APPENDIX G

ICCVAM RECOMMENDED HET-CAM TEST METHOD PROTOCOL

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ICCVAM Recommended Protocol for Future Studies Using the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) Test Method

PREFACE

The protocol was adapted from the protocol previously described by Spielmann and Liebsch (INVITTOX 1992). Examples of use of the protocol can be found in Luepke (1985), Balls et al. (1995), Gilleron et al. (1996, 1997), and Spielmann et al. (1996). Future studies using the HET-CAM test method could include further characterization of the usefulness or limitations of the HET-CAM test method 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 to describe the components and procedures used to evaluate the potential ocular irritancy of a test substance as measured by its ability to induce toxicity in the chorioallantoic membrane of a chicken. Effects are measured by the onset of: (1) hemorrhage; (2) coagulation; and (3) vessel lysis. These assessments are considered individually and then combined to derive a score, which is used to classify the irritancy level of the test substance.

The focus of this protocol is on the use of the HET-CAM test method for the detection of ocular corrosives and severe irritants, as defined by the U.S. Environmental Protection Agency (EPA 1996), the European Union (EU; EU 2001), and in the United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (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 HET-CAM test method have not yet been formally evaluated for the other classes of ocular irritancy defined by the EPA (1996), the EU (EU 2001), and the UN (2003).

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures with chicken eggs should follow the institution's applicable regulations and procedures for handling of human or animal materials, 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 Chicken Eggs

Fertile White Leghorn chicken eggs should be obtained from commercial sources. Fresh (not older than seven days), fertile, clean eggs weighing between 50 and 60 grams should be used. Eggs should be candled prior to use and nonviable or defective eggs should be discarded. Excessively misshapen eggs or eggs with cracked or thin shells should not be used. Transport of eggs should occur under conditions that will not affect embryo viability or development.

3.2 Equipment and Supplies

- Candling light
- Deionized/Distilled Water
- Dentist's rotating saw blade
- Incubator with an automatic rotating device
- Micropipette(s) and disposable tips appropriate for recommended volumes

- Mortar and pestle (or comparable grinding tools for test substances)
- Stop clock or electronic chronometer
- Standard general biological laboratory equipment and supplies (e.g., microcentrifuge tubes for measurement of substance volume), as needed
- Tapered forceps
- Volumetric flasks

3.3 Solutions

The manufacturer's recommendations should be followed with regard to storage temperature and shelf life of stock solutions. Solutions should be prepared volumetrically.

- 0.9% (w/v) sodium chloride (NaCl) in deionized/distilled water
- 1% (w/v) sodium dodecyl sulfate (SDS) in deionized/distilled water
- 0.1 N sodium hydroxide (NaOH) in deionized/distilled water

4.0 TEST SUBSTANCE PREPARATION

All test substances should be evaluated undiluted unless dilution is justified. If dilution is justified, then 0.9% NaCl or olive oil should be used as the diluent, depending on substance solubility. Use of a different solvent/vehicle should be justified. Dilutions should be prepared on the same day as the test.

Paste, particulate, or granular test substances or formulations should be evaluated without dilution. Solid test substances should be ground to a fine dust to obtain a volume of 0.3 mL after gentle compaction of the particulates in a measuring container (e.g., microcentrifuge tube).

5.0 CONTROLS

5.1 Negative Control

A 0.9% NaCl negative control should be included in each experiment in order to provide a baseline for the assay endpoints and to ensure that the assay conditions do not inappropriately result in an irritant response.

5.2 Solvent/Vehicle Control (if appropriate)

If the test substance is diluted in olive oil, then this solvent/vehicle should be included as a control substance in order to provide a baseline for the assay endpoints and to ensure that the assay conditions do not inappropriately result in an irritant response. If a solvent/vehicle other than 0.9% NaCl or olive oil is used, than both the solvent/vehicle and 0.9% NaCl should be included as controls to ensure that the alternative solvent/vehicle does not result in an irritant response.

5.3 Positive Control

A known ocular irritant should be included in each experiment to verify that an appropriate response is induced. If the HET-CAM assay is being used only to identify corrosive or severe irritants, then the positive control should be a substance (e.g., 1% SDS, NaOH) that induces a severe response *in vivo* as well as in HET-CAM. 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.

5.4 Benchmark Control (if appropriate)

Benchmark controls may be useful in demonstrating that the test method is functioning properly for detecting the ocular irritancy potential of chemicals of a specific chemical class or a specific range of responses, or for evaluating the relative irritancy potential of an ocular irritant. Appropriate benchmark controls 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 Treatment Groups

Use at least three eggs per group (negative and positive controls, test substance, and, if included, benchmark and solvent/vehicle controls). To the extent possible, eggs from the same hen should be randomized among treatment groups.

