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Validation Status of the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) Test Method

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Abstract

Concerns about animal welfare and interest in higher throughput testing have led researchers to develop alternative *in vitro* test methods for the current rabbit eye irritation test. NICEATM evaluated four *in vitro* ocular test methods for their ability to identify substances that cause irreversible or severe irritation or corrosion. One of these test methods, HET-CAM, is a model that is proposed to mimic the mucosal tissues of the eye. The ability of HET-CAM to correctly identify ocular corrosives and severe irritants using available HET-CAM data that evaluated the time to appearance of endpoints and corresponding *in vivo* eye irritation data was evaluated according to current hazard classification schemes for the U.S. EPA (n=54), the European Union (n=86), and the UN Globally Harmonized System (n=52). Depending on the classification scheme used, HET-CAM had a false positive rate of 20-27%, and a false negative rate of 0-7%. Lack of published intra- and inter-laboratory data for this analysis method precluded an evaluation of test method reliability. 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 HET-CAM for the detection of ocular corrosives and severe irritants. HET-CAM 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 by identifying substances that are severe irritants/corrosives. ILS staff supported by NIEHS contract N01-ES 35504.

Introduction

The ocular irritation or corrosion potential of substances, to which humans may be exposed, has been evaluated since 1944 by the Draize rabbit eye test (Draize et al. 1944). In recent years, increased efforts have focused on the development of *in vitro* alternatives to this *in vivo* test. Although progress has been made, there is currently no validated and accepted nonanimal alternative test method for ocular irritancy in the U.S. Recently, the U.S. Environmental Protection Agency (EPA) formally nominated to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) four *in vitro* test methods for evaluation of their ability to identify potential ocular corrosives and severe irritants in a tiered testing strategy. Adequate validation[1] of a test method is a prerequisite for it to be considered for regulatory acceptance (ICCVAM 1997, 2003).

One of the four test methods nominated for review was the Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) test method. The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) worked with the ICCVAM Ocular Toxicity Working Group (OTWG) and with the European Centre for the Validation of Alternative Methods (ECVAM) to compile information and data to support the ICCVAM technical evaluation the test method.

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

- describe the current validation status of the HET-CAM 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 Harmonised System; GHS [UN 2003])[2]
- identify the usefulness and limitations of the HET-CAM test method, based on existing data, for identifying ocular corrosives and severe irritants in a regulatory tiered testing strategy
- propose a standardized HET-CAM test method protocol
- propose additional test method protocol optimization studies that might enhance the accuracy and/or reliability of the HET-CAM 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 HET-CAM test method, and other data and information submitted in response to a *Federal Register* (*FR*) request (*FR* Vol. 69, No. 57, pp. 13859-13861, March 24, 2004); available at http://iccvam.niehs.nih.gov/methods/eyeirrit.htm). The HET-CAM test method was reviewed by an independent Expert Panel on January 11-12, 2005 and their report, including conclusions and recommendations, will be available in mid-March at this website. After considering the Expert Panel's report and any public comments, ICCVAM will prepare final recommendations on the HET-CAM test method, which will be published in a report that is expected to be available in August 2005.

[1]Validation is the process by which the reliability and relevance of a test method are established for a specific purpose (ICCVAM 1997, 2003).

[2]In a regulatory tiered testing strategy, substances positive in a validated *in vitro* test method could be classified and labeled as ocular corrosives or severe irritants, while negative substances would undergo additional testing in the *in vivo* rabbit eye test or a validated alternative test method capable of detecting ocular corrosives and severe irritants that were false negatives in the validated *in vitro* test method.

Test Method Overview

The HET-CAM test method uses the chicken embryo chorioallantoic membrane (CAM), which is a vascular fetal membrane composed of the fused chorion and adjacent wall of the allantois. The HET-CAM test method was proposed by Luepke (1985) and Luepke and Kemper (1986). It was assumed that acute effects induced by a test substance on the CAM are similar to effects induced by the same substance in the eye of a treated rabbit.

