Performance of the Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) Test Method For Detecting Ocular Corrosives and Severe Irritants

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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). There have been widespread efforts to develop and validate *in vitro* alternatives that might reduce or replace the use of animals for ocular irritancy assessments. The U.S. EPA[1] formally nominated to ICCVAM[1] four *in vitro* test methods, including the HET-CAM[1] assay, for evaluation of their ability to identify ocular corrosives and severe irritants in a tiered testing strategy.

NICEATM[1], in conjunction with the ICCVAM Ocular Toxicity Working Group, prepared a comprehensive BRD[1] reviewing the available data and information on the HET-CAM test method[2]. NICEATM released the draft HET-CAM BRD for public comment on November 1, 2004. On January 11-12, 2005, ICCVAM convened an Expert Panel to independently evaluate the validation status of HET-CAM and three other *in vitro* test methods for identifying ocular corrosives or severe irritants[3]. Public comments at that meeting indicated that additional data was available. The Expert Panel recommended that any additional data that could be obtained should be considered for a reanalysis of the accuracy and reliability of each test method.

In response to the Expert Panel recommendation, an *FR*[1] notice requesting the submission of all available *in vitro* HET-CAM test data and corresponding *in vivo* rabbit eye test data was reissued on February 28, 2005 (*FR* Vol. 70, No. 38, pp. 9661-9662). In addition to considering any HET-CAM data received in response to the *FR* notice, the reanalysis of the accuracy and reliability of this test method took into account (1) changes that occurred in the ocular irritancy classification of a few substances in response to clarification of the EU[1] (2001) and UN GHS[1] (UN 2003) ocular irritation classification rules, (2) a decision to use classifications based on *in vivo* rabbit eye test data only, and (3) revised chemical class assignments for some substances. Additional information on the reanalysis can be obtained at http://iccvam.niehs.nih.gov/methods/ocudocs/reanalysis.htm.

[1]BRD = Background Review Document; *FR* = Federal Register; GHS = Globally Harmonized System; HET-CAM = Hen's Egg Test – Chorioallantoic Membrane; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; UN = United Nations; U.S. EPA = U.S. Environmental Protection Agency.

[2]The draft HET-CAM BRD can be obtained at http://iccvam.niehs.nih.gov/methods/ocudocs//ocu brd.htm

[3]The Expert Panel Report can be obtained at http://iccvam.niehs.nih.gov/methods/ocudocs/EPreport/ocureport.htm

Test Method Overview

During a HET-CAM study, a test substance is applied to the chorioallantoic membrane (CAM) as a single dose. Adverse effects on the CAM are measured up to 300 sec after application of the test substance and damage to the CAM is assessed by visual inspection. Each endpoint (e.g., hyperemia, hemorrhage, and coagulation) evaluated is used to develop an overall irritancy score that is used to assign an *in vitro* irritancy classification.

Test Method Database

The following studies were used for this reanalysis:

- 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)
- Gilleron et al. (1996)
- Spielmann et al. (1996)
- Gilleron et al. (1997)
- Hagino et al. (1999)

These studies included a number of variations in test method protocol (e.g., relative humidity of eggs during incubation, endpoints evaluated) and methods of data analysis (i.e., IS(A), IS(B), Q-Score, S-Score, mtc10, and IS & ITC[4]). Due to these variations, not all studies were suitable for the accuracy and reliability analyses reported here.

[4]Analysis methods: **IS(A):** Irritation responses are evaluated at 0.5, 2, and 5 minutes and time-dependent scores are assigned to each endpoint. The total score is calculated by adding assigned scores. **IS(B):** Time of first appearance of endpoint is noted after application of test substance. Total score is calculated by using empirically derived formula. **Q-Score:** Calculated as ratio of test substance irritation score to investigator determined reference standard irritation score. **S-Score:** Calculated as the highest total score for any endpoint evaluated. **mtc10:** Mean detection time for appearance of coagulation endpoint when using a 10% solution. **IS &ITC:** Two different analysis methods used. IS value calculated as IS(A) or IS(B) (described above). ITC defined as lowest concentration required to produce a slight response after application of test substance.

