

## 1.0 INTRODUCTION AND RATIONALE FOR THE PROPOSED USE OF *IN VITRO* TEST METHODS TO IDENTIFY OCULAR CORROSIVES AND SEVERE IRRITANTS

### 1.1 Introduction

#### 1.1.1 Historical Background of *In Vitro* Ocular Irritation/Corrosion Test Methods and Rationale for Their Development

The location of the eye and its anatomy predisposes it to exposure to a variety of environmental conditions (e.g., ozone, pollen) and substances on a daily basis. Injury from ocular exposure to a variety of chemical agents can lead to a range of adverse effects with the most extreme being blindness. Societal concern for evaluating consumer products for ocular irritation and/or corrosion was heightened in 1933 when a 38 year old woman went blind after her eyelashes and eyebrows were tinted with a product containing paraphenylenediamine, a chemical with the potential to cause allergic blepharitis, toxic keratoconjunctivitis, and secondary bacterial keratitis<sup>1</sup> (Wilhelmus 2001).

In 1938, the U.S. Congress responded to these concerns by enacting the Federal Food, Drug, and Cosmetic Act of 1938, which included extending the regulatory control of the U.S. Food and Drug Administration (FDA) to cosmetics (FDA 1938). This legislation required manufacturers to evaluate product safety before marketing their products (Wilhelmus 2001). Several additional legislative statutes were later enacted to enable government agencies to regulate a variety of substances that could pose a risk to ocular health. **Table 1-1** provides a synopsis of current U.S. regulatory laws that pertain to eye irritation and corrosion.

**Table 1-1 Summary of Current U.S. Legislation Related to Ocular Health<sup>1</sup>**

Legislation (Year of Initial Enactment)	Agency	Substance
Food, Drug and Cosmetic Act (1938)	FDA	Pharmaceuticals and cosmetics
FIFRA (1947) and Federal Environmental Pesticide Control Act (1972)	EPA	Pesticides
FHSA (1964)	CPSC	Household products
FHSA (1964) and TSCA (1976)	Department of Agriculture and EPA	Agricultural and industrial chemicals
Occupational Safety and Health Act (1970)	OSHA	Occupational materials
Clean Air Act Amendments (1990)	Chemical Safety and Hazard Investigation Board and EPA	Accidentally released chemicals and air pollutants

<sup>1</sup>Adapted from Wilhelmus (2001).

Abbreviations: CPSC = U.S. Consumer Product Safety Commission; EPA = U.S. Environmental Protection Agency; FDA = U.S. Food and Drug Administration, FHSA = Federal Hazardous Substances Act; FIFRA = Federal Insecticide, Fungicide, and Rodenticide Act; TSCA = Toxic Substances Control Act.

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<sup>1</sup> Allergic blepharitis (also referred to as blepharitis): inflammation of the eyelids; Toxic keratoconjunctivitis (also referred to as contact, irritative, or chemical keratoconjunctivitis): inflammation of the cornea and conjunctiva due to contact with an exogenous agent; Secondary bacterial keratitis: inflammation of the cornea that occurs secondary to another insult that compromised the integrity of the eye (Vaughn et al. 1999; Chambers W, personal communications).

Exposure of the eye of a rabbit to a test substance is the primary method for assessing the hazard potential of substances that may come in contact with or be placed near the eye of a human. The rabbit eye test method currently accepted by U.S. Federal and international regulatory agencies (FHSA 1964; EPA 1998; OECD 2002) is based on a method developed by Draize and colleagues in 1944 (Draize et al. 1944). This technique involves placing a test substance into the lower conjunctival sac of one eye of a rabbit. The contralateral eye serves as a negative control. The rabbit is then observed at selected intervals for up to 21 days after exposure for adverse effects to the conjunctiva, cornea, and iris.

The current rabbit eye test method identifies both irreversible (e.g., corrosion) and reversible ocular effects. It also provides scoring that allows for relative categorization of severity for reversible effects such as mild, moderate, or severe irritants (e.g., see U.S. Environmental Protection Agency [EPA] Ocular Classification System discussed below). Current EPA ocular testing guidelines and the United Nations (UN) Globally Harmonized System (GHS) of Classification and Labeling of Chemicals (UN 2003) indicate that if serious ocular damage is anticipated (e.g., irreversible adverse effects on day 21), then a test on a single animal may be considered. If serious damage is observed, then no further animal testing is necessary (EPA 1998; UN 2003). If serious damage is not observed, additional test animals (1 or 2 rabbits) may be evaluated sequentially until concordant irritant or nonirritant responses are observed (UN 2003).

Depending on the legislative mandate of various regulatory agencies and their goals for protecting human health, the classification of irritant responses evaluated by each agency varies (**Table 1-2**). The EPA ocular irritation classification regulation and testing guidelines (EPA 1996, 1998) are based on the most severe response in one animal in a group of three or more animals. This classification system takes into consideration the kinds of ocular effects produced, as well as the reversibility and the severity of the effects. The EPA classifies substances into four ocular irritant categories, ranging from I to IV (**Table 1-2**) (EPA 1996). Category I substances are defined as corrosive or severe irritants, while classification from II to IV is based on decreasing irritation severity, as well as the time required for irritation to clear. Irritation that clears in 8 to 21 days is classified as Category II, while irritation that clears within seven days is classified as Category III. For Category IV substances, irritation clears within 24 hours. The U.S. Federal Hazardous Substances Act (FHSA) guideline for ocular irritation classification (CPSC 1995) categorizes a test substance as corrosive, irritant, or nonirritant. The definition of a corrosive, according to the FHSA, is a substance that causes visible destruction or irreversible alterations in the tissue at the site of contact (CPSC 2004). FHSA classification depends on the incidence of test animals exhibiting a positive ocular response within 72 hours after application of the test substance in the conjunctival sac. Hazard classification of ocular irritants in the European Union (EU) corresponds to two risk phrases: 1) R36 denotes "Irritating to eyes"; 2) R41 denotes "Risk of serious damage to the eyes" (EU 2001). These risk phrases are based on whether the levels of damage, averaged across the 24-, 48- and 72-hours observation times for each ocular lesion, fall within or above certain ranges of scores. For the purpose of harmonizing the classification of ocular irritants internationally, the GHS (UN 2003) includes two harmonized categories, one for irreversible effects on the eye/serious damage to the eye (Category 1), and one for reversible effects on the eye (Category 2). Reversible effects are further subclassified, based on the duration of

