Johnson & Son study, respectively.
b. Historically, the most commonly used positive control for solid test substances has been 20% (w/v) imidazole prepared in saline. In the *in vivo* rabbit eye test, based on an ECETOC (1998) study, 20% (w/v) imidazole is classified as GHS Category 1 (GHS; UN 2003) and EU R41 (EU 2001).

5.4 Benchmark Substances (if appropriate)

Benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative irritancy potential of an ocular irritant within a specific range of irritant responses. Appropriate benchmark substances should have the following properties:

- a consistent and reliable source(s)
- structural and functional similarity to the class of the substance being tested
- known physical/chemical characteristics
- supporting data on known effects in the *in vivo* rabbit eye test
- known potency in the range of the desired response

6.0 EXPERIMENTAL DESIGN

6.1 Collection and Transport Conditions of Bovine Eyes

Bovine eyes are typically obtained from a local cattle slaughterhouse, where the eyes are excised by a slaughterhouse employee as soon as possible after slaughter. Care should be taken to avoid damaging the cornea during the enucleation procedure. Eyes are collected in a suitable container in which they are immersed in HBSS containing the antibiotics penicillin (100 IU/mL) and streptomycin (100 µg/mL) The container is maintained on ice at all times throughout collection of the eyes and transportation to the testing facility (NOTE: antibiotics may not be necessary if the eyes are kept below 4°C throughout transport). The eyes are used within five hours after slaughter.

Under conditions where contamination of the bovine eyes with yeast occurs, immersion of the eyes in HBSS containing fungizone should be evaluated.

6.2 Preparation of Corneas

a. Carefully examine all eyes macroscopically. Those exhibiting unacceptable defects, such as opacity, scratches, pigmentation, and neovascularization are rejected.
b. Carefully remove the cornea from each selected eye by making an incision with a scalpel 2 to 3 mm outside the cornea, then by cutting around the cornea with dissection scissors, leaving a rim of sclera to facilitate handling. Carefully peel off the iris and lens, ensuring no fragments of these tissues are remaining on the cornea. Take care to avoid damaging the corneal epithelium and endothelium during dissection.
c. Store the isolated corneas in a petri dish containing HBSS until they are mounted in holders. Examine the corneas before use, and discard those with defects.
d. Mount the corneas in holders (one cornea per holder), by placing the endothelial side of the cornea against the O-ring of the posterior chamber. Place the anterior chamber over the cornea and join the chambers together by tightening the chamber screws. Care should be taken not to shift the two chambers to avoid damaging the cornea.
e. Fill both chambers with fresh complete MEM (about 5 mL), always filling the posterior chamber first to return the cornea to its natural curvature. Care should be taken when adding or removing liquid from the posterior chamber to avoid the formation of bubbles and to minimize shear forces on the corneal endothelium.
f. Seal each chamber with plugs provided with the holders.
g. Incubate the holders in a vertical position at 32 ± 1°C for at least 60 minutes.
h. At the end of the initial 1-hour incubation period, examine each cornea for defects, such as tears or wrinkling. Discard corneas with any observed defects.

6.3 Control Cornea Selection and Opacity Reading

a. After the 1-hour incubation period, remove the medium from both chambers of each holder (anterior chamber first) and replace with fresh complete MEM.
b. Take and record an initial opacity reading for each cornea, using an opacitometer or equivalent instrument that has been appropriately calibrated according to the manufacturer's specifications. This initial opacity reading will be used to calculate the final opacity value for each cornea. The testing facility should ensure the opacitometer is functioning properly each day it is used.
c. Calculate the average opacity value for all corneas.
d. Select a minimum of three corneas with opacity values close to the average value for all corneas as negative (or solvent/vehicle) control corneas.

e. Corneas that display an initial opacity reading significantly greater (+ 2 standard deviations [SDs]) than the average opacity for all corneas in the batch of eyes collected the day of testing should not be used in the assay.

6.4 Treatment Groups

A minimum of three corneas are treated with each test substance solution or suspension. In addition, three corneas per assay are treated with the positive control and three corneas per assay are treated with the negative control. If a benchmark substance is used the day of testing, three corneas should be treated with the benchmark.

Different treatment methods are used depending on the physical nature and chemical characteristics (liquid or surfactant versus nonsurfactant solid) of the test substance. The controls used depend on which method is used.