The CAM is evaluated for the development of irritant endpoints (e.g., hyperemia, hemorrhage, and coagulation). Depending on the test method protocol used, several protocol variations have been noted (see **Table 1**). These variations include:

- relative humidity of eggs during incubation
- quanitity of test substance applied
- number of replicate eggs used
- use of concurrent positive and/or negative controls
- endpoints evaluated

In addition to these noted test method protocol variations, different analysis methods were identified (**Table 2**).

During a HET-CAM study, a test substance is applied to the CAM as a single dose. The test substance may or may not be rinsed from the CAM prior to evaluation of irritancy potential. Adverse effects on the CAM are measured up to 300 seconds after application of the test substance and damage to the CAM is assessed by visual inspection. Depending on the type of data compiled and the analysis method used, the response could be considered qualitative or semi-quantitative.

Each endpoint evaluated is used to develop an overall irritancy score that is used to assign an *in vitro* irritancy classification (**Table 3**).

Table 1. Protocol Variations Among HET-CAM Test Method Studies

Study	# Eggs			Inc. Temp/ Humidity	Quantity Tested	Rinsing	Endpoints Evaluated**	Method of Analysis
	Neg	Treat	Pos					
CEC (1991)	-	6	-	37.5/62.5%	0.3 mL or 0.1 g	20 secs after	H, L, C	IS(B)
Gettings et al. (1991)	-	-	-	-	-	-	H, VL, C	IS(B)
Bagley et al. (1992)	2	4	-	37.5/62.5%	0.3 mL or 0.1 g	20 secs after	HY, H, C	IS(A)
Gettings et al. (1994)	-	3	-	38/60%	0.3 mL	-	H, L, C	IS(A) IS(B)
Vinardell and Macián (1994)	2	6	2	-	0.3 mL	-	H, V, C	IS(B)
Balls et al. (1995)	-	-	-	-	-	3 mins after*	H, L, C	S-Score, Q-Score
Kojima et al. (1995)	-	4	-	37.6/~70%	0.2 mL	20 secs after	HY, H, C	IS(A)
Gettings et al. (1996)	-	- 3	-	- 38/60%	0.1 mL 0.3 mL	-	D, H, C H, L, C	IS(A) IS(B)
Spielmann et al. (1996)	-	3	-	-	-	5 mins after*	H, L, C	IS & ITC
Hagino et al. (1999)	-	4	-	37.6/~70%	0.2 mL or 0.2 g	20 secs after	HY, H, C	IS(A)

Inc. = incubation; Neg = negative control; Pos = positive control; Treat = treatment.
* For non-transparent substances only
** C = coagulation; D = dilation; H = hemorrhage; HY = hyperemia; L = lysis; VL = vascular lysis; V = vasoconstriction.

Table 2. HET-CAM Analysis Methods

Analysis Method	Description	Reference
IS(A)	- Irritation responses are evaluated at 0.5, 2, and 5 minutes - Time-dependent scores are assigned to each endpoint - Scores is calculated by adding assigned scores	Luepke (1985)
IS(B)	- Time of first appearance of endpoint is noted after application of test substance - Score is calculated by using empirically derived formula: $\left(\left(\frac{(301-\text{Hemorrhage time})}{300}\right)\times 5\right)+\left(\left(\frac{(301-\text{Lysis time})}{300}\right)\times 7\right)+\left(\left(\frac{(301-\text{Coagulation time})}{300}\right)\times 9\right)$ *Hemorrhage time* = time until appearance of blood hemorrhages *Lysis time* = time until appearance of vessel lysis *Coagulation time* = time until appearance of protein coagulation	Kalweit et al. (1987) Kalweit et al. (1990)
Q-Score	- Calculated as ratio of test substance irritation score to investigator determined reference standard irritation score	Balls et al. (1995)
S-Score	- Calculated as the highest total score for any endpoint evaluated	Balls et al. (1995)
IS and ITC	- Two different analysis methods used - IS value calculated as IS(A) or IS(B) - ITC defined as lowest concentration required to produce a slight response after application of test substance	Spielmann et al. (1996)

IS = irritation score; ITC = irritation threshold concentration.