**Table 1-2 In Vivo Ocular Irritancy Classification Systems**

Regulatory Agency (Authorizing Act)	Number of Animals	Minimum Observation Times (after treatment)	Mean Score Taken?	Positive Response	Irritant/Nonirritant Classification
EPA (FIFRA; TSCA; and The Federal Environmental Pesticide Control Act)	At least 3	1 hour, 1, 2, 3, 7, 14, and 21 days	No	- Maximum score in an animal used for classification  - Opacity or Iritis $\geq 1$ or Redness or Chemosis $\geq 2$	One or more positive animals needed for classification in categories below.  <u>Category:</u> I = Corrosive, corneal involvement, or irritation persisting more than 21 days II = Corneal involvement or irritation clearing in 8 to 21 days III = Corneal involvement or irritation clearing in 7 days or less IV = Minimal effects clearing in less than 24 hours
European Union	<b>Current Directive:</b> 1 if severe effects are suspected or 3 if no severe effects are suspected  <b>Prior Directive:</b> 3 or 6 animals used to assign risk phrases	1, 2, 3 days (observation until Day 21)	Yes	(1) <u>6 animals</u> Mean study values (scores averaged over all animals in study over Days 1, 2, and 3) of: Opacity or Chemosis $\geq 2$ , Redness $\geq 2.5$ , or Iritis $\geq 1$  <b>OR</b>  (2) <u>3 animals</u> Individual animal mean values (scores for each endpoint are averaged for each animal over Days 1, 2, and 3) of: Opacity or Chemosis $\geq 2$ , Redness $\geq 2.5$ , or Iritis $\geq 1$	<b>R36 Classification</b> (1) Mean study value (when more than 3 animals are tested) where: $2 \leq \text{Opacity} < 3$ or $1 \leq \text{Iritis} < 1.5$ or $\text{Redness} \geq 2.5$ or $\text{Chemosis} \geq 2$ (2) If 2 of 3 tested animals have individual animal mean values that falls into one of the following categories: $2 \leq \text{Opacity} < 3$ $1 \leq \text{Iritis} < 2$ $\text{Redness} \geq 2.5$ $\text{Chemosis} \geq 2$  <b>R41 Classification</b> (1) Mean study value (when more than three animals are tested) where: $\text{Opacity} \geq 3$ or $\text{Iritis} > 1.5$ (2) If 2 of 3 tested animals have individual animal mean values that fall into one of the following categories: $\text{Opacity} \geq 3$ or $\text{Iritis} = 2$ (3) At least one animal where ocular lesions are still present at the end of the observation period, typically Day 21
GHS-Irreversible Eye Effects	3	1, 2, 3 days (observation	Yes	Mean animal values (over Days 1, 2, and 3) of:	- At least 2 positive response animals = Eye Irritant Category 1 - At least 1 animal where Opacity, Chemosis, Redness, or Iritis

Regulatory Agency (Authorizing Act)	Number of Animals	Minimum Observation Times (after treatment)	Mean Score Taken?	Positive Response	Irritant/Nonirritant Classification
		until Day 21)		Opacity $\geq 3$ and/or Iritis $\geq 1.5$	> 0 on Day 21 = Eye Irritant Category 1
GHS-Reversible Eye Effects	3	1, 2, 3 days (observation until Day 21)	Yes	Mean animal values (over Days 1, 2, and 3) of: Opacity or Iritis $\geq 1$ or Redness or Chemosis $\geq 2$ and the effect fully reverses in 7 or 21 days	- At least 2 positive response animals and the effect fully reverses in 21 days = Eye Irritant Category 2A - At least 2 positive response animals and effect fully reverses in 7 days = Eye Irritant Category 2B
CPSC (FHSA [provided under the authority of the Consumer Products Safety Act]), FDA (Food, Drug, and Cosmetics Act), and OSHA (Occupational Safety and Health Act)	6 (12, 18 possible)	1, 2, 3 days	No	Opacity or Iritis $\geq 1$ or Redness or Chemosis $\geq 2$ for any animal on any day	1 or more animals with destruction or irreversible alterations in the tissue at the site of contact = Corrosive  <u>1<sup>st</sup> Tier:</u> 4 or more positive animals = Irritant 2-3 positive animals = Go to <u>2<sup>nd</sup> Tier</u> 1 positive animal = Negative  <u>2<sup>nd</sup> Tier</u> 3 or more positive animals = Irritant 1-2 positive animals = Go to <u>3<sup>rd</sup> Tier</u>  <u>3<sup>rd</sup> Tier</u> 1 positive animal = Irritant

Abbreviations: CPSC = U.S. Consumer Products Safety Commission; EPA = U.S. Environmental Protection Agency; FDA = U.S. Food and Drug Administration; FIFRA = Federal Insecticide, Fungicide, and Rodenticide Act; GHS = United Nations Globally Harmonized System; OSHA = Occupational Safety and Health Administration; TSCA = Toxic Substances Control Act

persistence as Category 2A (“irritating to eyes”) (reverses within 21 days) and Category 2B (“mildly irritating to eyes”) (reverses within seven days). The GHS (UN 2003) categories are based on severity of the lesions and/or the duration of persistence. The GHS, the US, and the EU *in vivo* ocular irritancy classification systems are described in greater detail in **Section 4.1.3**.

Concerns about animal welfare, the cost and time to conduct ocular irritation assessments, the reproducibility of the currently used *in vivo* rabbit eye test, as well as scientific interest in understanding eye injury at the tissue and cellular level have led researchers to develop and evaluate alternative *in vitro* test methods. Recently, the EPA requested the evaluation of four *in vitro* test methods -- Isolated Chicken Eye (ICE), Isolated Rabbit Eye (IRE), Hen’s Egg Test – Chorioallantoic Membrane (HET-CAM) and Bovine Corneal Opacity and Permeability (BCOP) -- for their ability to identify ocular corrosives and severe irritants. As part of this evaluation process, a Background Review Document (BRD) has been prepared for each test method that describes the current validation status of the *in vitro* test method, including what is known about its reliability and accuracy, its applicability domain, the numbers and types of substances tested, and the availability of a standardized protocol.

This BRD evaluates the ability of the IRE test method to identify ocular corrosives and severe irritants. The IRE test method was developed by Burton et al. (1981) and proposed as a preliminary *in vitro* screen for the assessment of severe eye irritants. The principal advantage of the IRE test is to eliminate the use of live animals for ocular irritancy testing and eliminate the pain and suffering potentially associated with the *in vivo* Draize rabbit eye test. Another advantage of the IRE test method is that it typically uses eyes isolated from rabbits previously euthanized in other laboratories for research purposes in which the eyes were not specifically used or are culled from animals sacrificed commercially as a food source. In the IRE, liquid or solid substances are placed directly on the corneal surface of isolated rabbit eyes, which are held and maintained in a temperature-controlled chamber. After a 10-second exposure, followed by rinsing, the corneal thickness score, corneal swelling measurement, fluorescein penetration score, and effects on the corneal epithelium are taken over a four-hour observation period. Substances that exceed a defined cut-off score for any single parameter are considered severe irritants.

