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Validating and Troubleshooting Ocular *In Vitro* Toxicology Tests

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Abstract

In vitro organotypic models for testing ocular irritants have warranted sufficient interest as methods to replace *in vivo* ocular testing. The *in vitro* organotypic models claim to maintain short-term normal physiological and biochemical function of the mammalian cornea in an isolated system. In these test methods, damage by the test substance is assessed by quantitative measurements of changes in corneal opacity and permeability using opacitometry and spectrophotometry, respectively. Both measurements are used quantitatively for irritancy classification for prediction of the *in vivo* ocular irritation potential of a test substance. Examples of organotypic models that incorporate these criteria include: the bovine corneal opacity and permeability (BCOP) assay, the isolated chicken eye (ICE) test method and the isolated rabbit eye (IRE) assay. A fourth method, the hen's egg test-chorioallantoic membrane (HET-CAM) assay, differs in the evaluation criteria but is also normally included among this class of *in vitro* protocols. Each of these protocols is discussed in detail as representative candidate *in vitro* methods for assessing ocular irritation and corrosion. The methodologies, protocol details, applications, and their validation status are discussed. A brief historical perspective of the development of original *in vitro* ocular testing models is also mentioned. More importantly, improvement and troubleshooting the current techniques, in order to present the models as stand-alone *in vitro* tools for ocular toxicity assessment, is emphasized.

Keywords

alternative toxicology testing; ECVAM; ICCVAM; *in vitro* cytotoxicity; methods; ocular models; organotypic

1. Introduction

No other field in *in vitro* toxicology testing has driven academic, industrial, and government resources to develop cell modeling systems as much as the need for alternatives to local toxicity testing. This is principally due to the ubiquitous presence of cosmetics, toiletries, dermal and ocular pharmaceuticals that are marketed internationally. Historically, results obtained with the original *in vivo* animal models for dermal and ocular toxicology are not acceptable in predicting effects encountered with humans (Draize *et al.* 1944). This is primarily because of the inconsistencies between the data generated from the animal models and human risk

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exposure. In addition, there is significant pain and distress encountered with testing animals for local toxicity. This is particularly important since the application of analgesic or anesthetic agents are incompatible with the nature of the ocular test. Nevertheless, animal testing has continued in spite of the objections. Recently, technical advances and improved knowledge of cell culture technology has allowed for progress in the development of cellular systems, with some tests undergoing full validation evaluation in the United States (U.S.) and the European Union (E.U.).

As a result of the support behind the development of alternative tests for local toxicity testing, most of the initial *in vitro* tests that were introduced involved isolated mortal and immortal continuous cell lines and tissue cultures of human or animal origin (Burton 1972, Burton *et al.* 1981). The systems were used to evaluate the effect of substances in isolated environments devoid of hormonal, immune or neural influence. These advantages have also been noted as shortcomings–that is, the elimination of other biological factors in the cell culture system does not allow the method to mimic interactions occurring in the whole organism, particularly with a specialized organ such as the eye. Yet, many *in vitro* methods have been developed since the early 1980s; widespread regulatory acceptance, however, especially in the U.S., was limited (Barile and Cardona 1998; Casterton *et al.* 1996; Gautherton *et al.* 1992; Harbell and Curren 1998; Kalweit *et al*. 1987; Luepke 1985; Leupke and Kemper 1986; Sterzel *et al*. 1990). Besides the lack of scientific comparability between the *in vitro* and *in vivo* systems, the problems also centered on the time and cost necessary to develop the tests and the arduous validation process (Balls, *et al*., 2006; Ekwall and Barile, 1994; Hartung *et al*., 2004)—a process that establishes a specific purpose of an assay by measuring its reliability and relevance. Relevance is the extent to which an assay will correctly predict or measure the biological effect of interest. It is the limiting factor in the validation scheme. Reliability is the degree to which the data in the protocol is reproducible within the guidelines of the method. Therefore, validation should not be a procedure for sanctioning a system but should be focused as a rigorous set of policies for challenging an *in vitro* assay.

