Validation Status of the Isolated Rabbit Eye (IRE) 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 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, IRE, is an organotypic model that maintains normal physiological and biochemical function of the isolated eye. The ability of IRE to correctly identify ocular corrosives and severe irritants using available IRE and corresponding in vivo eve irritation data was evaluated from a database containing a total of 149 substances according to current hazard classification schemes for the U.S. EPA (n=113), the European Union (n=141), and the UN Globally Harmonized System (n=117); accuracy (48-86%), and false positive (22-59%) and false negative (0-57%) rates varied widely, but consistently across systems. When fluorescein penetration and/or epithelial integrity assessment were included (EU system only; n=66), accuracy was 77-86%; false positive and false negative rates were 23-34% and 0%, respectively. Lack of published intra- and interlaboratory data for this assay version precluded an evaluation of reliability. proposed standardized test method protocol and a recommended list of reference substances have been developed for future optimization/ validation studies to further assess the accuracy, reliability, and the applicability domain of IRE for the detection of ocular corrosives/severe irritants. IRE 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. ILS staff supported by NIEHS contract

Introduction

The Draize rabbit eve test (Draize et al. 1944), in one form or another, has been used since 1944 to evaluate the ocular irritation or corrosion potential of substances to which humans might be exposed. In recent vears, increased efforts have focused on the development of in vitro alternatives to this in vivo test. Although progress has been made, there irritancy. 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 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 Isolated Rabbit Eve (IRE) test. The National Toxicology Program (NTP) 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 of the test method.

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

- describe the current validation status of the IRE 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 IRE test method, based on existing data, for identifying ocular corrosives and severe irritants in a regulatory tiered testing strategy
- propose a standardized IRE test method protocol
- propose additional test method protocol optimization studies that might enhance the accuracy and/or reliability of the IRE test method and validation studies to further characterize its usefulness and
- 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 IRE test method, and other data and information submitted in response to a 2004 Federal Register (FR) request (FR Vol. 69, No. 57, pp. 13859-13861; available at http://iccvam.niehs.nih.gov/methods/eyeirrit.htm). The IRE 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 IRE test method, which will be published in a report that is expected to be available in August 2005.

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

²In 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 IRE test method is an *in vitro* organotypic model that provides shortterm maintenance of the rabbit eye in an isolated, controlled system with preservation of biochemical and physiological function. The current IRE test method protocols are similar to or modifications of the original protocol described by Burton et al. (1981) in which two endpoints, corneal opacity and corneal swelling, were used. In a prevalidation study of IRE supported retention was added as an endpoint. For the European Commission/British Home Office validation study of IRE, only corneal opacity and swelling were evaluated (Balls et al. 1995). In the Gettings et al. (1996) Cosmetic, Toiletries and Fragrance Association (CTFA) validation study of IRE, only corneal swelling was evaluated. Guerriero et al. (2004) evaluated the use of four ocular parameters in IRE for the identification of ocular corrosives or severe irritants. These endpoints included corneal opacity and area, fluorescein penetration area and intensity, corneal swelling and evaluation of epithelial integrity. A comparison of protocol variations among IRE test method studies is shown in Table 1.

In the standardized IRE test method proposed in the BRD, eyes may be obtained from rabbits killed for food or, if permitted by regulatory considerations, from those used in a previous scientific experiment (e.g. mild irritant skin testing or negative control animals). The eyes are carefully, but rapidly, enucleated from a rabbit euthanized with an overdose of sodium pentobarbital, and equilibrated for 30 minutes in a temperature controlled Perspex superfusion apparatus. The superfusion apparatus contains blackened chambers to facilitate slit-lamp examination. Each chamber is fitted to permit insertion of eve holders that clamp the eve in place vertically such that a stainless steel drip tube can keep the eve moist with drops of warm saline delivered by stainless steel tubes inserted in the upper part of the chamber and fed by a peristaltic pump. Test substances are applied as a single dose (100 µL or 100 mg) for 10 seconds followed by a 20 mL rinse with isotonic saline. Historically, one positive controls have not been included. The proposed standardized protocol recommends that three eves should be used for each test group (i.e., concurrent positive and negative controls; benchmark substances) if applicable; the test substance). Using a slit-lamp examination, each eve is qualified for use prior to treatment. After substance application eves are examined at 30, 60, 120, 180, and 240 minutes. Scores for corneal opacity area and intensity and for fluorescein penetration area and intensity are obtained. Corneal thickness is measured using an optical or ultrasonic pachymeter. Corneal swelling is the difference in the post-treatment and pretreatment thickness measurements expressed as percent swelling. Histopathological assessments may also be included on a case-by-case basis. Decision criteria for identification of an ocular corrosive or severe irritant are shown in Table 2. Maximum scores (products of area x intensity) for corneal opacity equal to or exceeding 3, or for fluorescein penetration equal to or exceeding 4, or a mean corneal swelling equal to or greater than 25%, or any sign of pitting, mottling, stippling, sloughing, ulceration, or other effects on the cornea epithelium results in a severe irritant classification.