For current regulatory applications, the IRE test method could potentially be used to identify the irreversible, corrosive, and severe irritation potential of products, product components, individual chemicals, or substances in a tiered testing strategy (e.g., GHS [UN, 2003]). In the GHS stepwise approach, substances that are predicted by IRE as ocular corrosives/severe irritants could be classified as Category 1 eye irritants without the need for animal testing. Substances that are negative in the IRE test method for severe/irreversible effects would then undergo additional testing to confirm that they are not false negatives, and to determine the type, if any, of reversible effects that may occur. The test method also may be useful in a battery of *in vitro* eye irritation methods that collectively predicts the eye irritation potential of a substance *in vivo*. However, the predictivity of a battery approach will first require the assessment of the performance of each individual component.

The IRE test method is currently being used or contracted by some U.S. and European companies (e.g., pharmaceutical, cosmetic, and personal care product companies) as an in-house assay to assess the ocular irritation and corrosion potential of a wide range of substances for which there could be accidental exposures in the workplace or home. Substances are tested either individually, as mixtures, or in product formulations. The IRE test method is currently being used as an assay to evaluate industrial chemicals or products and formulations such as those used in cosmetics or in shampoos and conditioners (Balls et al. 1995; Chamberlain et al. 1997; Jones et al. 2001; Guerriero et al. 2004).

Although the IRE test method is not yet validated, the EU national regulatory authorities accept positive outcomes from this test method for eye irritation for classifying and labeling severe eye irritants (R41). Where a negative result is obtained, an *in vivo* test is subsequently required, as IRE has not been shown to adequately discriminate between eye irritants and nonirritants (European Communities 2004).

#### 1.1.2 Peer Reviews of the IRE Test Method

Studies have been conducted in recent years to assess the validity of the IRE test method as a complete replacement for the *in vivo* ocular irritation and corrosion test method (e.g., Balls et al. 1995; Gettings et al. 1996). Previous validation efforts may have failed because: 1) they attempted to support the utility of an *in vitro* alternative as a full replacement for the *in vivo* rabbit test, rather than as a component in a tiered testing strategy; and/or 2) data generated with the *in vitro* test method(s) have typically been compared to *in vivo* maximum average scores (MAS).

However, there have been no formal evaluations of the ability of the IRE test method to identify ocular corrosives and severe irritants, as defined by the GHS (UN 2003) and the EPA (EPA 1996). This BRD was prepared for use by an Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) expert panel review of IRE as a method to identify ocular corrosives and severe irritants. Parallel reviews of the ICE, HET-CAM, and BCOP test methods were conducted. Results of the Expert Panel Report, combined with the analyses presented in the BRDs, were used to support ICCVAM recommendations on the proposed standardized test method protocols, proposed list of recommended reference substances, and additional optimization and/or validation studies that may be necessary to further develop and characterize the usefulness and limitations of these methods.

## 1.2 **Scientific Basis for the IRE Test Method**

### 1.2.1 Purpose and Mechanistic Basis of the IRE Test Method

The IRE test method is an *in vitro* organotypic assay used to assess the ocular irritancy potential of substances that may be exposed topically to the eye (Burton et al. 1981; Köeter and Prinsen 1995; Chamberlain et al. 1997; Cooper et al. 2001; Jones et al. 2001). This test method was originally developed as a refinement of the *in vivo* rabbit eye test to avoid application of severe ocular irritants or corrosives to rabbits' eyes and to obviate associated pain and suffering (Burton et al. 1981). The IRE test method was later modified to identify less severe ocular irritants (Cooper et al. 2001; Jones et al. 2001).

In the IRE test method, the isolated eye is physiologically maintained and the corneal effects of an applied test substance are assessed over time (usually up to 4 hours) using multiple endpoints. These endpoints are corneal opacity (area and intensity), corneal swelling, fluorescein penetration (area and intensity), and an assessment of epithelial integrity (e.g., ulceration, mottling, stippling, etc.). If such data exist, corneal opacity in the IRE test method can be compared to that produced in the *in vivo* rabbit eye test. However, not all of the endpoints evaluated in the IRE test method are conducted in the *in vivo* rabbit eye test. For example, corneal thickness is measured quantitatively in the IRE using pachymetry, which may then be used for calculating corneal swelling (i.e., percent increase in corneal thickness over time). Because it is not required in regulatory testing (e.g., according to OECD TG 405 [OECD 2002]), corneal thickness is not typically measured in the *in vivo* rabbit eye test. Although qualitative, fluorescein staining is also measured in the IRE to estimate the degree of corneal penetration. Again, this endpoint is not typically measured in the rabbit eye test.

Histopathology or confocal microscopy would allow for a more accurate assessment of the extent of corneal injury. Maurer et al. (2002) proposed that the extent of ocular injury, as measured by confocal microscopy, has the greatest impact on the outcome of such an injury. Live/dead cell staining methods evaluated with confocal microscopy have also been used to determine the extent or depth of corneal injury *in vivo* (Maurer et al. 1997) and in an *ex vivo* corneal button assay (Jester et al. 2001). These studies prompted the authors to suggest that the extent of corneal injury could be used as the basis for developing alternative methods to predict the level of damage produced by ocular irritants.

### 1.2.2. Similarities and Differences of Modes and Mechanisms of Action Between the IRE Test Method and Ocular Irritancy in Humans and/or Rabbits

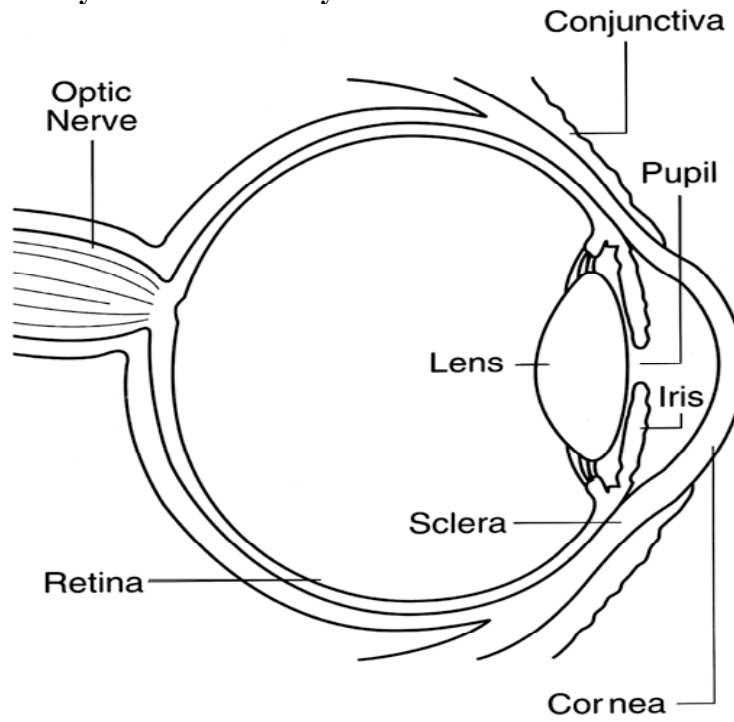
#### 1.2.2.1 *The Mammalian Eye: Common Anatomy of the Human and Rabbit Eye*

