5.0   THE HET-CAM TEST METHOD

5.1   HET-CAM Technical Summary

The following technical summary provides a synopsis of the performance analysis described in the HET-CAM BRD, which reviewed the available data and information for the test method.[16] The BRD describes the current validation status of the HET-CAM test method, including what is known about its reliability and accuracy, the scope of the substances tested, and a standardized protocol. The BRD may be obtained from the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov/).

5.1.1   Test Method Description
The HET-CAM test method uses the chorioallantoic membrane (CAM), which is a vascular fetal membrane, composed of the fused chorion and allantois. It is assumed that acute effects induced by a test substance on the small blood vessels and proteins of this soft tissue membrane are similar to effects induced by the same test substance in the eye of a treated rabbit. The CAM has been proposed as a model for a living membrane (such as the conjunctiva) since it comprises a functional vasculature. Additionally, evaluation of coagulation (i.e., protein denaturation) may reflect corneal damage that may be produced by the test substance. The CAM is evaluated for the development of irritant endpoints (hyperemia, hemorrhage, and coagulation). Depending on the method used to collect data on the endpoints (e.g., time to development, severity of observed effect) qualitative assessments of the irritation potential of test substances are made.

The HET-CAM test method protocols used in the various studies evaluated are similar, but not identical. Examples of some of the test method components that differed among the HET-CAM protocols used to generate data include:
- relative humidity during egg incubation ranged from 52.5% to 62.5%,
- volume or quantity of the test substance applied to the CAM (when reported) was either 0.1 mL or 0.3 mL for liquids and 0.3 g for solids,
- number of replicate eggs per test substance ranged from three to six, and
- some studies included concurrent positive control substances, while others did not.

5.1.2   Validation Database
There were several HET-CAM analysis methods used by the various studies.[17] For the Irritation Score (IS)(A)[18] and IS(B)[19] analysis methods, data were available to conduct additional sub-analyses (ICCVAM 2006d). For these sub-analyses, substances tested at a 10% concentration or 100% concentration *in vitro* were compared to responses observed at a 100% concentration tested *in vivo* (e.g., IS(A)-10, IS(B)-10, IS(B)-100).

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[16]Comparison of the performance analysis for HET-CAM to the other three *in vitro* test methods evaluated can be reviewed in **Section 6.0** and **Appendix B**.
[17]For additional information on this evaluation, please see the HET-CAM BRD (http://iccvam.niehs.nih.gov/methods/ocudocs/ocu_brd.htm#hetcam).
[18]Analysis method described in Luepke (1985).
[19]Analysis method described in Kalweit et al. (1987).

A total of 24 and 20 substances were evaluated for the IS(A)-10 and IS(A)-100 analysis methods, respectively, using the decision criteria of Luepke (1985). For the IS(B)-10 and IS(B)-100 analysis methods, using the decision criteria of Luepke (1985), 101 and 138 substances were evaluated, respectively. The chemical classes tested included, but were not limited to, alcohols, amines, esters, ethers, formulations, heterocyclic compounds, inorganic salts, ketones, and organic salts. The product classes tested included, but were not limited to, cosmetics, solvents, shampoos, flavor ingredients, and pharmaceutical synthetics.

5.1.3   Test Method Accuracy

For the IS(A) analysis method, accuracy increased when substances were evaluated *in vitro* at 100% concentration (IS(A)-100) compared to the 10% concentration (IS(A)-10) and where *in vivo* data were classified according to the EPA (1996), EU (2001), and GHS (UN 2003) classification systems. The opposite pattern was observed for the IS(B) analysis method; test method accuracy increased when substances were evaluated *in vitro* at 10% concentration (IS(B)-10) compared to the 100% concentration (IS(B)-100) and where *in vivo* data were classified according to the EPA (1996), EU (2001), and GHS (UN 2003) classification systems.

Chemical classes that were overpredicted by the HET-CAM IS(B) analysis methods, when testing substances at either a 10% or at 100% concentration, include alcohols (IS(B)-10: 89% [8/9]; IS(B)-100: 88% [14/16]), ethers (IS(B)-10: 50% [5/10]; IS(B)-100: 50% [6/12]), amines (IS(B)-10: 60% [3/5]; IS(B)-100: 83% [5/6]), organic salts (IS(B)-10: 57% [4/7]; IS(B)-100: 86% [6/7]), and heterocyclic compounds (IS(B)-10: 86% [6/7]; IS(B)-100: 78% [7/9]). Formulations appeared to have the lowest false positive rates for both IS(B)-10 and IS(B)-100 (**Table 5-1**). Chemical classes that were underpredicted by both analysis methods were amines and ethers.