6.5 Treatment of Corneas and Opacity Measurements

6.5.1 Closed chamber method for nonviscous to slightly viscous liquid test substances

a. Record the initial opacity readings and label each chamber with the appropriate control or test substance identification. Just prior to treatment, remove the medium from the anterior chamber through the dosing holes using an appropriate aspiration technique (e.g., blunt needle attached to a vacuum pump).
b. Add 0.75 mL of the control or test substance to the anterior chamber through the dosing holes using a micropipettor. The dosing holes are then resealed with the chamber plugs.
c. Rotate the holders such that the corneas are in a horizontal position. The holders should be gently tilted back and forth to ensure a uniform application of the control or test substance over the entire cornea.
d. Incubate the holders in a horizontal position at 32 ± 1°C for 10 ± 1 minutes. If other exposures times are used, justification must be provided.
e. Remove the control or test substance from the anterior chamber through the dosing holes and rinse the epithelium at least three times with approximately 2 to 3 mL of fresh complete MEM with phenol red. Perform one last rinse of the epithelium using fresh complete MEM. If it is not possible to remove all visible signs of the test substance, document the observation in the study notebook. Refill the anterior chamber with fresh complete MEM.
f. Perform a post-treatment opacity reading for each cornea and record the results. Observe each cornea visually and, if applicable, record pertinent observations (e.g., dissimilar opacity patterns, tissue peeling or residual test article).
g. Incubate the holders in a vertical (anterior chamber facing forward) position at 32 ± 1°C for 120 ± 10 minutes. If other post-exposure incubation times are used, justification should be provided.
h. Record a post-incubation opacity reading for each cornea, which will be used to calculate the final corneal opacity value. Observe each cornea visually and

record pertinent observations in the study notebook. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test substance, etc.

6.5.2 Open chamber method for semiviscous and viscous liquid test substances and surfactant preparations

a. Record the initial opacity readings and label each chamber with the appropriate control or test article identification. Just prior to treatment, remove the medium from the anterior chamber through the dosing holes.
b. Remove the window-locking ring and glass window from all appropriate anterior chambers and place the holders into a horizontal position (anterior chamber facing up).
c. Add test substance to each chamber successively at a constant rate of 15 to 30 seconds between each chamber. Apply approximately 0.75 mL of the control or test substance (or enough test substance to completely cover the cornea) directly to the epithelial surface of the cornea using a micropipettor or other appropriate device, such as a spatula. Maintain the holders in a horizontal position (anterior chamber up).
d. If necessary, to aid in filling the pipette with substances that are viscous, the test article may first be transferred to a syringe. Insert the pipette tip of the positive displacement pipette into the dispensing tip of the syringe, so that the substance can be loaded into the displacement tip under pressure. Simultaneously, depress the syringe plunger as the pipette piston is drawn upwards. If air bubbles appear in the pipette tip, the test article should be expelled and the process repeated until the tip is filled without air bubbles. This method should be used for any substances that cannot be easily drawn into the pipette (e.g., gels, toothpastes, and face creams).
e. If necessary, immediately upon dosing, slightly tilt the holders to achieve a uniform application of the test article over the entire cornea.
f. After all of the chambers are dosed, replace the glass windows and window-locking rings.
g. Incubate the holders in a horizontal position at 32 ± 1°C for 10 ± 1 minutes. If other exposure incubation times are used, justification should be provided.
h. Prior to the end of the exposure period, remove the window-locking ring and glass window from each appropriate chamber.
i. At the completion of the exposure period, successively rinse each cornea in the exposure group according to the intervals that they were dosed. Using a syringe, add fresh complete MEM with phenol red to the inside wall of the anterior chamber creating a “whirlpool or vortex effect”, which causes the test article to be rinsed off the cornea. Take special care not to spray the medium directly onto the cornea. Residual test article that cannot be removed from the cornea by the “whirlpool method” is removed by placing a layer of medium over the cornea (added to the inside wall of the chamber). Spray a gentle stream of medium through the medium layer, directing it towards the residual test article. If after several tries the test article cannot be removed, document this in the study notebook, and proceed to the next step.

j. Once each cornea is completely rinsed of test article, replace the glass window and window-locking ring. Continue rinsing as stated previously for the "closed chamber method" (see **Section 6.5.1, step e**).
k. Perform a post-treatment opacity reading for each cornea and record the results. Observe each cornea visually and, if applicable, record pertinent observations (e.g., dissimilar opacity patterns, tissue peeling or residual test article).
l. Incubate the holders in a vertical (anterior chamber facing forward) position at 32 ± 1°C for 120 ± 10 minutes. If other post-exposure incubation times are used, justification should be provided.
m. Record a post-incubation opacity reading for each cornea, which will be used to calculate the final corneal opacity value. Observe each cornea visually and record pertinent observations in the study notebook. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test substance, etc.