The eyeball is a fibrovascular globe, which is surrounded by a bony orbit that is impenetrable to light (Bruner 1992). The anterior portion of the eyeball is the only portion that is exposed to the environment, while the remainder of the eye is protected by the eyelids and the bony orbit. The eyeball is composed of three concentric tunics (the fibrous tunic, the vascular tunic, and the neuroectodermal tunic) that can be further subdivided. The fibrous tunic is the outermost layer of the eye comprised of the transparent cornea and the opaque sclera. The middle vascular tunic is comprised of the choroids, the ciliary body, and the iris (which can be referred to as the uvea). The neuroectodermal tunic is the innermost layer and is comprised of the retina, which contains photoreceptors and is connected to the central nervous system (Wilkie and Wyman 1991; Bruner 1992).

The fibrous tunic provides the primary framework for the eye. The cornea is the transparent surface of the eye, and is comprised of three major layers: the epithelium, the stroma, and the endothelium (**Figure 1-1**). The human cornea is a hydrated, nonvascularized structure. Corneal stroma contains 78% water and hydration is a requisite for the capacity of the stroma to swell in response to an irritant (Duane 1949). The cornea is nutritionally maintained in a homeostatic state by the aqueous humor, tear film, and the surrounding vascularized tissues.

Proper function of squamous or cuboidal cells in the endothelial layer is required to remove water from the cornea.

**Figure 1-1 Anatomy of the Human Eye**



Figured obtained at <http://www.nei.nih.gov/photo/eyean/index.asp>

The cornea is the major refracting element in the optical path, which flows from the light source through the cornea (70% of refractive power) to the lens (30% of refractive power) and into the retina (Duane 1949; Mishima and Hedbys 1968a). Therefore, corneal transparency is an important factor in optimal eye functioning. For maximum refractive power, the anterior surface of the cornea, composed of layers of translucent epithelial cells, is maintained in a smooth configuration by the tear film. The corneal stroma, composed of translucent keratocytes interspersed with collagen fibrils, requires uniformity and proper spacing of the collagen fibrils to maintain an appropriate corneal refractive index with minimal light scattering (Maurice 1957). This combination of structure and cellular morphology serves to maintain corneal transparency.

The eye is critically dependent on the highly vascularized middle coat (uvea) for regulation of blood and ocular permeability barriers, maintenance of intraocular pressure in the aqueous humor, and drainage of ocular fluid (Unger 1992). The uveal tract is richly innervated by somatic sensory neurons, derived from the ophthalmic division of the trigeminal nerve. Importantly, alterations to any of these features (e.g., edema, cell destruction, vascularization, cell proliferation) can cause corneal opacity and concomitant loss of function (Parish 1985; Wilkie and Wyman 1991; Bruner 1992).



The sclera is comprised primarily of three layers of irregularly arranged collagen fibrils of varying diameter. The irregular arrangement of the fibrils produces the white color that is seen on eyeballs. The conjunctiva is a mucous membrane that covers the exposed scleral surface (bulbar conjunctiva) and the inner surface of the eyelids (palpebral conjunctiva). The conjunctiva contains blood vessels, nerves, conjunctival glands, and inflammatory cells. As part of the inflammatory response in the conjunctiva, dilation of the blood vessels, fluid leakage and cellular leakage occurs (Bruner 1992).

The major component of the vascular tunic is the iris. The iris sits in front of the lens and the ciliary body, which also are considered part of the vascular tunic. Contraction of the iridal muscles alters the diameter of the pupil and thus regulates the amount of light entering the eye (Bruner 1992).

#### 1.2.2.2 *Differences Between Human and Rabbit Eyes*

There are several anatomical and physiological differences between the rabbit eye and the human eye. One difference is the presence of a nictitating membrane, or third eyelid, in the rabbit. As this membrane slides horizontally across the eye, it is proposed that it aids removing and/or excluding irritating substances from the corneal surface (Calabrese 1983). It also is proposed that the kinetic removal of a substance from a rabbit eye may occur at a rate different than in humans, due to the presence of the nictitating membrane, although this has not been documented in comparative studies (Curren and Harbell 1998). Another difference is the larger conjunctival sac in the rabbit, which allows for larger test volumes to be instilled, perhaps more than could be accounted for on accidental exposure (Curren and Harbell 1998).

The rabbit cornea is thinner than that found in humans, and rabbits tend to have less tear production (Curren et al. 1998; Cooper et al. 2001). The thicknesses of structural components of the cornea also are different between the two species. For example, Descemet's membrane is proposed to be about 5 to 10  $\mu\text{m}$  in humans and 7 to 8  $\mu\text{m}$  in rabbits (Calabrese 1983). Furthermore, the area of the cornea in relation to the total surface of the globe varies significantly between species; in humans the relationship is 7%, while in rabbits the relationship is 25% (Swanston 1985). Finally, young rabbits have the ability to regenerate damaged corneal endothelium, while humans do not (Chambers W, personal communication).

The relationship between species differences in eye anatomy and physiology and the sensitivity to ocular irritants has not been clearly established. It has been proposed that the larger conjunctival sac, thinner cornea, larger proportion of the cornea to the eyeball as well as other differences in the rabbit eye lead to an increased sensitivity to irritants (Calabrese 1983; Swanston 1985). However, other differences (e.g., the presence of the nictitating membrane, low blink frequency rate) indicate that the rabbit is as sensitive as humans to irritants. Comparisons of human exposure experiences to results in the *in vivo* test method indicate that in some cases the rabbit eye is more sensitive to some irritants, while in other cases the human eye is more sensitive (McDonald et al. 1987).

### 1.2.2.3 *The In Vivo Rabbit Eye Test Method*

The current *in vivo* rabbit eye irritation test method evaluates the cornea, the iris, and the conjunctiva for adverse effects after exposure to a potential irritant (See **Section 4.0** for a discussion of the *in vivo* scoring system for lesions at these sites). The cornea is visually observed both for the degree of corneal opacity and the area of the cornea in which opacity is involved. The iris is assessed for inflammation, iridal folds, congestion, swelling, circumcorneal injection, reaction to light, hemorrhage, and gross destruction. The conjunctiva is evaluated for the degree of redness, chemosis (swelling), and discharge (Draize et al. 1944). Draize and colleagues (1944) developed an analysis method where the severities of the effects are weighted differently, with corneal effect being weighted the most. The effects of a test substance on the cornea, conjunctiva, and iris play a role in severe ocular irritant and corrosive labeling and classification in classification systems used by some regulatory agencies (CPSC 1995; EPA 1998; EU 2001; UN 2003).