An evaluation based on the physical form of the test substance *in vivo* depended on the analysis method being evaluated. For the IS(B)-100 analysis method, substances tested as solids *in vivo* had a false positive rate of 67% (16/24) and substances tested as liquids *in vivo* had a false positive rate of 65% (33/51) (**Table 5-1**). For the IS(B)-100 analysis method, substances tested as liquids *in vivo* had a false negative rate of 0% (0/9) and substances tested as solids *in vivo* had a false negative rate of 24% (4/17). For the IS(B)-10 analysis method, liquids had a false positive rate of 19% (3/16) and false negative rate of 37% (7/19) while solids had false positive and false negative rates of 58% (11/19) and 13% (1/8), respectively.

An analysis of the ability of the HET-CAM test method to identify ocular corrosives and severe irritants, depending on the nature of the *in vivo* ocular lesions (i.e., severity and/or persistence) responsible for classification of a substance as an ocular corrosive/severe irritant, indicated that, for IS(B)-10, the underpredicted substances were more likely to be substances classified as corrosive or severely irritating *in vivo* based on persistent lesions, with a false negative rate of 37% (10/27) compared to 15% (2/13) for substances classified as corrosive or severely irritating *in vivo* based on severity. For the IS(B)-100 analysis method, the underpredicted substances were more likely to be substances classified as corrosive or severely irritating *in vivo* based on severe lesions, with a false negative rate of 11% (2/19)

Table 5-1   False Positive and False Negative Rates of the HET-CAM Test Method, by Chemical Class and Properties of Interest, for the GHS Classification System

Category	N[1]	False Positive Rate[2]		False Negative Rate[3]	
		%	No.[4]	%	No.
Overall IS(B)-10 (Entire database)	101	33	20/61	30	12/40
Overall IS(B)-100 (Entire database)	138	59	58/99	13	5/39
Chemical Class-IS(B)-10[5]					
Alcohol	16	89	8/9	25	2/7
Aldehyde	5	0	0/4	100	1/1
Amine	7	60	3/5	50	1/2
Ether	14	50	5/10	50	2/4
Formulation	24	0	0/8	44	7/16
Heterocyclic Compound	7	86	6/7	-	0/0
Organic salt	7	57	4/7	-	0/0
Chemical Class-IS(B)-100[5]					
Alcohol	24	88	14/16	13	1/8
Aldehyde	6	80	4/5	0	0/1
Amine	9	83	5/6	33	1/3
Carboxylic acid/Carboxylic acid salt	11	60	3/5	17	1/6
Ester	12	90	9/10	0	0/2
Ether	16	50	6/12	25	1/4
Formulation	27	26	6/23	0	0/4
Heterocyclic Compound	12	78	7/9	33	1/3
Inorganic salt	5	100	2/2	0	0/3
Ketone	6	67	4/6	-	0/0
Organic salt	9	86	6/7	0	0/2
Properties of Interest					
Physical Form: IS(B)-10 **Liquids/Solutions** **Solids** **Unknown**	35 27 39	19 58 23	3/16 11/19 6/26	37 13 31	7/19 1/8 4/13
Physical Form: IS(B)-100 **Liquids** **Solids** **Unknown**	60 41 37	65 67 38	33/51 16/24 9/24	0 24 8	0/9 4/17 1/13
Surfactant – Total IS(B)-100 **-nonionic** **-anionic** **-cationic**	2 2 0 0	50 50 - -	1/2 1/2 - -	- - - -	0/0 0/0 - -
Surfactant-Based Formulation – IS(B)-10	24	0	0/8	44	7/16
pH – IS(B)-10[6] **- acidic (pH < 7.0)**	35	58	11/19	13	2/16