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Test Method Accuracy Analysis

HET-CAM test method accuracy for the various data analysis methods, when compared to *in vivo* classifications using the GHS classification system (UN 2003), are provided in **Table 1**. The analysis methods with the largest database are the IS(B) analysis method when the *in vitro* concentration tested is 10% (IS(B)-10 method) or 100% (IS(B)-100 method). **Table 2** indicates that higher accuracy and lower false positive rates are achieved with a lower concentration of the test substance (i.e., the IS(B)-10 analysis method has a higher accuracy rate than the IS(B)-100 analysis method).

The small number of substances representing most chemical classes allows for only limited conclusions with respect to the accuracy of the HET-CAM IS(B)-10 and IS(B)-100 test methods by chemical class or property of interest (e.g., solids vs. liquids, basic vs. acidic pH, surfactants)(**Table 3**). However, among classes with at least six substances for analysis, alcohols, ethers, heterocycles, and organic salts appear to be overpredicted in HET-CAM IS(B)-10 while alcohols, amines, esters, heterocycles, ketones and organic salts appear to be overpredicted in HET-CAM IS(B)-100. For IS(B)-100, formulations appear to be underpredicted. With regard to properties of interest, surfactant-based formulations appear to be underpredicted by IS(B)-10. The underprediction rate was independent of whether substances were classified *in vivo* as ocular corrosives/severe irritants based on ocular lesion severity or lesion persistence (data not shown).

Table 1. HET-CAM Test Method Accuracy of Four Analysis Methods for Predicting GHS Classification

Analysis Method	*In Vitro* Conc. Tested	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
IS(A)	100%	85% (17/20)	100% (2/2)	83% (15/18)	17% (3/18)	0% (0/2)
	10%	50% (12/24)	25% (4/12)	100% (8/8)	0% (0/8)	75% (12/16)
IS(B)	100%	53% (76/143)	85% (35/41)	40% (41/102)	60% (61/102)	15% (6/35)
	10%	68% (69/101)	70% (28/40)	67% (41/61)	33% (20/41)	30% (12/40)
Q-Score		63% (27/43)	100% (12/12)	43% (12/28)	57% (16/28)	0% (0/12)
S-Score		44% (7/16)	36% (4/11)	60% (3/5)	40% (2/5)	64% (7/11)

Abbreviations: Conc. = concentration; GHS = Globally Harmonized System.
IS(A) = Luepke (1985); IS(B) = Kalweit et al. (1987, 1990); Q-Score = Balls et al. (1995); S-Score = Balls et al. (1995).

Table 2. HET-CAM Test Method Accuracy for IS(B)-10 and IS(B)-100 Analysis Methods

Statistic	IS(B)-10 Analysis Method			IS(B)-100 Analysis Method		
	GHS (n=101)	EPA (n=98)	EU (n=95)	GHS (n=143)	EPA (n=138)	EU (n=178)
Accuracy	68% (69/101)	65% (64/98)	67% (64/95)	53% (76/143)	51% (70/138)	54% (96/178)
Sensitivity	70% (28/40)	68% (21/31)	70% (23/55)	85% (35/41)	87% (26/30)	89% (31/35)
Specificity	67% (41/61)	64% (43/67)	66% (41/62)	40% (41/102)	41% (44/108)	45% (65/143)
False Positive Rate	33% (20/41)	36% (24/67)	34% (21/61)	60% (61/102)	59% (64/108)	55% (78/143)
False Negative Rate	30% (12/40)	32% (10/31)	30% (10/33)	15% (6/35)	13% (4/30)	11% (4/35)

Abbreviations: EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = Globally Harmonized System.
IS(B) = Kalweit et al. (1987, 1990)

Table 3. False Negative and False Positive Rates of the HET-CAM Test Method, by Chemical Class and Properties of Interest, for the GHS[1] Classification System