Table 1. Protocol Variations Among IRE Test Method Studies

64-1-			# Eyes		Exposure	60	CC	FP	Epith.	Method of
Study	n	Neg	Treat	Pos	Duration	со	CS	FF	Integ.	Analysis
CEC (1991)	21	1	3	-	10 s	Y	Y	Y	N	Draize
Balls et al. (1995)	59	1	3	-	10 s	Y	Y	N	N	Draize
Gettings et al. (1996)	25	1	3	-	10 s	N	Y	N	N	Draize
Guerriero et al. (2004)	44	1	3	-	10s	Y ¹	Y	Y ¹	Y	McDonald Shadduck

¹Area and intensity scored
CO: Corneal opacity; CS: Corneal swelling; FR: Fluorescein penetration; Epith. Integ.:
Epithelial integrity; n: Number of substances tested; Neg: Negative control; Pos: Positive control; Treat: Test substance treated

Table 2. IRE Decision Criteria for Classifying Ocular Corrosives and Severe

Ocular Parameter	Cutoff Value to Detect Severe Eye Irritants
Maximum Corneal Opacity (Cloudiness x Area)	Greater than or equal to a score of 31
Maximum Fluorescein Uptake (Intensity x Area)	Greater than or equal to a score of 41
Mean Corneal Swelling ²	Greater than or equal to 25%
Corneal Epithelial Observations ³	Any pitting, mottling, sloughing, or ulceration of epithelium

Represents maximum score obtained in three eyes
Represents mean calculated for three eyes
Represents information obtained for any single eye

A total of 149 substances in four studies (CEC 1991; Balls et al. 1995; Gettings et al. 1996; Guerriero et al. 2004) were used to evaluate the accuracy of the IRE test method; data for 80 of these substances could also be used to evaluate interlaboratory reproducibility (Table 3). Fifteen different chemical classes were represented among these substances (heterocyclics/aromatics, acetates/esters, and formulations were the represented (industrial chemicals, chemical intermediates, and soaps and surfactants were the most commonly represented). Much of the published in vivo rabbit eve test data on the substances used to evaluate the accuracy of IRE for detecting ocular corrosives and severe irritants were limited to average score data or the reported irritancy classification. However, 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 were necessary to calculate the appropriate EPA (1996), European Union (EU 2001), o GHS (UN 2003) ocular irritancy hazard 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 3. Primary IRE Data Sources

Test Method Database

			Accu	racy		In	tralab	Interlab		
Study		GHS	EPA	EU	Total	CVs	GHS Class.	CVs	GHS Class.	
CEC	S			8	8	-	-		-	
CEC (1991)	NS			13	13	-	-	21	-	
()	Total			21	21	-	-		-	
	S	22	18	21	22	-	-		22	
Balls et al. (1995)	NS	34	34	38	38		-	59	34	
(1773)	Total	56	52	59	59	-	-		56	
C. W	S	16	17	7	17	-	-	-	-	
Gettings et al. (1996)	NS	9	8	18	18	-	2	-	-	
(1770)	Total	25	25	25	25	-	-	-	-	
	S	12	12	15	15	-	-	-	-	
Guerriero et al. (2004)	NS	24	24	29	29	-	-	-	-	
an. (2004)	Total	36	36	44	44	-	-		-	
	S	12	12	12	12	-	-	-	-	
Guerriero et al. (2004) ¹	NS	24	24	24	24	-	-	-	-	
ai. (2004)	Total	36	36	36	36	-	-	-	-	

Class.: Classification; CV: Coefficient of variation; EPA: Environmental Protection Agency; EU: European Union; GHS: Globally Harmonized System; NS: Nonsevere irritants or nonirritants; S: Severe irritant or ocular corrosive

1 For comparative purposes, did not include eight substances available for EU analysis that were classified on the basis of pH extremes or skin corrosivity test results

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



Test Method Accuracy Analysis

The ability of the IRE test method to correctly identify ocular corrosives and severe irritants, as defined by the GHS (UN 2003), EPA (1996), and EU (2001) classification systems³, was evaluated. Accuracy statistics were calculated for each IRE test method protocol by report and, where appropriate:

- classifications were pooled into one classification per substance (i.e., based on the majority call among studies).
- using individual studies, where a balanced design existed (multiple) substances in multiple labs as in Balls et al. [1995] and CEC [1991]). The overall IRE test method accuracy was consistent across all three classification systems, but varied widely from study to study. Test method accuracy appears to be increased by the inclusion of fluorescein penetration and/or evaluation of epithelial integrity (CEC 1991; Guerriero et al. 2004) For example, in the Gettings et al. (1996) and Balls et al. (1995) studies accuracy for the EU classification was 52% (44/84), whereas in the CEC (1991) and Guerriero et al. (2004) studies, accuracy was 80% (52/65) (**Table 4**). **Table 5** lists the false negative and false positive rates of the IRE test method for identifying ocular corrosives/severe irritants based on chemical class and physicochemical properties. Regardless of the number of endpoints evaluated, acids/alcohols, acetates, and ketones appear to be overpredicted in the IRE test. However, more data are needed to substantiate this pattern. In regard to physical properties. liquids were commonly overpredicted (75% [6/8]), but this may be more

There were notable limitations of the IRE test method accuracy analysis. A lack of individual rabbit test data prevented an accuracy evaluation for all 149 substances using the GHS and EPA classification schemes. In addition, the small number of substances representing each chemical class allows for only limited conclusions with respect to the accuracy of IRE by chemical class or physicochemical property.

related to chemical class than to physical property.

³For 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).

Table 4. IRE Test Method Accuracy

Data Source	Statistic	G	HS	E	PA	EU	
Data Source	Statistic	%	n¹	%	n ¹	%	n ¹
CEC (1991)	Accuracy	-	-	-	-	86	18/21
	Sensitivity	-	-	-	-	100	8/8
	Specificity	-	-	-	-	77	10/13
	False Positive Rate	-	-	-	-	23	3/13
	False Negative Rate	-	-	-	-	0	0/8
Balls et al. (1995)	Accuracy	50	28/56	48	25/52	53	31/59
	Sensitivity	64	14/22	61	11/18	67	14/21
	Specificity	41	14/34	41	14/34	45	17/38
	False Positive Rate	59	20/34	59	20/34	55	21/38
	False Negative Rate	36	8/22	39	7/18	33	7/21
	Accuracy	64	16/25	60	15/25	52	13/25
80 80 80	Sensitivity	56	9/16	53	9/17	43	3/7
Gettings et al. (1996)	Specificity	78	6/8	75	6/8	56	10/18
()	False Positive Rate	22	2/9	25	2/8	44	8/18
	False Negative Rate	44	7/16	47	8/17	57	4/7
	Accuracy	78	28/36	78	28/36	77	34/44
	Sensitivity	100	12/12	100	12/12	100	15/15
Guerriero et al. (2004)	Specificity	68	17/25	67	16/24	66	19/29
(2001)	False Positive Rate	33	8/24	33	8/24	34	10/29
	False Negative Rate	0	0/12	0	0/12	0	0/152

n: Proportion of substances fitting statistic to the total number available for classification
Additional eight substances available for EU analysis that permitted use of physicochemical properties or pH extremes in classification; otherwise statistic identical to GHS and EPA (n=44 versus n=36)

Table 5. IRE Test Method Accuracy According to Chemical Class and Physical Property for GHS Classification System¹

Class	# o	f Substa	nces		Negative ate ²	False Positive Rate ³	
Class	Total	Cat 1	Cat 2A, 2B, NI ⁴	%	n ⁵	%	n ⁵
OVERALL	36	12	24	0	0/12	33	8/24
Amine	5	2	3	0	0/2	33	1/3
Heterocyclic	7	1	6	0	0/1	33	2/6
Amide	6	2	4	0	0/2	25	1/4
Sulfur-containing	4	1	3	0	0/1	0	0/3
Acetate	2	0	2	-	-	100	2/2
Ketone	2	0	2	-	027	100	2/2
Acid; Alcohol	2	0	2	-	-	100	2/2
Aldehyde; Inorganic; Hydrocarbon; Surfactant	4	1	3	0	0/1	0	0/3
Solids	28	12	16	0	0/12	13	2/16
Liquids	12	4	8	0	0/4	75	6/8
	9 9		4				

Guerriero et al. (2004)

False negative rate is the proportion of all positive substances that are falsely identified by a test method as negative (obtained from a 2 x 2 table)

False positive rate is the proportion of all negative substances that are falsely identified by a test method as positive (obtained from a 2 x 2 table)

⁵ n: Proportion of positive or negative substances to the total number in each chemical class

Test Method Reliability Analysis

Due to the lack of available IRE test data, analyses of intralaboratory repeatability and reproducibility were not conducted. However, an assessment of interlaboratory reproducibility was feasible using data from a single study (Balls et al. 1995) in which 59 substances were tested among four different laboratories. Two separate analyses were conducted:

- a qualitative analysis, in which the extent of agreement between testing laboratories when identifying ocular corrosives and severe irritants was compared.
- a quantitative analysis using a coefficient of variation (CV) calculation to compare variability in measurements of the primary IRE test method endpoints.