Gradual improvement to the methodologies involved the development of cell models of local toxicity using the establishment of primary cultures from specialized tissues, especially cells of ocular epithelial origin. These primary cultures possess specific functional markers that can distinguish selective toxic effects. In particular, the effects of a chemical on a specific functional target, which is unique for ocular epithelia, is assessed, and distinguished from the chemical's effect on ocular cells or any other cell type. Other isolated *in vitro* models used to screen for local toxicity incorporate tissue types that are not limited to the organ of origin but include supportive structures, such as keratinocyte epithelial cells, connective tissue cells, or *in vitro* compartmental models (Barile 1997; Calabro *et al.* 2008; Konsoula and Barile 2007). These cells retain sufficient physiological properties similar to the mammalian eye that allow for a response to a test substance. For instance, corneal epithelial cell culture models have been introduced to test for chemicals with potential for detecting ocular effects.

More recently, organotypic models have warranted sufficient interest as methods that maintain short-term normal physiological and biochemical function of the mammalian cornea in an isolated system (Barile 2007; Cooper *et al.* 2001; Gettings *et al.* 1996; Muir 1985). In these test methods, damage by the test substance is assessed by quantitative measurements of changes in corneal opacity and permeability using opacitometry and spectrophotometry, respectively. Both measurements are used quantitatively for irritancy classification for prediction of *in vivo* ocular irritation potential of a test substance. Examples of organotypic models that incorporate these criteria include: the bovine corneal opacity and permeability (BCOP) assay; the isolated chicken eye (ICE) test method (or the chicken enucleated eye test method, CEET); and, the isolated rabbit eye (IRE) assay. A fourth method, the hen's egg test-chorioallantoic

membrane (HET-CAM) assay, differs in the evaluation criteria but is also normally included among this class of *in vitro* protocols.

Each of these protocols is discussed below in detail as representative candidate *in vitro* organotypic methods for assessing ocular irritation and corrosion. The methodologies, protocol details, applications, and their validation status are outlined. A brief historical perspective of the development of original *in vitro* ocular testing models is also mentioned. More importantly, improving upon and troubleshooting the current techniques, in order to present the models as stand-alone *in vitro* tools for ocular toxicity assessment, is emphasized.

2. Methods for Acute *In Vitro* Ocular Toxicology Testing

2.1. *In vivo* Draize rabbit eye test

The current *in vivo* Draize rabbit eye test method identifies both irreversible (e.g., corrosion) and reversible ocular effects (Draize *et al.* 1944). It also provides scoring that allows for relative categorization of severity for reversible effects such as mild, moderate, or severe irritants. Despite its scientific endurance in toxicity testing however (significant pain inflicted on the rabbits notwithstanding), the Draize eye test has been justifiably criticized for several shortcomings: its lack of reproducibility, its subjective nature of assessment, the variable interpretation of results, the high dose of test material used, and the over prediction of human response (Christian and Diener, 1996; Lordo *et al.*, 1999; Ohno *et al.*, 1999; Weil and Scala, 1971).

The normal endpoints of toxicity for the *in vivo* rabbit eye test include corneal opacity, inflammation, and cytotoxicology. Current U.S. Environmental Protection Agency (EPA 1998) ocular testing guidelines and the United Nations (U.N.) Globally Harmonized System (GHS) of Classification and Labeling of Chemicals (U.N. 2003) indicate that if serious ocular damage is anticipated[1], then a test on a single animal is considered. When anticipated or unanticipated damage is observed, then no further animal testing is necessary (EPA 1998;U.N. 2003). If it is not observed, additional test animals may be evaluated sequentially until concordant irritant or nonirritant responses are detected.

2.2. Development of *in vitro* ocular toxicology testing methods

One of the original models developed in the early 1990s as a biochemical procedure to evaluate ocular irritation was the EYTEX™ system (Balls *et al.*, 1995). The test was based on the concept that the normal transparent state of the cornea depends on the relative degree of hydration and organization of corneal proteins. Corneal opacification, therefore, is a result of a decrease in protein hydration associated with changes in protein conformation and aggregation. The EYTEX™ test simulated corneal opacification by using alterations in the hydration and conformation of an ordered macromolecular matrix to predict *in vivo* ocular irritancy. Although the model did not prove reliable as an *in vitro* prescreening system for determining eye irritation potential of cosmetic formulations, it set the foundation for further development of ocular toxicology testing models based on production of corneal alterations.