Category	N[2]	False Positive Rate[3]	False Negative Rate[3]
Chemical Class-IS(B)-10			
Overall	101	33% (20/61)	30% (12/40)
Alcohols	17	90% (9/10)	25% (2/7)
Amines	7	60% (3/5)	50% (1/2)
Ethers	14	50% (5/10)	50% (2/4)
Heterocycles	6	83% (5/6)	-
Organic salts	7	57% (4/7)	-
Chemical Class-IS(B)-100			
Overall	143	60% (61/102)	15% (6/41)
Alcohols	20	91% (10/11)	11% (1/9)
Aldehydes	6	80% (4/5)	0% (0/1)
Amines	10	83% (5/6)	50% (2/4)
Esters	14	83% (10/12)	0% (0/2)
Ethers	20	60% (9/15)	20% (1/5)
Formulations	51	19% (6/31)	35% (7/13)
Heterocycles	10	75% (6/8)	-
Ketones	6	67% (4/6)	-
Onium compounds	7	100% (2/2)	0% (0/5)
Organic salts	8	88% (7/8)	-
Properties of Interest			
IS(B)-10 Physical Form:			
Liquid	101	33% (20/61)	30% (12/40)
Solid	-		-
IS(B)-100 Physical Form:			
Liquid	63	67% (36/54)	0% (0/9)
Solid	43	67% (16/24)	26% (5/19)
Unknown	37	38% (9/24)	8% (1/13)
IS(B)-10 Surfactant-Based Formulations	24	0% (0/8)	44% (7/16)

[1]GHS = Globally Harmonized System (UN 2003).
[2]N = number of substances.
[3]False Positive Rate = the proportion of all negative substances that are falsely identified as positive *in vitro;* False Negative Rate = the proportion of all positive substances that are falsely identified as negative *in vitro.* The data used to calculate the percentage are provided in parenthesis.
Highlighted cells indicate those chemical classes and properties of interest where the rate of misclassification is (a) greater than the overall rate, (b) is based on a sufficient number of substances (N ≥ 6 substances), and (c) would be expected to have an appreciable effect on the overall rate, if excluded from the database when conducting an accuracy analysis.

Test Method Reliability Analysis

***Intralaboratory Repeatability*[5]**

In both studies evaluated (**Table 4**), the hemorrhage endpoint had a high percent coefficient of variation (%CV) value (104%-118%) and the coagulation endpoint had the lowest %CV of the three endpoints evaluated in the HET-CAM test method.

***Intralaboratory Reproducibility*[5]**

Similar to the results for the intralaboratory repeatability analysis, the %CV values were highest for the hemorrhage endpoint and were the lowest for the coagulation endpoint (**Table 5**).

***Interlaboratory Reproducibility*[5]**

Two interlaboratory reproducibility 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 %CV calculation to compare variability in IS(B) values

Qualitative Analysis

All the laboratories were in 100% agreement for the ocular irritancy classification (GHS corrosive/severe irritant or GHS nonsevere irritant/nonirritant) for 79% (85/107) of substances tested using the IS(B)-10 analysis method and for 82% (81/99) of substances tested using the IS(B)-100 analysis method (data not shown).

Quantitative Analysis

The evaluation of interlaboratory reproducibility is shown in **Table 6**. Mean %CV values ranged from 34.63% to 60.17%, while median %CV values ranged from 26.22% to 42.65%.

[5]Intralaboratory Repeatability=The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period. Intralaboratory Reproducibility=A determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times. Interlaboratory Reproducibility=A measure of whether different qualified laboratories using the same protocol and test substances can produce qualitatively and quantitatively similar results.