Table 6 summarizes the results of the qualitative analysis for each of the three regulatory classification systems. When corneal swelling, opacity, and fluorescein retention were scored, the participating laboratories were in 100% agreement in regard to the ocular irritancy classification (corrosive/severe irritant or nonsevere irritant/nonirritant) of 81% (17/21) of the substances tested. When the qualitative analysis was based on at least 75% of the participating laboratories being in agreement for a test substance, the percentage of laboratories in agreement increased from 81% to 95% (20/21). However, agreement among laboratories for a test substance does not signify concordance between the *in vitro* and the *in vivo* classifications.

Table 7 summarizes the quantitative analyses using a CV calculation for two studies -- CEC (1991) and Balls et al. (1995). Mean and median %CV values were calculated to provide an assessment of overall variability. A wide range of %CV values was evident for all endpoints evaluated at four hours in the IRE test method. When all test data for each study were considered, mean and median %CV values ranged from 38 to 64% and 24 to 43%, respectively, for corneal opacity, and from 54 to 57% and 40 to 50%, respectively, for corneal swelling. Mean/median %CV values for fluorescein penetration were 59 and 28%, respectively. When only severe irritants (based on *in vivo* classification) were considered, mean/median %CV values were reduced for corneal opacity, swelling, and fluorescein penetration.

Draft BRD Proposals

Based on this evaluation of IRE test method performance, an IRE test method protocol that evaluates corneal opacity, swelling, fluorescein penetration, and epithelial effects, was proposed. A proposed standardized protocol, based on the method of Guerriero et al. (2004) and SafePharm Laboratories (Derby, United Kingdom) has been provided (NICEATM 2004). The only difference between the current SafePharm protocol and the proposed standardized protocol is the number of eyes serving as negative controls, and the inclusion of a concurrent positive control, and, when appropriate, benchmark controls (all tested using three eyes per group). Additional optimization studies are proposed that may enhance the performance of the IRE test method for identifying ocular

corrosives and severe irritants. These studies include

- a retrospective analysis of the decision criteria used to classify substances as ocular corrosives and severe irritants to possibly reduce the false positive rate
 additional evaluation of interlaboratory variability for specific
- additional evaluation of interlaboratory variability for specific chemical classes based on increasing the number of representative substances (i.e., alcohols, acetates/esters, ketones)
- evaluation of exposure time
- determination of the feasibility of introducing quantitative measurement for corneal opacity
- consideration of additional endpoints (e.g., depth of field measurements using confocal microscopy)
- standardization of ocular histopathology scoring and defined conditions for use

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.

Table 6. Qualitative Analysis of IRETest Method Interlaboratory Reproducibility

% Interlaboratory		HS ¹ Labs)	3500	PA ¹ Labs)		EU ¹ Labs)		EU² Labs)
Agreement	%	n	%	n	%	n	%	n
100% (All)	59	35/59	59	35/59	61	36/59	81	17/2
≥75% (All)	85	50/59	85	50/59	85	50/59	95	20/2
100% (Severes) ³	81	18/22	85	17/20	85	18/21	75	6/8
≥75% (Severes) ³	100	22/22	100	20/20	100	21/21	88	7/8

Table 7. Quantitative Analysis of IRE Test Method Interlaboratory Reproducibility: Coefficient of Variation (CV)

			%CV	
		СО	CS	FP
2005-018 1 2 2 7 7 5	Mean	63.8	53.5	-
Total (59 Substances) ¹	Median	43.4	49.7	
	Range	0-200	10-118	-
NASS YOUR ASSESSMENT SHOOL IS	Mean	40.5	36.9	-
GHS Category 1 (22 Substances) ¹	Median	40.6	36.0	-
	Range	0-200	1-118	-
500,000 100 ±0 1	Mean	37.7	57.3	58.9
Total (21 Substances) ²	Median	24.0	40.0	28.0
(======================================	Range	0-141	7.2-173	0-175
	Mean	15.5	35.4	22.1
GHS Category 1 (8 Substances) ²	Median	15.4	35.5	21.0
(o zubstances)	Range	0-40	20-61	0-78

¹Balls et al. (1995); ²CEC (1991)
CO: Corneal opacity; CS: Corneal swelling; CV: Coefficient of variation = standard deviation/mean; %CV: CV, expressed as a percentage; FU: Fluorescein penetration; Interlaboratory %CV values based on results from three or four laboratories

Proposed Reference Substances For Optimization and Validation Studies

A common list of reference substances proposed for future optimization and validation studies of IRE and other alternative test methods intended to detect ocular corrosives/severe irritants has been developed⁴. 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.

⁴See IRE BRD (http://iccvam.niehs.nih.gov/methods/ocudocs/ocu_brd.htm)

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