Although progress was slow initially, by the next decade, the race to develop ocular and dermal *in vitro* toxicity testing methods gained sufficient momentum. In October, 2003, the U.S. EPA nominated several *in vitro* ocular toxicity test methods for evaluation by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, National Institutes for Environmental Health and Safety [NIEHS]). Of the many nominations received by ICCVAM (ICCVAM, NIH Publication No. 03-4508, 2003), the Committee determined that

[1]Serious ocular damage to the rabbit eye is described as irreversible adverse effects still occurring on day 21 after exposure.

four of the *in vitro* test methods proposed for identifying potential ocular corrosives and severe irritants in a tiered-testing strategy should have the highest priority for evaluation (ICCVAM, NIH Publication No. 97-3981, 1997). These included:

1. the hen's egg test-chorioallantoic membrane (HET-CAM) assay;
2. the bovine corneal opacity and permeability (BCOP) assay;
3. the isolated chicken eye test method (ICE); and,
4. the isolated rabbit eye (IRE) assay.

Working closely with the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM, NIEHS), and with the European Centre for the Validation of Alternative Methods (ECVAM), ICCVAM established an ocular toxicity working group to complete the test method evaluations. After reviewing the available data and information, comprehensive background review documents (BRDs) were prepared for each of the four methods. The BRDs described the current validation status of the *in vitro* test methods, including their reliability and accuracy, the scope of the substances tested, and the availability of standardized protocols. Subsequently, ICCVAM organized an international independent expert panel in January 2005 to assess the validation status of the methods. By March 2005, ICCVAM coordinated the production of draft reports, addenda, modifications of the original BRDs, and evaluation by a public advisory group (Scientific Advisory Committee on Alternative Toxicological Methods, SACATM; ICCVAM, NIH Publication No. 06-4511, 2006). Although ICCVAM endorsed the pre-evaluation of these methods by NICEATM (ICCVAM, NIH Publication No. 06-4511, 2006), the Committee concluded that none of the four *in vitro* test methods could be considered to be replacements for the *in vivo* Draize rabbit eye test (ICCVAM, NIH Publication No. 06-4512 to 06-4515, 2006). However, results of preliminary validation suggested that BCOP and ICE could be used as screening tests for the detection of ocular corrosives and severe irritants in a tiered-testing strategy, where positive substances meet criteria for classification as ocular corrosives as part of a weight-of evidence (WoE) approach. At that time, HET-CAM and IRE were not considered to have sufficient performance or an adequate amount of robust data to substantiate their use for regulatory hazard and classification purposes. Consequently the latter were not recommended as screening tests for the detection of ocular corrosives and severe irritants (ICCVAM, NIH Publication No. 06-4511, 2006), but were deemed useful for other objectives.

Similarly, and a few years before, ECVAM recommended the BCOP and ICE *in vitro* test procedures for use as part of a preclinical battery of tests, which have since been integrated in test protocols by many E.U. countries for identification of ocular irritants (U.N. GHS of Classification and Labeling of Chemicals Category 1, E.U. R41, 2003). More importantly, the Organization for Economic Co-operation and Development (OECD) has formally adopted test guidelines (TG) 437 and TG 438 that describe the BCOP and ICE test methods, respectively (OECD 2009, TG 437 & 438). Consequently, these regulatory documents promulgate the international use of the test methods for identifying ocular corrosives and severe irritants without relying on incorporation of animals in a toxicity testing study. Furthermore, at its 26th meeting (27 April 2007), the ECVAM Scientific Advisory Committee (ESAC) issued a statement on the conclusions of the ICCVAM retrospective study on organotypic *in vitro* assays as screening tests to identify potential ocular corrosives and severe irritants. The Committee unanimously endorsed that the BCOP and ICE test methods had sufficient data to support their use for identifying substances as ocular corrosives and severe irritants in a tiered-testing strategy. In addition, the Committee could not comment on the validity of IRE or HET-CAM until further work was performed (ECVAM Scientific Advisory Committee, 2007).

OECD continuously updates existing test guidelines as restructured draft proposals for future adoption. For instance, TG 430 and TG 431 outline testing guidelines for *in vitro* skin corrosion using the transcutaneous electrical resistance (TER) test based on isolated rat skin (OECD 2004, TG 430) and reconstructed human epidermis (RhE) (OECD 2004, TG 431), respectively. Also, document TG 435 represents a draft proposal for a new guideline incorporating an *in vitro* membrane barrier test method for skin corrosion based on the commercially available Corrositex® (OECD 2006, TG 435). And, ICCVAM and its Dermal Corrosivity and Irritation Working Group are currently participating in the development of other OECD TGs using *in vitro* human skin model systems, like EpiDerm™ and EpiSkin™, for the identification of substances with the potential to cause skin irritation.