Table 4. Intralaboratory Repeatability Evaluation for Substances Tested Using the IS(B) Analysis Method

Study		Hemorrhage	Lysis	Coagulation	Overall IS(B) Score
Gilleron et al. (1996)	Mean (SD) for All Substances[1]	1.64 (1.93)	2.68 (2.88)	3.59 (3.44)	7.92 (5.84)
	%CV for All Substances[2]	**117.56**	**107.52**	**95.69**	**73.74**
	Mean (SD) Excluding Nine Substances[1,3]	1.63 (1.90)	1.87 (2.57)	2.83 (3.25)	6.33 (5.43)
	%CV Excluding Nine Substances[2,3]	**116.13**	**137.49**	**115.07**	**85.84**
Gilleron et al. (1997)	Mean (SD) for All Substances[1]	1.94 (2.12)	5.60 (2.31)	6.42 (2.68)	13.96 (4.89)
	%CV for All Substances[2]	**109.10**	**41.24**	**41.78**	**34.99**
	Mean (SD) Excluding Four Substances[1,3]	2.07 (2.16)	5.75 (2.19)	6.60 (2.49)	14.42 (4.48)
	%CV Excluding Four Substances[2,3]	**104.43**	**38.04**	**37.78**	**31.05**

Abbreviations: CV = coefficient of variation, SD = standard deviation.
[1]Mean calculated using the values from the mean of 3 eggs tested for each substance for each endpoint and the Overall IS(B) Score. SD was based on the values in these individual columns.
[2]To avoid eliminating data for which the %CV could not be calculated (i.e., where the mean and SD both equaled 0), the %CV values were calculated using the mean and SD calculated as described in footnote 1.
[3]For some compounds (nine compounds in Gilleron et al. (1996) and four compounds in Gilleron et al. (1997)) the data used in the publication could not be traced in detail by the authors. Therefore, substitute test data for these substances were provided. The results provided exclude these substances.

Table 5. Intralaboratory Reproducibility Evaluation for Substances Tested Using the IS(B) Analysis Method

Study		Hemorrhage	Lysis	Coagulation	Overall IS(B) Score
Gilleron et al. (1996)	Mean (SD) for All Substances[1]	1.60 (1.70)	2.51 (2.28)	3.40 (2.89)	7.51 (5.28)
	%CV for All Substances[2]	**106.43**	**91.00**	**84.89**	**70.35**
	Mean (SD) Excluding Nine Substances[1,3]	1.63 (1.71)	1.87 (1.98)	2.83 (2.73)	6.33 (5.06)
	%CV Excluding Nine Substances[2,3]	**104.49**	**106.22**	**96.63**	**79.92**
Gilleron et al. (1997)	Mean (SD) for All Substances[1]	197 (2.04)	5.64 (2.14)	6.46 (2.44)	14.07 (4.62)
	%CV for All Substances[2]	**103.34**	**37.92**	**37.80**	**32.86**
	Mean (SD) Excluding Four Substances[1,3]	2.07 (2.07)	5.75 (2.06)	6.60 (2.28)	14.42 (4.31)
	%CV Excluding Four Substances[2,3]	**100.01**	**35.00**	**34.54**	**29.87**

Abbreviations: CV = coefficient of variation, SD = standard deviation.
[1]Mean was calculated using the values from the mean of 3 eggs tested for each substance for each endpoint and the Overall IS(B) Score. The SD was calculated based on the values in these individual columns.
[2]To avoid eliminating data for which the %CV could not be calculated (i.e., where the mean and SD both equaled 0), the %CV values were calculated using the mean and SD calculated as described in footnote 1.
[3]For some compounds (nine compounds in Gilleron et al. (1996) and four compounds in Gilleron et al. (1997)) the data used in the publication could not be traced in detail by the authors. Therefore, substitute test data for these substances were provided. The results provided exclude these substances.

Table 6. HET-CAM IS(B) Test Method Interlaboratory Reproducibility: Coefficient of Variation

Coefficient of Variation Analysis	CEC (1991) IS(B) (n=12)	Spielmann et al. (1996) IS(B)-10 (n = 103)	Spielmann et al. (1996) IS(B)-100 (n = 95)
Mean (all substances)	34.63	60.17	35.21
Median (all substances)	33.05	42.65	26.22
Range (all substances)	6.6-74.9	0-141.42	0-141.42
Mean (only substances tested in 2 laboratories)	NA	58.07	34.62
Median (only substances tested in 2 laboratories)	NA	31.85	21.57
Range (only substances tested in 2 laboratories)	NA	0-141.42	0-141.42

Abbreviation: NA = not applicable.

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