Irritation responses and the degree of the response in the cornea, iris, and conjunctiva differ due to the specific functions and anatomy of each structure. Development of slight corneal opacity can be due to loss of superficial epithelial cells and epithelial edema. Comparatively, more severe corneal opacity may be observed if an ocular irritant produces its effects deeper into the cornea. The ensuing repair process can lead to scar development on the cornea and vision impairment. Irritation responses in the iris are typically due to direct exposure to a substance, which has passed through the cornea and sclera, or due to extension of significant surface inflammation. Acute inflammation of the uvea tract is characterized by edema, vessel dilation, and the presence of exudates, while severe inflammation of the uvea tract is characterized by accumulation of blood or leukocytes in the anterior chamber. Conjunctival inflammatory responses can produce vasodilation, edema, subconjunctival hemorrhage, and lacrimal secretions (Bruner 1992).

The extent of corneal injury resulting from an ocular irritant also is dependent on the physicochemical characteristics (e.g., acids and bases with pH extremes, solvent-induced protein or DNA precipitation, surfactant-induced saponification of membranes), and chemical reactivity of the substances when in contact with individual ocular cells or structures (e.g., alkylation, hydrolysis, oxidation, reduction, hydroxylation) (Grant 1974; McCulley 1987; Berta 1992; Nourse et al. 1995; Fox and Boyes 2001). Direct or indirect ocular injury may result from the impact of these physicochemical effects on normal homeostatic cellular mechanisms and from consequent edema, inflammation, apoptosis, necrosis, and reparative processes (e.g., collagen deposition and scarring) (Unger 1992; Pfister 2005). In the normal eye, test substances may disrupt the tear film, reach the epithelium, and penetrate through Bowman's layer into the stroma, through Descemet's membrane, and into the endothelium (Pasquale and Hayes 2001). Damage to the endothelium may be irreparable.

The tear film consists of an inner layer of mucous, a middle layer of water, and an outer film of oil. The tear film contains lactoferrin, peroxidase, lysozyme, immunoglobulins and complement factors to eliminate potentially offensive material (Unger 1992). In conjunction with the neurogenically controlled blink reflex and tear producing cells, the tear film serves as a protective barrier against an ocular irritant for the corneal epithelium. The

physicochemical properties (e.g., hydrophilicity, hydrophobicity, hypertonicity, hypotonicity, oxidation, reduction) in addition to the chemical and biochemical properties of an applied test substance impact its ability to breach the tear film, or interact with its components and impact the corneal epithelium. The tear film and the aqueous humor also provide nourishment (e.g., glucose and oxygen) to the nonvascularized cornea. The extent of damage to the tear film by an applied substance therefore impacts the ability of the tear film to nourish dependent corneal tissue. Changes in the distribution, physical structure, or secretion rate of the tear film by an applied test substance might have significant nutritional, refractory, chemical and physical impacts on corneal tissue (Mishima and Hedbys 1968a; Mishima and Hedbys 1968b).

Either direct (e.g., caustic or corrosive) or indirect (e.g., inflammatory mediator release) effects of chemicals in contact with the anterior corneal surface may result in perturbation of the optical elements needed to maintain the appropriate index of refraction in the cornea (e.g., uniformity and proper spacing of collagen fibrils), resulting in significant light scattering and impairment of vision (McCulley 1987; Berta 1992; Nourse et al. 1995; Wilson et al. 2001). Corneal injury may result in opacification, swelling, damage extending from the epithelium into the stroma or possibly through the endothelium, and changes in corneal morphology (e.g., ulceration, scarring, pitting, mottling).

Opacification of the cornea may result from: 1) direct or indirect damage to the epithelial cells with or without penetration into the stroma; 2) protein denaturation of the epithelial cells such as that produced by alcohols, alkalis, or organic solvents; 3) alkylation of protein or DNA; 4) membrane saponification by surfactants; 5) inflammatory cell infiltration; 6) collagen deposition; 7) swelling of corneal epithelial cells or corneal stroma; 8) displacement or rearrangement of collagen fibrils; or 9) degradation of the extracellular matrix (Grant 1974; McCulley 1987; Thoft 1979; York et al. 1982; Fox and Boyes 2001; Kuckelkorn et al. 2002; Eskes et al. 2005; Pfister 2005).

Corneal swelling results from disruption of the anterior barrier membrane formed by the epithelial cell layer and Bowman's layer. This results in disruption of stromal collagen fibril uniformity, loss of proteoglycans, and cell death, which leads to bullae formation, stromal cloudiness, and increased hydrostatic pressure (which may extend posteriorly throughout the corneal stroma, penetrating into Descemet's layer and into the endothelium) (Mishima and Hedbys 1968a; Mishima and Hedbys 1968b). Osmotic changes induced by these effects may further damage keratocytes and the collagen matrix.

Corneal damage also may be characterized by morphological changes (e.g., described as stippling, ulceration, mottling, pannus, neovascularization).

Corneal injury also is dependent on the type and concentration of applied chemical. Alkalis penetrate more readily than acids do, and the depth of penetration is dependent on alkali concentration (McCulley 1987). With alkali injury, the hydroxyl ion saponifies the fatty acid components of the cell membrane, disrupting cellular contents and resulting in cell death. The cation is responsible for the penetration process (Grant 1974). Acids tend to penetrate less deeply than alkalis, with the exception of hydrofluoric and sulfuric acids. The hydrogen

ion causes damage due to pH alteration, while the anion precipitates and denatures protein in the corneal epithelium and superficial stroma (Freidenwald et al. 1946). Limbal ischemia is a significant consequence of even mild alkali or acid burns (Kuckelkorn et al. 2002).

While not in the direct optical path, the Palisades of Vogt, located in the sclero-corneal limbus, are thought to house corneal stem cells and serve as a generative organ for normal replacement of dead corneal epithelial cells for re-epithelialization during repair of corneal injury. Depletion or partial loss of the limbal stem cell population may result in corneal vascularization due to loss of the barrier function of the limbus, which serves to prevent conjunctival epithelial cells from migrating to the corneal surface (Dua and Azuara-Blanco 2000).

Neutrophils are recruited in response to acid and alkali injury as well as in response to other ocular toxicants (Pfister 2005). Neutrophil migration is stimulated by the release of chemotactic factors (e.g., interleukins, growth factors, etc.) from damaged or chemically activated local resident epithelial cells or stromal keratocytes (Wilson et al. 2001). Loss of keratocytes following either chemical or mechanical epithelial injury may be mediated by apoptosis, perhaps by release of IL-1 and TNF $\alpha$  (Wilson et al. 2001). Resident mast cells may release biogenic amines that perturb the hydrostatic balance and permit inflammatory or edemagenic mediators into the locally inflamed area. Migrated neutrophils release additional cytokines (e.g., IL-1 and TNF- $\alpha$ ) and enzymes such as proteases, collagenases, kinases, and phospholipaseA<sub>2</sub> (PLA<sub>2</sub>). PLA<sub>2</sub> produces edemagenic and vasoactive mediators such as prostaglandins and leukotrienes from arachidonic acid in cellular membranes.