6.5.3 Solid and liquid surfactant test substances

Surfactant test substances are administered following one of the previously described procedures, with one exception, which is noted below:

- Surfactant test substances are tested on the cornea as a 10% (w/v) solution or suspension prepared in an appropriate solvent/vehicle (e.g., sterile deionized water).

6.5.4 Solid nonsurfactant test substances

Solid nonsurfactant test substances are administered following one of the previously described procedures, with a few exceptions, which are noted below:

- Solid test substances are tested on the cornea as a 20% (w/v) solution or suspension prepared in an appropriate solvent/vehicle (e.g., sterile deionized water).
- Solid test substances are incubated at 32 ± 1°C for 240 ± 10 minutes.
- There is no post-treatment incubation period. Thus, immediately following the rinsing process, both chambers are refilled (posterior chamber first) with fresh complete MEM, and the post-treatment opacity readings are taken. During the post-treatment opacity reading, visual observations are performed for each cornea and, if necessary, are recorded in the workbook. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test article, etc. Immediately following these opacity readings and visual observations, the permeability experiment is performed.

6.6 Application of Sodium Fluorescein

Following the final opacity measurement, permeability of the cornea to Na-fluorescein is evaluated. The Na-fluorescein solution is applied to the cornea by one of two methods, depending on the nature of the test substance:

a. Liquid and surfactant test substances and surfactant preparations: Remove the medium from both chambers (anterior chamber first). Fill the posterior

chamber with fresh complete MEM, and add 1 mL of a 4 mg/mL Na-fluorescein solution to the anterior chamber using a micropipettor. Reseal the dosing holes in the top of both chambers with the chamber plugs.

b. Solid nonsurfactant test substances: Remove the medium from the anterior chamber only and replace with 1 mL of a 5 mg/mL Na-fluorescein solution. Reseal the dosing holes in the top of both chambers with the chamber plugs.

6.7 Permeability Determinations

a. After adding the Na-fluorescein to the anterior chamber and sealing the chambers, rotate the holders into a horizontal position with the anterior chamber facing up. Tilt the holders slightly, if necessary, to achieve a uniform application of the Na-fluorescein over the entire cornea. Incubate the holders in a horizontal position for 90 ± 5 minutes at 32 ± 1°C.

b. After the 90-minute incubation period, remove the medium in the posterior chamber of each holder and place into sample tubes prelabeled according to holder number. It is important to remove most of the medium from the posterior chamber and mix it in the tube so that a representative sample can be obtained for the OD_{490} determination.

c. After completing the Na-fluorescein penetration steps, the corneas should be fixed in an appropriate fixative (e.g., 10% neutral buffered formalin) at room temperature for at least 24 hours, so that the tissues are available if histology is necessary or requested at a later time. It is important that the corneas not be allowed to dry between transfer from the holders and fixation (submersion in the fixative).

d. If using a microplate reader to measure optical density, transfer 360 µL of the medium from each sample tube into its designated well on a 96-well plate. The standard plate map provides two wells for each cornea. The first well receives an undiluted sample from each cornea tested. When all of the media samples have been transferred onto the plate, measure and record their OD_{490}. Any OD_{490} value (of a control or test substance sample) that is 1.500 or greater must be diluted to bring the OD_{490} into the acceptable range. A dilution of 1:5 is generally sufficient but higher dilutions may be required. Prepare the dilution from the original sample of medium and transfer 360 µL into the second well designated for that cornea. Reread the plate and record the data from both the undiluted and diluted OD_{490} values. Use the values from this second reading in all calculations. The OD_{490} values of less than 1.500 will be used in the permeability calculation.