Table 3. HET-CAM Decision Criteria for Classifying Ocular Corrosives and Severe Irritants

Analysis Method	Decision Criteria
IS(A)	IS(A) ≥ 9
IS(B)	IS(B) ≥ 9
Q-Score	Q-Score ≥ 2
S-Score	S-Score ≥ 15
IS and ITC	ITC ≤ 1%
	IS ≥ 16 and 1% < ITC ≤ 2.5%

IS = irritation score; ITC = irritation threshold concentration.

Test Method Database

A total of 246 substances in the following studies were used to evaluate the accuracy and interlaboratory reproducibility of the HET-CAM test method (**Table 4**):

- CEC (1991)
- Gettings et al. (1991)
- Bagley et al. (1992)
- Gettings et al. (1994)
- Vinardell and Macián (1994)
- Balls et al. (1995)
- Kojima et al. (1995)
- Gettings et al. (1996)
- Spielmann et al. (1996)
- Hagino et al. (1999)

Twenty different chemical classes (with three or more substances) were represented; alcohols, carboxylic acids, amines, and formulations were the most commonly represented. Fifteen different product classes were represented; cosmetics, solvents, hair shampoos, soaps/surfactants were the most commonly represented.

Detailed *in vivo* data, consisting of cornea, iris and conjunctiva scores for each animal at 24, 48, and 72 hours and/or assessment of the presence or absence of lesions at 7, 14, and 21 days was necessary to calculate the appropriate EPA (1996), European Union (EU 2001), and GHS (UN 2003) ocular irritancy hazard classification.

Much of the published *in vivo* rabbit eye test data on the substances used to evaluate the accuracy of HET-CAM for detecting ocular corrosives and severe irritants was limited to average score data or the reported irritancy classification. Thus, many of the test substances for which there was only limited *in vivo* data could not be used for evaluating test method accuracy and reliability for all three ocular irritancy classification systems.

Table 4. Primary HET-CAM Data Sources

Study	Accuracy (Severes/Total)			Intralab (Severes/Total)		Interlab (Severes/Total)	
	GHS	EPA	EU	CVs	GHS Classific.	CVs	GHS Classific.
CEC (1991)	-	-	11/32	-	-	14	-
Gettings et al. (1991)	3/9	3/9	2/9	-	-	-	-
Bagley et al. (1992)	0/3	0/3	0/3	-	-	-	-
Gettings et al. (1994)	1/18	1/18	1/18	-	-	-	-
Vinardell and Macián (1994)	0/2	0/2	0/2	-	-	-	-
Balls et al. (1995) (Q)	15/45	10/45	14/48	-	-	40	15/29
Balls et al. (1995) (S)	11/17	6/12	11/19	-	-	8	11/5
Kojima et al. (1995)	3/5	3/5	3/5	-	-	-	-
Gettings et al. (1996)	8/23	10/25	6/25	-	-	-	-
Spielmann et al. (1996)	-	-	45/118	-	-	-	-
Hagino et al. (1999)	8/16	6/14	7/17	-	-	8	8/8

classific. = classification; CV = coefficient of variation; EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = Globally Harmonized System; Intralab = intralaboratory; Interlab = interlaboratory

More information on ICCVAM and NICEATM can be accessed at:
http://iccvam.niehs.nih.gov/

ICCVAM
NICEATM

Test Method Accuracy Analysis

The ability of the HET-CAM test method to correctly identify ocular corrosives and severe irritants, as defined by the GHS, EPA, and EU classification systems (EPA 1996; EU 2001; UN 2003)[3], was evaluated. Accuracy statistics were calculated for each HET-CAM test method protocol by report 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

The overall HET-CAM test method accuracy, when compared to the GHS classification system, for four analysis methods ranged from 47% to 85%, while the false positive and false negative rates ranged from 20% to 57% and 0% to 64%, respectively (**Table 5**). The analysis method with the highest accuracy and the lowest false positive and false negative rates was the IS(B) analysis method (**Table 5**).

Table 6 provides the accuracy statistics for the IS(B) analysis method when compared to the GHS, EPA, and EU classification systems. **Table 6** also provides accuracy statistics for two sets of concentrations (10% and 100%) for substances evaluated in Spielmann et al. (1996). These results indicate that higher accuracy and lower false positive rates are achieved with a lower concentration of the test substance.