Category	N[1]	False Positive Rate[2]		False Negative Rate[3]	
		%	No.[4]	%	No.
- basic (pH > 7.0)	24	50	7/14	20	2/10
	11	80	4/5	0	0/6
pH – IS(B)-100[6]	35	68	13/19	13	2/16
- acidic (pH < 7.0)	23	69	9/13	10	1/10
- basic (pH > 7.0)	12	67	4/6	17	1/6
Category 1 Subgroup-IS(B)-10[7]					
- Total	40	-	-	30	12/40
- 4 (CO=4 at any time)	13	-	-	15	2/13
- 3 (severity/persistence)	0	-	-	-	-
- 2 (severity)	0	-	-	-	-
- 2-4 combined[8]	13	-	-	15	2/13
- 1 (persistence)	27	-	-	37	10/27
Category 1 Subgroup-IS(B)-100[7]					
- Total	38[9]	-	-	11	4/38
- 4 (CO=4 at any time)	19	-	-	11	2/19
- 3 (severity/persistence)	1	-	-	100	1/1
- 2 (severity)	2	-	-	0	0/2
- 2-4 combined[8]	22	-	-	14	3/22
- 1 (persistence)	16	-	-	6	1/16

Abbreviations: CO = corneal opacity; GHS = Globally Harmonized System (UN 2003); HET-CAM = Hen's Egg Test – Chorioallantoic Membrane.
[1]N=number of substances.
[2]False Positive Rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.
[3]False Negative Rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.
[4]Data used to calculate percentages.
[5]Chemical classes included in this table are represented by at least five substances tested in the HET-CAM test method and assignments are based on the MeSH categories (www.nlm.nih.gov/mesh).
[6]Total number of GHS Category 1 substances for which pH information was obtained.
[7]NICEATM-defined subgroups assigned based on the lesions that drove classification of a GHS Category 1 substance. 1: based on lesions that are persistent; 2: based on lesions that are severe (not including CO=4); 3: based on lesions that are severe (not including CO=4) and persistent; 4: CO = 4 at any time.
[8]Subcategories 2 to 4 combined to allow for a direct comparison of GHS Category 1 substances classified *in vivo* based on some lesion severity component and those classified based on persistent lesions alone.
[9]The number of substances evaluated in the Category 1 subgroup analysis may be less than the number of *in vivo* Category 1 substances evaluated, since some substances could not be classified into the subgroups used in the evaluation.

compared to 6% (1/16) for substances classified as corrosive or severely irritating *in vivo* based on persistence. However, two substances that were classified based on severe lesions (i.e., CO=4) were underpredicted by the HET-CAM IS(B)-10 and IS(B)-100 analysis methods.

The performance statistics for the EPA and EU classification systems are similar to those discussed for the GHS classification system. Additional information on the performance characteristics of the HET-CAM test method for the EPA and EU classification systems can be obtained from **Section 6.0**, **Appendix B**, and the HET-CAM BRD.

5.1.4　　Test Method Reliability (Inter- and Intra-Laboratory Reproducibility)
The analysis of intralaboratory repeatability was evaluated using data from two different publications for the IS(B) analysis method. In both studies, the hemorrhage endpoint had the

highest CV value (109.10%-117.56%). The CV values for the coagulation endpoint ranged from 41.78% to 95.69%. The difference in the numbers may be due to several factors including test substances evaluated and differences in the test method protocols used between the two studies. The calculated variability for the endpoints and the overall test method may be exaggerated because of the relatively small dynamic ranges for each of the endpoints (0.02 to 5 for hemorrhage, 0.02 to 7 for lysis, and 0.03 to 9 for coagulation). Similar results were obtained from the analysis of intralaboratory reproducibility.

A qualitative analysis of interlaboratory reliability also was conducted. For the IS(B)-10 analysis method, the participating laboratories were in 100% agreement for 84 to 85 (79% to 81%) of 104 to 107 substances evaluated, when compared to all three hazard classification systems. For the IS(B)-100 analysis method, the participating laboratories in a study were in 100% agreement for 80 to 81 (82% to 84%) of the 95 to 99 substances evaluated, when compared to all three hazard classification systems. There was 100% agreement with regard to the ocular irritancy classification for 11 (64% to 69%) of the 16 to 17 substances evaluated in five laboratories using the IS(A) analysis method, when compared to all three hazard classification systems.

The overall reliability statistics, arranged by HET-CAM data analysis method, were consistent with what was observed for the individual studies evaluated. For the IS(B)-10, the statistics were identical to what was discussed previously. For the IS(A) and IS(B)-100 analysis methods, additional laboratory data was available for a subset of the substances tested for each analysis method. For both of these analysis methods, the addition of the results from additional testing laboratories yielded a concordance pattern consistent with that described above.