This cascade of events ultimately facilitates repair by stimulating fibrin deposition and granuloma formation. However, migrating inflammatory cells such as neutrophils also may be involved in the release of collagenases (e.g., matrix metalloproteinases [MMPs]), which have been implicated in corneal ulcer formation. Acetylcysteine, L-cysteine, and EDTA have been shown to reduce corneal ulceration in response to alkali injury while inhibiting MMPs (Pfister 2005). Other inflammatory cells such as macrophages and T-lymphocytes may be found up to 24 hours after injury. Once an area is damaged and devoid of keratocytes, proliferation and migration occurs as part of the wound healing process. This process may be mediated in part by numerous growth factors (Wilson et al. 2001).

Although variable responses occur among species, neuropeptides (e.g., Calcitonin Gene Related Peptide [CGRP] and substance P) have profound effects on the anterior portion of the highly innervated eye, particularly in lower mammals such as the rabbit (Unger 1992). CGRP appears to affect vascular smooth muscle (Oksala and Stjernschantz 1988), whereas substance P may be involved in meiosis (Unger 1990). Loss of functional sympathetic innervation reduces or eliminates presynaptic catecholamine reuptake sites resulting in denervation supersensitivity. This also may result in enhanced sensitivity to noxious stimuli.

Applied test substances also can adversely affect homeostasis within the cornea. As oxygen is absorbed into the cornea from the atmosphere, interference with oxygen uptake may lead to corneal swelling (Mishima and Hedbys 1968a). The cellular respiratory needs of the endothelium and epithelium are similar, both requiring carbohydrate metabolism. Glucose

metabolism in the cornea occurs by glycolysis and oxidation through the tricarboxylic acid cycle as well as through the hexose-monophosphate shunt (Kinoshita 1962). Glucose within the cornea is used to supply glycogen, which is stored in the epithelium. Applied substances that modulate any of these processes may be associated with ocular toxicity.

#### 1.2.2.4 Comparison of the IRE Test Method with the In Vivo Rabbit Eye Test Method

Both the IRE test method and the Draize rabbit eye irritation test focus primarily on irritation or corrosion to the corneal surface. The degree of penetration of damage to the corneal surface by an irritant or corrosive into the epithelium, stroma or endothelium can be assessed in the IRE, but is difficult to assess in the *in vivo* test. In the isolated eye, fluorescein retention and histology can also be assessed to confirm the degree of penetration within the corneal tissue. In addition, the morphological abnormalities of the corneal surface (i.e., stippling, mottling, pitting, etc.) and the area of corneal involvement can be evaluated in the IRE as it can in the *in vivo* test method. The primary disadvantage of the IRE test method in comparison to the *in vivo* test method is that the iridal tissue *in vitro* is devoid of blood flow and nerves controlling the musculature and blood vessels may be compromised. Thus, iridal damage due to inflammatory or neuromuscular components cannot be evaluated. Similarly, the conjunctival tissue is not present in the isolated eye. However, in the Draize test, the corneal score is weighted to represent 73% of the total score. The iris is weighted at 9% of the total ocular score and the conjunctiva is weighted at 18%. The combined contribution of the iridal and conjunctival tissue to the *in vivo* score is 27%. Thus, for the identification of a severe irritant or corrosive, damage to the cornea would be expected to have the greatest impact on the Draize score. Furthermore, other *in vitro* test methods can be used in a tiered-testing approach to assess vascular changes produced by an irritant or corrosive (e.g., HET-CAM) which would be more likely related to conjunctival damage. The isolated rabbit eye cannot be used to assess inflammatory changes induced by an irritant or corrosive, since the vascular components are not functional. Substances that might produce irritancy through a delayed inflammatory response may not be picked up in the IRE test method, since the test is only run for four hours.

In the isolated rabbit eye, neurogenic components that drive tear film production are not functional. Although the cornea is constantly hydrated with a saline drip in the IRE test method, the lack of a tear film is considered a limitation. In fact, the saline drip eventually removes the residual tear film, which cannot subsequently be regenerated. When compared with an *in vivo* rabbit eye study, application of a test substance in the absence of this protective barrier might be expected to cause an increase in false positive outcomes. One of the conclusions from a workshop on mechanisms of eye irritation highlighted the need for additional research on the impact of chemicals on tear film and the consequences of tear film disruption (Bruner et al. 1998).

Corneal opacification in both the *in vivo* rabbit eye test and the IRE test method is visually observed or may be assessed using a slit-lamp.

In the IRE test, corneal swelling is assessed quantitatively, using an ultrasonic or optical pachymeter to measure the increase in corneal thickness during an experiment. It is

expressed as a percent increase in corneal thickness over time relative to the pre-treatment measurement.

### 1.2.3 Intended Range of Substances Amenable to the IRE Test Method and/or Limits of the IRE Test Method

Studies indicate that the IRE test method is amenable to use with a broad range of solid and liquid substances. However, the IRE appears to be less predictive with solids than it is for liquid substances (Whittle et al. 1992). Substances amenable to testing include, but are not limited to, inorganic, aliphatic, aromatic, and heterocyclic chemicals, surfactants, polymers, and mixtures/formulations.

The major limitation of the IRE test method is the inability to evaluate the effect of test substances on the iris and conjunctival tissue. For the evaluation of severe irritants, this is not a major limitation of the test method, since most severe irritants damage the corneal tissue.

Another potential limitation of the test method is that it can be used only for short-term assessments of the irritancy of a test substance. The currently accepted *in vivo* test method usually observes the rabbits for up to 21 days after treatment to assess reversibility of any of the observed endpoints and to evaluate test substances that produce eye effects over an extended time period. Comparatively, the observation period for evaluating effects in the IRE test method post-treatment is up to four hours. Therefore, potential reversibility of the affected endpoint beyond four hours, or an effect with a delayed onset (e.g., slow-acting irritants) due to inflammation or other factors cannot be adequately evaluated with this test method.

## 1.3 **Regulatory Rationale and Applicability**

### 1.3.1 Current Regulatory Testing Requirements and ICCVAM Prioritization Criteria

The following section reviews and summarizes the extent to which the five ICCVAM prioritization criteria apply to the IRE test method (ICCVAM 2003).