In addition, an extensive number of *in vitro* eye irritation models have been developed and proposed as alternatives to the rabbit Draize eye test. Eskes *et al.* (2005) advance these methods from the perspective of relevance, role of developers, known users, applications, limitations, recommendations, as well as inclusion of short descriptions. Even the selection of test chemicals has been coordinated for the ECVAM-funded skin irritation validation study (SIVS), whose aim is to evaluate whether the EpiDerm™, EPISKIN™ and the mouse skin integrity function test (SIFT) are able to reliably identify skin irritant and non-irritant chemicals (Eskes *et al.*, 2007). The protocols include: isolated organ methods (BCOP, IRE, CEET), organotypic models (HET-CAM, CAM-VA, CAM-TB), reconstituted human tissue models (MatTek EpiOcular™, SkinEthic HCE™, HCE-TER), cell-based cytotoxicity methods (NRU, NRR, RBC hemolysis), and cell function-based assays (FL, SM)[2].

2.3. Tiered-testing approach

Tiered testing, standardization and validation of methods, and risk assessment are concepts which have been discussed and reviewed extensively, particularly since they must be continuously updated to accommodate the large influx of chemicals, methods and data into the regulatory arena. Although it is beyond the scope of this review to completely discuss the role of tiered testing, a brief overview of the process and its participation in the utilization of *in vitro* testing assays is appropriate.

The tiered testing approach relies on a systematic sequence of ranked levels used in a testing strategy. A positive result from a test indicator at the first or primary level qualifies the substance for confirmation of that positive result at the subsequent level, usually in order of complexity of effects detected with the indicator. For instance, analysis of a test agent for structure-activity relationship and physicochemical properties is frequently employed as a screening test in the first tier. The analysis of structural features of a chemical may suggest similar mechanisms of toxicity of chemicals in structurally related classes. This is followed by a second set (second tier) of tests incorporating short-term bacterial or mammalian assays, after which a decision point is determined, based on the evaluation of results from data accumulated at levels 1 and 2. The process continues through a third level in assigned categories of irritation toward the evaluation of human and animal risk assessment.

Specifically, current alternative approaches to the assessment of eye irritation make routine use of tiered testing strategies based initially on the WoE approach (McNamme *et al.*, 2009). The principle is that all available information is considered in the evaluation of eye irritation, which may include physicochemical properties of the test chemical, historical *in vivo* animal data, *in vitro* and human data, and exposure. A tiered testing strategy using a decision tree

[2]CAM-VA = chorioallantoic membrane vascular assay; CAM-TB = chorioallantoic membrane trypan blue staining test; CEET = chicken enucleated eye test; FL = fluorescein leakage; HCE = human corneal epithelium; HET-CAM = hen's egg test-chorioallantoic membrane; HET-TER = hen's egg test-transepithelial electrical resistance; ICE = isolated chicken eye; IRE = isolated rabbit eye; NRU/NRR = neutral red uptake/release; RBC = red blood cell; SM = silicon microphysiometer.

approach follows if the data from the WoE approach is deemed insufficient. The 3-step decision tree evaluates ingredients incorporating all available data (step 1), an *in vitro* severe eye irritancy test (step 2), and an *in vitro* mild to moderate irritancy test (step 3). A more complex alternative decision tree evaluates non-irritants, irritants and severe eye irritants using validated *in vitro* assays as well as scientifically applicable *in vitro* methods with acceptable industrial experience (McNamme *et al.*, 2009).

Similarly, Scott *et al.* (2010) propose an *in vitro* eye irritation testing strategy using bottom–up and top–down approaches. Based on an expert meeting convened by ECVAM in February 2005, the working group evaluated and categorized assays based on their proposed applicability domains (e.g., categories of chemical irritation severity, modes of action, chemical class, and physicochemical compatibility). This tiered approach for risk assessment parallels the tiered testing strategy mentioned above while incorporating controlled human testing if needed. The group proposed that the test method used, in the bottom–up and top–down testing strategy, should be relative to the anticipated severity of the eye irritation based on the applicability domains. Thus, if the test material is expected to be a non- to low-eye irritant, the bottom–up approach is initiated. Conversely, the top–down approach is obligatory if the test material is expected to be a moderate to severe eye irritant. A default EU R36/GHS category 2 classification is assigned if neither a non-classified or severe irritancy assignment is made. Relative to the organotypic models, therefore, the group concluded that isolated organ methods have the greatest potential to distinguish severe eye irritants (EU R41/GHS category 1) from other classes (EU R36/GHS category 2, or not labeled). Epithelial models (EpiOcular™ or HCE) and test methods which assess the potential of a test material to disrupt cellular membranes (RBC, HET-CAM), have the greatest potential to distinguish non-classified substances from irritants (Scott *et al.*, 2010).