   Note: The linear range of absorbance of different microplate readers can vary. Thus, each laboratory must determine the upper limit of absorbance (in the linear range) for the microplate reader used in its facility.

e. If using a UV/VIS spectrophotometer to measure optical density, adjust the spectrophotometer to read at OD_{490}, and zero the spectrophotometer on a sample of complete MEM. Prior to reading samples from the BCOP assay,

prepare and read two quality control samples of Na-fluorescein solution to ensure the Na-fluorescein calibration curve (see note below) conducted for the spectrophotometer is still acceptable. If the average of the quality control samples does not fall within the accepted range of the Na-fluorescein calibration curve, then prepare a Na-fluorescein calibration curve prior to running samples from the BCOP assay. If the average of the quality control samples falls within the accepted range of the calibration curve, then proceed to read samples from the BCOP assay. Transfer an aliquot of the mixed medium from the posterior chamber of the BCOP holder into a cuvette, then take and record an absorbance reading using the spectrophotometer. Any solutions giving an OD_{490} beyond the linear range of the spectrophotometer must be diluted in complete MEM, and another reading taken, repeating these steps until the OD_{490} is within the linear range of the spectrophotometer. Repeat these procedures for each sample from the BCOP assay, rinsing the cuvette(s) thoroughly between each sample, until all samples have been read and results recorded.

Note: If conducting this assay for the first time, a calibration curve for the spectrophotometer must be performed, using a series of dilutions of Na-fluorescein solution in complete MEM. A calibration curve should be prepared and used to determine the linear range of the testing facility's spectrophotometer and thus determine the upper limit of absorbance.

6.8 Histopathology

A histopathological evaluation of the corneal tissue might be useful when the standard BCOP endpoints (i.e., corneal opacity and permeability) produce borderline results. A standardized scoring scheme using the formal language of pathology to describe any effects should be used. However, such an evaluation may not be necessary if the test substance belongs to class of materials known to be accurately predicted using only corneal opacity and permeability measurements.[2]

6.9 Maintenance of the Corneal Holders

Following completion of the assay, clean the disassembled parts of each holder as follows:

a. Soak the posterior and anterior chambers in a solution of warm tap water and a dime-size or greater amount of Liquinox (or equivalent).
b. Soak the chamber plugs, O-rings, and handle screws in 70% ethanol. Rinse the chamber plugs, O-rings, and handle screws thoroughly in hot tap water and air dry prior to reassembling the chambers.

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[2] The ICCVAM Test Method Evaluation Report recommends for the BCOP test method "histopathological evaluation of the corneal tissue, using a standardized scoring scheme, should be conducted. Such data will allow for the development of standardized decision criteria and a more comprehensive evaluation of the usefulness of this endpoint for classifying and labeling substances, especially those that may otherwise produce borderline or false negative results."

c. Clean the interior and exterior surfaces of each pre-soaked posterior and anterior chamber by using a scrubbing sponge. Rinse each posterior and anterior chamber thoroughly in warm tap water and air dry prior to reassembling the chambers.
d. Match up each numbered posterior chamber with its corresponding anterior chamber, insert an O-ring into the appropriate place, attach a chamber handle screw to the anterior chamber, and finally insert the chamber screws into the anterior chamber.

7.0 EVALUATION OF TEST RESULTS

Results from the two test method endpoints, opacity and permeability, should be combined in an empirically derived formula that generates an *In Vitro* Irritancy Score for each test substance.

7.1 Opacity

a. Calculate the change in opacity for each individual cornea (including the negative control) by subtracting the initial opacity reading from the final post-treatment opacity reading. Then calculate the *average* change in opacity for the negative control corneas.
b. Calculate a corrected opacity value for each treated cornea, positive control, and solvent/vehicle control (if applicable) by subtracting the average change in opacity of the negative control corneas from the change in opacity of each treated, positive control, or solvent/vehicle control cornea.
c. Calculate the mean opacity value of each treatment group by averaging the corrected opacity values of the treated corneas for each treatment group.

7.2 Permeability

Microplate Reader Method

a. Calculate the mean OD_{490} for the blank wells (plate blanks). Subtract the mean blank OD_{490} from the raw OD_{490} of each well (blank corrected OD_{490}).
b. If a dilution has been performed, correct the OD_{490} for the plate blank before the dilution factor is applied to the reading. Multiply each blank corrected OD_{490} by the dilution factor (e.g., a factor of 5 for a 1:5 dilution).
c. Calculate the final corrected OD_{490} value for each cornea by subtracting the mean OD_{490} value for the negative control corneas from the OD_{490} value of each treated cornea.