6.2 CAM Preparation

a. Select fresh (not older than 7 days), clean, fertile 50-60 g White Leghorn chicken eggs. Candle the eggs and discard any eggs that are nonviable or defective. Excessively misshapen eggs or eggs with cracked or thin shells should not be used. Shaking, unnecessary tilting, knocking, and all other mechanical irritation of the eggs should be avoided when preparing.
b. Place eggs in an incubator with a rotating tray. Incubate eggs at 38.3 ± 0.2°C and 58 ± 2% relative humidity when incubating in a still-air incubator or at 37.8 ± 0.3°C and 58 ± 2% relative humidity when incubating in a forced-air incubator. Hand rotate eggs five times per day until the day 8.
c. Candle the eggs on incubation day 8 and remove any nonviable or defective eggs. Eggs are returned to the incubator (without hand rotation) with the large end of the eggs upwards for an additional day.
d. Remove eggs from the incubator on day 9 for use in the assay. Candle eggs and discard any nonviable or defective eggs.

e. Mark the air cell of the egg. Cut the section marked as the air cell with a rotating dentist saw blade and then pare it off. Care should be taken when removing the eggshell to ensure that the inner membrane is not injured.
f. Moisten the inner membrane with 0.9% NaCl. A disposable glass pipette can be used to apply the solution. Place the egg into the incubator for a maximum of 30 minutes.
g. Remove the egg from the incubator, prior to its use in the assay, and decant the 0.9% NaCl solution. Carefully remove the inner membrane with forceps, ensuring that the inner membrane is not injured.

6.3 Treatment of Eggs with Test Substances

Depending on the physical form of the test substance, the following form-specific application protocols should be followed.

6.3.1 Liquid or Diluted Test Substances or Formulations
Apply 0.3 mL of liquid substances or diluted substances directly onto the CAM surface.

6.3.2 Solid, Particulate, or Granular Test Substances or Formulations
Apply 0.3 mL of solid, particulate, or granular substances (which have been ground to a fine dust) directly onto the CAM, ensuring that at least 50 % of the CAM surface area is covered. In cases where the total weight of the test substance at this volume is greater that 0.3 g, 0.3 g of the solid, particulate, or granular test substance should be used. In either case, the weight of the test substance should be recorded.

6.3.3 Paste Test Substances or Formulations
Apply 0.3 mL of paste substances or formulations directly onto the CAM, ensuring that at least 50% of the CAM surface area is covered. In cases where the total weight of the test substance at this volume is greater that 0.3 g, 0.3 g of the paste test substance should be used. In either case, the weight of the test substance should be recorded.

6.4 Observations

Observe the reactions on the CAM over a period of 300 seconds. The time for the appearance of each of the noted endpoints should be monitored and recorded, in seconds. Endpoints that should be observed are:

- hemorrhage (bleeding from the vessels)
- vascular lysis (blood vessel disintegration)
- coagulation (intra- and extra-vascular protein denaturation)

Collection of additional information and data may be useful in further analyses and conducting retrospective studies. To maximize the likelihood of obtaining reproducible results, reference photographs for all endpoints should be available.

7.0 EVALUATION OF TEST RESULTS

By collection of time to appearance of each of the noted endpoints, a variety of analysis methods may be used to assess irritancy potential of test substances. One analysis method that has been used extensively is an irritation score (IS). The formula used to generate an IS value is:

$$\left(\left(\frac{(301 - \textit{Hemorrhage time})}{300}\right)\times 5\right)+\left(\left(\frac{(301 - \textit{Lysis time})}{300}\right)\times 7\right)+\left(\left(\frac{(301 - \textit{Coagulation time})}{300}\right)\times 9\right)$$

Hemorrhage time = observed start (in seconds) of hemorrhage reactions on CAM
Lysis time = observed start (in seconds) of vessel lysis on CAM
Coagulation time = observed start (in seconds) of coagulation formation on CAM

8.0 CRITERIA FOR AN ACCEPTABLE TEST

A test is considered acceptable if the negative and positive controls each induce a response that falls within the classification of nonirritating and severely irritating, respectively. Historical control studies indicate that using 0.9% NaCl, as a negative control, the IS value was 0.0. Historical control studies indicate that using 1% SDS and 0.1 N NaOH, as positive controls, the IS values ranged between 10 and 19, respectively.

9.0 DATA INTERPRETATION

When using the IS analysis method, the severe irritancy classification for a test substance is used when the value is greater than nine.

10.0 STUDY REPORT

Information and data that should be included in study reports for the HET-CAM test method include, but are not limited to:

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)
- 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

- Experimental starting and completion dates
- 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., irritancy scores for the test substance and the various controls, including data from replicate repeat experiments as appropriate, and means and ± the standard deviation for each test)

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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