The current review focuses on and describes the principles, detailed protocols and validation status of the organotypic models (Table 1). The epithelial models (EpiOcular™ and HCE™) are briefly summarized after this section since they are currently part of intensive validation investigations with ECVAM. Contemporary and updated U.S. validation status of each test application is summarized in the **Conclusions** section. It is important to note that no single *in vitro* test method protocol has gained wide acceptance as representative of standard procedures for any of the methods.

3. Hen's egg test-chorioallantoic membrane (HET-CAM) test

3.1. Background

The HET-CAM provides information on the effects that may occur in the conjunctiva following exposure to a test substance. The test method was an extension of traditional chicken embryo models used by embryo-toxicologists and virologists (Luepke and Kemper 1986; Parish 1985) and was based on the observation that the chorioallantoic membrane (CAM) of an embryonated hen's egg is similar to the vascularized mucosal tissues of the human or rabbit eye. Initial results in the 1990s, using modifications of the current HET-CAM test, showed high rank order correlation between data from testing chemicals and cosmetic formulations with Draize *in vivo* results. Later studies included classification of several classes of irritants, ranging from non-irritant to severe, with high correlations between the systems. This development was encouraging since it suggested that *in vitro* models could represent valid alternative constructs to predict *in vivo* ocular toxicity. Thus, it was reasonable to conclude that adverse effects on the CAM could correlate to irritation or corrosion *in vivo*. Consequently, the HET-CAM was validated for its ability to predict ocular corrosives and severe or irreversible effects as defined by the U.S. EPA (1996), the E.U. (2001), and the U.N. GHS of Classification and Labeling of Chemicals (UN, 2003).

3.2. Overview of the Protocol

The major components of the HET-CAM test method involve exposing and treating the CAM of chicken eggs, with a test substance. The CAM is a vascular fetal membrane composed of the fused chorion and allantois. The development of endpoints are observed and scored, and the data is evaluated in relation to a prediction model. The endpoints monitor the changes that occur in blood vessels in the CAM.

The allantois functions in maintaining chick embryo viability by serving as an embryonic respiratory, excretory, and storage organ. During chicken embryo development, the allantois increases in size, wraps around the embryo and fuses with the chorion to form the CAM (Tufan and Satiroglu-Tufan 2005). The fusion of the two membranes allows for a free exchange of gases between the embryo and the environment, after which there is a rapid increase in the surface area for the first 10 days of incubation (Sinn-Harlon 1998).

3.3. Details of the Typical Protocol

Fertilized White Leghorn hen's eggs are incubated at 37°C±1°C, at an average relative humidity of 62.5%, for 9 or 10 days. The eggs usually weigh between 50 and 80 grams and are rotated several times daily until the procedure is performed (Spielmann 1997). In general, based on the observation of the development of the CAM, the earliest incubation day for test application is day 7. On day 10, the eggs are surgically cut with a rotary saw or tool with a cutting disk, a portion of the eggshell is removed and the inner membrane and CAM are exposed.

A 200 to 300 μl of test substance is applied to the surface of the CAM for 20-seconds and rinsed with water. Appropriate test substance controls are incorporated, including positive, negative, solvent, and benchmark controls. At 0.5, 2, and 5 minutes after rinsing, visual inspection is used for evaluating development of irritant endpoints, including hyperemia, hemorrhage, and coagulation. The development of hemorrhage and coagulation is evaluated in combination with other endpoints including injection (mild hemorrhage), vasoconstriction, dilation, and lysis (Budai and Várnagy 2000; CEC 1991; Gettings *et al.* 1996; Luepke and Kemper 1986; Macián *et al.* 1996; Sterzel *et al.* 1990).

Scoring is assigned based on the time required for development of each endpoint and evaluated according to a variety of user-defined scoring scales. In general, the scores range from 0 (no effect) to 3 (severe irritant effect) (Balls *et al.* 1995; Steiling *et al.* 1999). For instance, the *irritation score (IS)* represents the *irritation potential* (*IP*) of the test substance based upon the time (seconds) of the first appearance of blood hemorrhage (*hemorrhage time*), vessel lysis (*lysis time*), or protein coagulation (*coagulation time*).