#### **Criteria 1. The extent to which the proposed test method is (a) applicable to regulatory testing needs and (b) applicable to multiple agencies/programs.**

The IRE assay has been proposed as a method to identify ocular corrosives or severe irritants, as is required by several U.S. laws. **Table 1-1** identifies the U.S. agencies and programs, which classify and label substances for eye irritation and corrosion. These agencies are the FDA, the EPA, Department of Agriculture, Department of Labor, the Consumer Product Safety Commission (CPSC), and the Chemical Safety and Hazard Investigation Board. Therefore, the IRE test method is applicable to the regulatory testing needs of multiple U.S. Federal agencies and programs.

#### **Criteria 2. Warranted, based on the extent of expected use or application and impact on human, animal, or ecological health.**

Current regulatory testing needs require the *in vivo* assessment of the eye irritancy or corrosivity hazard associated with the use of chemicals/products for labeling purposes.

These testing needs require the use of laboratory rabbits. Alternative *in vitro* eye irritation and corrosion test methods could be applied to these testing needs.

**Criteria 3. The potential for the proposed test method, compared to current test methods accepted by regulatory agencies, to (a) refine animal use (decrease or eliminate pain and distress), (b) reduce animal use, or (c) replace animal use.<sup>2</sup>**

The IRE test method has the potential to refine or reduce animal use in eye irritation testing. Substances that are identified as ocular corrosives or severe irritants would be excluded from testing *in vivo*, which would reduce the number of rabbits used for ocular testing and also spare animals the pain and distress of exposure to severe eye irritants.

**Criteria 4. The potential for the proposed test method to provide improved prediction of adverse health or environmental effects, compared to current test methods accepted by regulatory agencies.**

Based on its long history of use and acceptance by U.S. Federal and international regulatory agencies, the current system of ocular hazard assessment, which is based on the rabbit eye test (i.e., CPSC 1995; EPA 1998; OECD 2002), appears to have adequately protected public health. However, use of the rabbit eye test to predict the ocular irritation potential of substances for humans is not without controversy (e.g., intra- and inter-laboratory variability, qualitative evaluation of ocular lesions). The accuracy of the currently used *in vivo* rabbit eye test for predicting severe eye irritants in humans and the limitations of the method for predicting the irritancy of specific chemical and/or product classes are not known due to the lack of comparative data. Therefore, the potential of the proposed test method to provide improved prediction of adverse human health effects is unknown.

**Criteria 5. The extent to which the test method provides other advantages (e.g., reduced cost and time to perform) compared to current methods.**

The IRE test method could reduce the time needed to assess a substance, when compared to the currently accepted *in vivo* rabbit eye test method. The *in vivo* Draize rabbit eye test is typically carried out for a minimum of one to three days and can be extended up to 21 days. The irritancy of a test substance in the IRE assay is measured over a four-hour time period. The complete assay with dissection and equilibration can be completed (after eye procurement) in eight hours. Regarding comparative costs (based on conducting Good Laboratory Practice [GLP] compliant studies), the standard IRE assay conducted with concurrent positive and negative controls costs \$1074 per test substance at SafePharm Laboratories (2004). The current cost of a GLP compliant EPA OPPTS Series 870 Acute Eye Irritation (EPA 1998) or OECD Test Guideline 405 (OECD 2002) test at MB Research Laboratories (Spinnerstown, PA) ranges from \$765 for a 3 day/3 animal study up to \$1665 for a 21 day/3 animal study (MB Research Laboratories, personal communication). While the cost of an IRE assay includes concurrent positive controls, the *in vivo* rabbit test method

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<sup>2</sup> Refinement alternative is defined as a new or revised test method that refines procedures to lessen or eliminate pain or distress to animals, or enhances animal well-being; Reduction alternative is defined as a new or revised test method that reduces the number of animals required; Replacement alternative is defined as a new or revised test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate) (ICCVAM 1997).

does not include equivalent controls. The cost of the IRE assay is reduced significantly by the reduction in time involved in running the assay relative to the *in vivo* test and by the use of donated rather than purchased animals.

### 1.3.2 Intended Uses of the Proposed IRE Test Method

*In vitro* ocular irritation testing methods (e.g., ICE, IRE, BCOP, and HET-CAM) have been proposed for identification of ocular corrosives and severe irritants (e.g., Ocular Irritant Class I per the EPA classification system [EPA 1996], Ocular Irritant Class R41 per the EU classification system [EU 2001], or Ocular Irritant Class 1 per the GHS classification system [UN 2003]).

### 1.3.3 Similarities and Differences in the Endpoints Measured in the Proposed Test Method and *In Vivo* References Test Method

As mentioned in **Section 1.1.1**, the *in vivo* rabbit eye test method in current use by U.S. Federal and international agencies is based on a method developed by Draize and colleagues in 1944. This test method involves instillation of the test substance into the lower conjunctival sac of the rabbit eye, and evaluates the cornea, the iris, and the conjunctiva for adverse effects after exposure to the potential irritant. The cornea is evaluated both for the degree of corneal opacity and the area of the cornea in which opacity is involved. The iris is assessed for inflammation, iridal folds, congestion, swelling, circumcorneal injection, reaction to light, hemorrhage, and gross destruction. The conjunctiva is evaluated for the degree of redness, chemosis (swelling), and discharge (Draize et al. 1944).

As detailed in **Section 1.2.1**, the IRE is an organotypic model that uses an isolated rabbit eye for ocular irritation testing. This *in vitro* assay is designed to identify the effect of severe irritants/corrosives on the corneal tissue. Corneal opacity, corneal swelling, penetration of fluorescein into the corneal tissue and assessment of the morphology of the corneal epithelium are measured or scored. In the *in vivo* Draize rabbit eye test, scores are obtained for corneal opacification and the area affected in response to an irritant, irritation of the iris and redness, swelling and discharge in the conjunctival tissue. The scores are weighted with the effects on the cornea receiving the highest weight (73% of total). The IRE test method is designed to measure corneal effects only, since the iris and conjunctival tissue are dissected out in the preparation of the eye. However, given the increased weight of the *in vivo* Draize score for corneal effects over iridal or conjunctival irritation and the general observation that most severe irritants have an effect on corneal opacity (Parish 1985; Sina and Gautheron 1994), the endpoints measured in the IRE test method are comparable to those used for evaluation of corneal damage in the *in vivo* method.

### 1.3.4 Use of Proposed Test Method in Overall Strategy of Hazard or Safety Assessment

The IRE test method is being considered for use in the identification of ocular corrosives and severe irritants in a tiered testing strategy (GHS [UN 2003]). The GHS proposes a tiered testing and evaluation strategy for serious eye damage and eye irritation using available data from dermal irritation studies, knowledge of structure activity relationships, and pH screening. As shown in **Figure 1-2**, the GHS also allows for use of validated and accepted *in vitro* methods to identify severe ocular irritants/corrosives without further testing. If a test substance is classified in a validated *in vitro* method as an ocular corrosive or severe irritant,



then no further testing would be required and the test substance would be appropriately labeled. If a test substance is not classified as an ocular corrosive or severe irritant using a validated *in vitro* method (i.e., the test substance remains unclassified), then current regulatory agency regulations for ocular testing would be followed. It is noted that the current testing strategy is proposed for use for regulatory classification and labeling purposes.