   Final Corrected OD_{490} = (raw OD_{490} – mean blank OD_{490}) - mean blank corrected negative control OD_{490}

d. Calculate the mean OD_{490} value for each treatment group by averaging the final corrected OD_{490} values of the treated corneas for a particular treatment group.

UV/VIS Spectrophotometer Method

a. Calculate the corrected OD_{490} value of each treated, positive control, or solvent/vehicle control cornea by subtracting the average value of the negative control corneas from the original OD_{490} value for each cornea.

   Final Corrected OD_{490} = raw OD_{490} - mean blank corrected negative control OD_{490}

b. Calculate the mean OD_{490} value for each treatment group by averaging the final corrected OD_{490} values of the treated corneas for a particular treatment group.

7.3 *In Vitro* Irritancy Score

Use the mean opacity and mean permeability values (OD_{490}) for each treatment group to calculate an *in vitro* score for each treatment group:

In Vitro Irritancy Score = mean opacity value + (15 x mean OD_{490} value)

Additionally, the opacity and permeability values should be evaluated independently to determine whether a test substance induced irritation through only one of the two endpoints.

8.0 CRITERIA FOR AN ACCEPTABLE TEST

A test is acceptable if the positive control gives an *In Vitro* Irritancy Score that falls within two SDs of the current historical mean, which is to be updated at least every three months. In the BCOP, 100% ethanol induces a moderate to severe response (*in vitro* score = 39.9 - 65.4 at IIVS [n = 632]; mean = 52.7, standard deviation [SD] = 6.4), while 20% (w/v) imidazole induces a severe response (*in vitro* score = 69.7 - 136.2 at IIVS [n=125]; mean = 103, SD = 16.6). The negative or solvent/vehicle control responses should result in opacity and permeability values that are less than the established upper limits for background opacity and permeability values for bovine corneas treated with the respective negative or solvent/vehicle control.

9.0 DATA INTERPRETATION

The following classification system was established by Sina et al. (1995) based on studies with pharmaceutical intermediates exposed for 10 minutes (liquids) or 4 hours (solids).

In Vitro Score: 55.1 and above = severe irritant

While this classification system provides a good initial guide to interpretation of these *in vitro* data, these specific ranges may not be applicable to all classes of substances. For example, the Sina et al. (1995) scoring scale is not appropriate for anionic and nonionic surfactants since they produce appreciable permeability while inducing little direct opacity.

For these and other substances that produce significant permeability with minimal opacity, it is recommended that permeability values > 0.600 be considered severe. Benchmark substances are recommended for assaying the responses of test substances of different product or chemical classes. Additionally, histological evaluation of the corneas can be instrumental in identifying occult changes (e.g., peroxide-induce stromal damage).

10.0 STUDY REPORT

The test report should include the following information, if relevant to the conduct of the study:

Test and Control Substances

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known
- The CAS Registry Number (RN), if known
- Purity and composition of the substance or preparation (in percentage(s) by weight), to the extent this information is available
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study
- Treatment of the test/control substances prior to testing, if applicable (e.g., warming, grinding)
- Stability, if known

Information Concerning the Sponsor and the Test Facility

- Name and address of the sponsor
- Name and address of the test facility
- Name and address of the Study Director

Justification of the Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time (e.g., periodic testing of proficiency substances, use of historical negative and positive control data)

Criteria for an Acceptable Test

- Acceptable concurrent negative control ranges based on historical data
- Acceptable concurrent positive control ranges based on historical data
- If applicable, acceptable concurrent benchmark control ranges based on historical data

Test Conditions

- Description of test system used
- Calibration information for measuring device used for measuring opacity and permeability (e.g., opacitometer and spectrophotometer)
- Supporting information for the bovine corneas used including statements regarding their quality
- Details of test procedure used
- Test concentration(s) used
- Description of any modifications of the test procedure
- Reference to historical data of the model (e.g., negative and positive controls, proficiency substances, benchmark substances)

- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples (e.g., opacity and $O.D._{490}$ values and calculated *in vitro* irritancy score for the test substance and the positive, negative, and benchmark controls, reported in tabular form, including data from replicate repeat experiments as appropriate, and means and ± the standard deviation for each experiment)

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement indicates all inspections made during the study, and the dates any results were reported to the Study Director. This statement also serves to confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

11.0 REFERENCES

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