3.4. Decision criteria

Several computations of relative scores are interpreted in the decision analysis. The *Q-score* represents a comparison of the *IP* of the test substance with that of a reference substance. This score is calculated using the *IP* of both the test substance and a reference substance. The *irritation threshold concentration* (*ITC*) is defined as the lowest concentration required to produce a slight or weak response after application of the test substance. The *S-score* (severity score) is the highest total score for any endpoint evaluated for a test substance. Based on a range of 0 to 3, severity scores assigned for each endpoint are totaled for all of the replicate eggs per test substance, and calculated to produce an endpoint total score (Demirci *et al.* 2003, 2004). The *severity irritation score* (*SIS*) is based on the potency of the anti-angiogenic effect of the test substance. The scores are summarized to yield an *IS* for the test substance (maximum score of 21), for which they are subsequently assigned to an irritancy category according to Table 2 (Kalweit *et al.* 1987; Luepke 1985).

Alternatively, the Federal Hazardous Substance Act (FHSA) Classification System (Gettings *et al.* 1996) assigns irritation classification based on *Q-scores*, mean coagulation detection time, combination of *IS* and *ITC*, and *S-scores*.

Based on the *IS* classification, the HET-CAM is proposed to provide information on the effects that may occur in the human or animal conjunctiva following exposure to a test substance as a response to local injury, rather than monitoring direct cytotoxicology. The test also assumes that acute effects induced by a test substance on capillaries and proteins in the membrane are similar to effects induced by the same test substance in the eye of a treated rabbit and, moreover, correlates to irritation and/or corrosion in human eyes.

3.5. Validation

The HET-CAM test method is currently used by a variety of toxicology testing consortia for the identification of ocular corrosives and severe irritants in a tiered testing strategy. In this approach, positive *in vitro* test results are considered in a WoE decision in order to classify the substance as an ocular corrosive or severe irritant. The ability of the test to correctly identify ocular corrosives and severe irritants, as defined by the EPA (1996), the EU (2001), and the GHS (UN 2003), has been investigated in controlled validation studies (ICCVAM, NIH Publication No. 06-4511, 2006).

In preliminary studies, ICCVAM evaluated several HET-CAM analysis methods and summarized the range of hazard classification accuracy rates across the EU, EPA, and GHS classification systems. The rates ranged from 65% to 68% and 52% to 57% for 2 analysis methods (IS(B)-10 and IS(B)-100, respectively), based on the decision criteria of Luepke (1985). The overall false negative and false positive rates of the methods ranged from 6% to 59%, respectively, depending on the classification system. Qualitative analysis of interlaboratory reliability for the analysis methods were in 100% agreement for approximately 80% of the substances evaluated. In addition, mean and median values of coefficient of variation (CV) calculated for quantitative evaluation of interlaboratory reproducibility for 14 substances at 100% concentrations, indicated that the data were not robust. Consequently, based on these results, the HET-CAM was not recommended for screening and identifying ocular corrosives and severe irritants, as a replacement for the Draize rabbit eye test (ICCVAM, NIH Publication No. 06-4511, 2006).

The HET-CAM test method, however, has shown potential to refine and reduce animal use in eye irritation testing, when used in the GHS tiered testing scheme. In addition, for assessing severe eye damage, this method reduces the numbers of animals subjected to testing and reduces the pain and suffering of rabbits by their exclusion from the testing of corrosives and severe irritants.

4. Bovine Corneal Opacity and Permeability (BCOP) assay

4.1. Background

The BCOP assay is a modified organotypic model (i.e., isolated whole organ) *in vitro* eye irritation test method that uses isolated eyes from slaughtered cattle (Gautheron *et al.* 1992; Muir 1985). The assay provides for short-term maintenance of normal physiological and biochemical function of the cornea in an isolated system. The basis of the test relies on the role of the cornea as an indicator of visual impairment resulting from damage occurring during accidental eye exposure to chemicals. In addition, results obtained with the BCOP assay are comparable to those obtained with the *in vivo* Draize eye test, since the corneal effects with the latter are of considerable significance in the scoring system for ocular irritancy.