Among the limited dataset of substances evaluated for accuracy in predicting the GHS Category 1 classification, surfactant based formulations appear to be accurately predicted (7% [1/15] false positive rate; 0% [0/8] false negative rate)[4]. Only liquids or solutions were tested by the IS(B) analysis method (**Table 7**).

Limitations of the HET-CAM test method accuracy analysis.

- The impact of the differences in the test methods protocol on results is unknown
- Most of the substances evaluated using the IS(B) analysis method were:
  - Nonsevere ocular toxicants
  - Formulations
  - Tested as solutions or liquids
- Limited conclusions with respect to the accuracy of HET-CAM by chemical class or physicochemical property because of the limited database

[3]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).

[4]Current analyses do not include a chemical class or physical property evaluation of the substances evaluated in Spielmann et al. (1996). Additional analyses, which will include Spielmann et al. (1996) and other recently received data, are planned.

Table 5. HET-CAM Test Method GHS Accuracy For Four Analysis Methods

Analysis Method	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
IS(A)	75% (46/61)	67% (12/18)	79% (34/43)	21% (9/43)	33% (6/18)
IS(B)	**85% (44/52)**	**100% (12/12)**	**80% (32/40)**	**20% (8/40)**	**0% (0/12)**
Q-Score	62% (28/45)	100% (15/15)	43% (13/30)	57% (17/30)	0% (0/15)
S-Score	47% (8/17)	36% (4/11)	67% (4/6)	33% (2/6)	64% (7/11)

GHS = Globally Harmonized System; IS(A) = Analysis method described by Luepke (1985); IS(B) = Analysis method described by Kalweit et al. (1987, 1990); Q-Score = Analysis method described by Balls et al. (1995); S-Score = Analysis method described by Balls et al. (1995).

Table 6. HET-CAM Test Method Accuracy For IS(B) Analysis Methods

Statistic	GHS (n=52)	EPA (n=54)	EU (n=86)*	EU-10% (n=112)**	EU-100% (n=108)**
Accuracy	85% (44/52)	83% (45/54)	73% (63/86)	68% (76/112)	57% (62/108)
Sensitivity	100% (12/12)	93% (13/14)	95% (19/20)	80% (32/40)	88% (35/40)
Specificity	80% (32/40)	80% (32/40)	67% (44/66)	61% (44/72)	40% (27/68)
False Positive Rate	20% (8/40)	20% (8/40)	33% (22/66)	39% (28/72)	60% (41/68)
False Negative Rate	0% (0/12)	7% (1/14)	5% (1/20)	20% (8/40)	12% (5/40)

IS(B) = Analysis method described by Kalweit et al. (1987, 1990)
EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = Globally Harmonized System.
* Additional 32 chemicals available for EU analysis only (individual animal data not available for GHS or EPA classification).
** Data analyzed from Spielmann et al. (1996) at 10% concentration (EU-10%) and 100% concentration (EU-100%).

Table 7. HET-CAM Test Method Accuracy (when compared to the GHS classification system) According to Chemical Class and Physical Property

Class	Number of Substances			False Positive Rate		False Negative Rate	
	Total	Cat 1	Cat 2A, Cat 2B, NI	%	n	%	n
Overall	52	12	40	20	8/40	0	0/12
Formulations	50	12	38	18	7/38	0	0/12
- Hydroalcoholic formulation	9	3	6	33	2/6	0	0/3
- Oil/Water emulsion	18	1	17	24	4/17	0	0/1
- Surfactant-based formulation	23	8	15	7	1/15	0	0/8
Surfactant	2	0	2	50	1/2	-	-
Liquids	52	12	40	20	8/40	0	0/12

Cat = Category; GHS = Globally Harmonized System; NI = nonirritant.

Test Method Reliability Analysis[5]

Intralaboratory Repeatability and Reproducibility

Due to the lack of available HET-CAM test data, analyses of intralaboratory repeatability and reproducibility were not conducted.