#### **1.4 Validation of *In Vitro* IRE Test Methods**

The ICCVAM Authorization Act (Sec. 4(c)) mandates that “[e]ach Federal Agency ... shall ensure that any new or revised ... test method ... is determined to be valid for its proposed use prior to requiring, recommending, or encouraging [its use].” (Public Law 106-545).

Validation is the process by which the reliability and relevance of an assay for a specific purpose are established (ICCVAM 1997). Relevance is defined as the extent to which an assay will correctly predict or measure the biological effect of interest (ICCVAM 1997). For the IRE test method described in this BRD, relevance is restricted to how well the assay identifies substances that are capable of producing corrosive or severe irritant effects to the eye. Reliability is defined as the reproducibility of a test method within and among laboratories and should be based on performance with a diverse set of substances that are representative of the types of chemical and product classes that are expected to be tested and the ranges of response that need to be identified. The validation process will provide data and information that will allow U.S. Federal agencies to develop guidance on the development and use of the IRE test method as part of a tiered-testing approach to evaluating the eye irritation potential of substances.

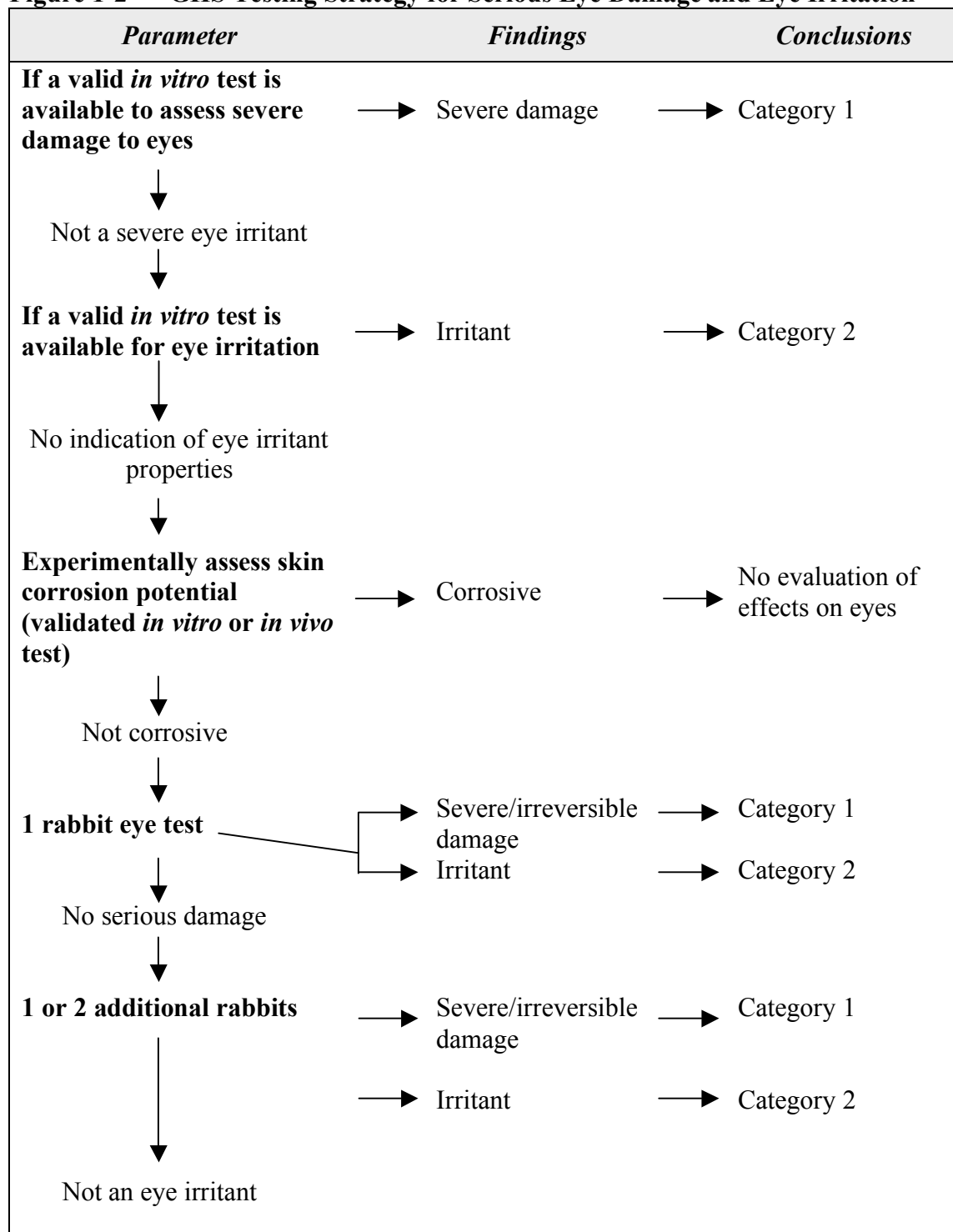
The first stage in this evaluation is the preparation of a BRD that presents and evaluates the relevant data and information about the assay, including its mechanistic basis, proposed uses, reliability, and performance characteristics (ICCVAM 1997). This BRD summarizes the available information on the various versions of the IRE test method that have been published. Where adequate data are available, the qualitative and quantitative performances of the assays are evaluated and the reliability of each version of the test method is compared with the reliability of the other versions. If there are insufficient data to support the recommendation of a standardized protocol for IRE, this BRD will aid in identifying essential test method components that should be considered during its development and validation.

#### **1.5 Search Strategies and Selection of Citations for the IRE BRD**

The IRE test method data summarized in this BRD are based on information found in the peer-reviewed scientific literature. An online literature search of entries in MEDLINE, TOXLINE, and Web of Science was conducted to retrieve database records on publications reporting on *in vitro* testing of substances for their ocular irritancy potential using the IRE test method. Specifically, records were sought containing the keywords “isolated” or “enucleated” and “rabbit” and “eye.” Each database record included authors, bibliographic

citation, and indexing terms. Most records also included abstracts. Of the 28 records obtained from the search last updated in February 2004, seven contained results from an IRE

**Figure 1-2 GHS Testing Strategy for Serious Eye Damage and Eye Irritation**



Adapted from UN (2003).

test method, four were review articles and two were background articles related to the IRE test method. Additional information including an abstract on the IRE test method was obtained at the Society of Toxicology Meeting in March, 2004. Abstracts of selected titles were reviewed, and the relevant articles were selected and retrieved from the literature for analysis. A database of the literature citations was established using bibliographic database software.

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