Quantitative evaluations of interlaboratory reproducibility were conducted for the same analysis methods. For one study, two different evaluations were conducted based on the concentration tested *in vitro* using the IS(B) analysis method. For 14 substances evaluated at 100% concentration, the mean and median CV values were 31.86% and 33.04%, respectively. In the same study, for 12 substances evaluated at 10% concentration, the mean and median CV values were 66.29% and 60.75%, respectively. For the substances evaluated in another study, which used the IS(B) analysis method, the mean and median CV values for substances tested at 10% concentration were 60.17% and 42.65%, respectively. For substances tested at 100% concentration in the same study, the mean and median CV values were lower: 35.21% and 26.22%, respectively. When substances that were tested in three different testing laboratories (instead of two) were removed from the assessment, little change was seen in the mean and median CV values for both concentrations tested. For a study using the IS(A) analysis method, the mean and median CV for substances classified as GHS Category 1 (UN 2003) were 26.09% and 27.08%, respectively. The mean and median CV for substances classified as EPA Category I (EPA 1996) were 25.86% and 26.43%, respectively.

5.2   ICCVAM Recommendations for the HET-CAM Test Method

5.2.1   Use of the HET-CAM Test Method

ICCVAM evaluated several HET-CAM analysis methods proposed for identifying substances that are ocular corrosives or severe irritants. These included one analysis method termed the IS(B)-10 and another analysis method termed IS(B)-100. The range of hazard classification accuracy rates across the EU, EPA, and GHS classification systems for these two analysis methods ranged from 65% (64/98) to 68% (69/101) for IS(B)-10 and 52% (69/133) to 57% (94/164) for IS(B)-100, when the decision criteria of Luepke (1985) were used. The overall false negative and false positive rates of the IS(B)-10 analysis method range from 30% (10/33 to 12/40) to 32% (10/31) and 33% (20/61) to 36% (24/67), respectively, depending on the classification system. The overall false negative and false positive rates for the IS(B)-100 analysis method range from 6% (2/33) to 13% (5/39) and 52% (68/131) to 59% (58/99), respectively, depending on the classification system. Based on these rates, the use of these analyses methods and decision criteria for screening and identifying ocular corrosives and severe irritants (i.e., EPA Category I, GHS Category 1, EU R41) in a tiered-testing strategy, as part of a weight-of-evidence approach, is not recommended.

Users should be aware that HET-CAM's performance characteristics could be revised as additional data become available. Therefore, prior to initiation of non-regulatory, validation, or optimization HET-CAM studies, investigators are encouraged to consult the ICCVAM/NICEATM website (see http://iccvam.niehs.nih.gov/methods/eyeirrit.htm) to review the most current validation database, overall performance characteristics, and chemical and physical class performance characteristics. Evaluation of the most current information will allow users to determine the appropriateness of this test method for evaluating substances that are within a specific chemical, physical, or product classes.

5.2.2   HET-CAM Test Method Protocol

When non-regulatory, validation, or optimization studies are conducted using the HET-CAM test method, the protocol should be based on the standardized protocol provided in **Appendix G**. This will facilitate collection of consistent data and expand the current validation database. Exceptions and/or changes to the test method protocol should be accompanied by a scientific rationale.

Users should be aware that HET-CAM's standardized test method protocol could be revised as additional data become available. Therefore, prior to initiation of HET-CAM studies, investigators are encouraged to consult the ICCVAM/NICEATM website (see http://iccvam.niehs.nih.gov/methods/eyeirrit.htm) to review the most current recommended standardized test method protocol.

ICCVAM recommends that, for all studies, raw data be collected and maintained. The availability of such data will allow for further retrospective evaluation of test method accuracy and/or reliability.

5.2.3   Optimization of the Current HET-CAM Test Method Protocol

ICCVAM recommends that additional studies should be conducted to further optimize the HET-CAM prediction models and the decision criteria (e.g., mtc10) that would be used to identify ocular corrosives and severe irritants for the EPA, GHS, or EU classification systems. Such studies could potentially improve the usefulness of the HET-CAM test method for identifying severe ocular irritants and corrosives and its possible future use for the identification of mild and moderate ocular irritants (e.g., EPA Category II, III, and IV; GHS Category 2; EU R36).