Interlaboratory Reproducibility

Assessment of interlaboratory reproducibility of the IS(B) analysis method was conducted using data from a single study (CEC 1991) in which 32 substances were tested among three to five different laboratories.

Two separate analyses were conducted:

- *Qualitative analysis*: Extent of agreement between testing laboratories when identifying ocular corrosives and severe irritants was compared
- *Quantitative analysis*: Evaluated using a coefficient of variation (CV) calculation to compare variability in IS(B) values

Qualitative Interlaboratory Reproducibility

All the laboratories were in 100% agreement in regard to the ocular irritancy classification (EU corrosive/severe irritant or EU nonsevere irritant/nonirritant) of 15 (47%) of the 32 substances tested **(Table 8)**. All laboratories were in at least 60% agreement for 29 (91%) of the 32 substances tested.

When only severe irritants (based on *in vivo* EU classification) were considered, the laboratories were in 100% agreement for 7 (70%) of the 10 substances tested. All the laboratories were in at least 60% agreement for 10 (100%) of the 10 substances tested (**Table 8**).

Quantitative Interlaboratory Reproducibility

When quantitative HET-CAM test data from substances tested in five laboratories were considered, median and mean CV values were approximately 34% (**Table 9**).

[5]Current analyses do not include an evaluation of the substances evaluated in Spielmann et al. (1996). Additional analyses, which will include Spielmann et al. (1996) and other recently received data, are planned.

Table 8. Qualitative Analysis of HET-CAM IS(B) Analysis Method Interlaboratory Reproducibility: Extent of Agreement Between Laboratories

% Interlaboratory Agreement	EU (3-5 labs, 32 substances)	
	%	n
100% (all)	47	15/32
≥60% (all)	91	29/32
100% (severes)	70	7/10
≥60%(severes)	100	10/10

EU = European Union.

Table 9. Quantitative Analysis of HET-CAM IS(B) Test Method Interlaboratory Reproducibility: Coefficient of Variation

Coefficient of Variation Analysis	CEC (1991)
Mean (all substances)	34.1 (n=14)
Median (all substances)	33.1 (n=14)
Range (all substances)	6.6-74.9 (n=14)

Draft BRD Proposals

Based on this evaluation of test method performance, a HET-CAM test method protocol that evaluates development of hemorrhage, lysis, and coagulation is proposed. A proposed standardized protocol, based on the method of Spielmann and Liebsch (INVITTOX 1992), was provided in the draft BRD[6]. The proposed test method protocol proposes to use:

- the IS(B) analysis method (Kalweit et al. 1987, 1990)
- concurrent positive and negative controls

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

- retrospective analysis of decision criteria used to classify substances as corrosives and severe irritants
- evaluation of additional endpoints (e.g., trypan blue) for potential inclusion in determining irritancy potential

Once these studies have been completed, additional validation studies will be necessary using substances from the proposed list of reference substances to further characterize the accuracy and reliability of the optimized method.

[6]See HET-CAM BRD (http://iccvam.niehs.nih.gov/methods/ocudocs/ocu_brd.htm#hetcam)

Proposed Reference Substances For Optimization and Validation Studies

A common list of reference substances proposed for future optimization and validation studies of HET-CAM and other alternative test methods intended to detect ocular corrosives/severe irritants has been developed[7]. 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 might be 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 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.

[7]See HET-CAM BRD (http://iccvam.niehs.nih.gov/methods/ocudocs/ocu_brd.htm#hetcam)

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Acknowledgments

We would like to especially thank the generous contributions of the companies and individuals who provided data for this review of the HET-CAM test method and comments on the draft HET-CAM BRD:

- Cosmetics, Toiletry, and Fragrance Association (Dr. Carol Eisenmann)
- ECVAM (Dr. Chantra Eskes)
- National Institute of Health Sciences (Dr. Yasuo Ohno)
- U.S. Food and Drug Administration (Ms. Donnie Lowther)
- Johnson & Johnson (Dr. Philippe Vanparys)
- ZEBET (Dr. med Horst Spielmann and Dr. Manfred Liebsch)

U.S. Department of Health and Human Services
National Institutes of Health
National Institute of Environmental Health Sciences