4.2. Overview of the Protocol

As an important transparent tissue in visual functioning, the cornea is the major refracting element in the optical path for light passage through the lens and toward the retina. The BCOP assay measures two important components of ocular irritation affecting the cornea—opacity and permeability (Gautheron *et al*., 1995). Opacity, which is experimentally determined by the amount of light transmission through the cornea, was initially investigated as the only corneal endpoint graded in many *in vivo* ocular irritancy assays. Irritant-induced opacity in the cornea is an indicator of protein denaturation, swelling, vacuolization, or damage in the epithelial or stromal layers (Millichamp, 1999). Permeability is measured by the amount of sodium fluorescein dye that passes through all corneal cell layers. However, since ocular irritation can also involve the iris and conjunctiva, this method originally underrepresented the complex response of the eye to irritants. To overcome the criticisms, several additional endpoints were added to the BCOP assay, including corneal swelling and histological evaluation of morphological alterations (Cooper *et al.* 2001; Curren *et al.* 2000). In addition, the use of opacity and permeability together were purported to better predict ocular irritancy.

4.3. Details of the Typical Protocol

The basic procedure has undergone some modifications (Sina and Gautheron, 1994, 1998) since first described by Gautheron *et al.* (1992). The BCOP assay uses isolated corneas from the eyes of freshly slaughtered cattle obtained from a local slaughterhouse. The abattoir should be in close proximity to allow for transport of the eyes to the laboratory within 2 to 4 hours after the animals are killed. Immersion in Hanks' Balanced salt solution (HBSS) is adequate for storage during transport at either ambient or cold temperatures.

Initial experimental preparatory steps require dissection of the cornea from the eye structure and mounting on a plastic holder fitted into a two-compartment cell capable of measuring light transmission. Corneas are surgically dissected to avoid epithelial and endothelial damage. A 2 to 3 mm rim of sclera should remain to assist in subsequent handling. Isolated corneas are mounted in corneal holders consisting of anterior and posterior compartments (5 ml volume) which interface with the epithelial and endothelial sides of the cornea, respectively[3]. Both chambers are filled with medium and then the cornea is mounted over an O-ring that is positioned around the opening of the posterior chamber. The chambers are clamped together and the dosing holes located on the top of each chamber allow the epithelial and endothelial sides of the cornea to be treated independently. The assembly is equilibrated at 32°C ± 1°C for 1 hour, after which the medium is changed and baseline opacity measurements are established (Casterton *et al.* 1996;Gautheron *et al.* 1992;Rachui *et al.* 1994). After the equilibration period, corneas are examined for defects and are discarded if baseline readings register above 10. With the epithelial side of the cornea in the anterior position, the test substance is introduced in the upper chamber, after which transmission is measured spectrophotometrically.

Changes in light passage through the cornea are commonly assessed with a white light, dual-beam opacitometer that provides a center-weighted reading of light transmission. The two compartments, each with its own light source and photocell, are individually used for treated corneas and for calibration and standardization.

Permeability measurements are based on the amount of sodium fluorescein (NaFL) that permeates through the cornea into the posterior chamber. Immediately after completing the final opacity measurements, 1 ml of NaFL (0.4% for liquids and surfactants, 0.5% for solids) is added to the anterior compartment of the corneal holder. The apparatus is incubated

[3]More recently, a redesigned corneal holder has been introduced that improves the maintenance of normal corneal morphology (Ubels *et al.* 2002).

horizontally for 90 minutes and the amount of dye that penetrates the cornea is determined by measuring the optical density (OD) of the medium in the posterior chamber (compared to controls) using a microplate reader or UV/VIS spectrophotometer set at 490 nm. Measurement of diffusion of dye to the lower chamber is correlated with the opacity index (see 4.4. Decision Criteria, below) and with *in vivo* Draize test scores.

Treatment protocols are separately employed depending on test substance characteristics (e.g. for liquids, surfactants, or solids). Application of 750 μl dilutions of the solutions are inserted through the dosing holes and corneas are incubated horizontally for 10 ± 1 minutes at 32°C ± 1°C. The chemical is removed from the anterior compartment and the epithelial surface is washed at least three times. After refilling both chambers with fresh medium, a second opacity measurement is similarly recorded, after which the corneas are incubated for an additional 2 hours prior to final opacity measurements. Solids are tested as solutions or suspensions for 4 hours at 32°C ± 1°C as 20% dilutions in saline or deionized water. After examination for visible insoluble or suspended particles, test materials are subsequently manipulated as with liquids.

Although the BCOP assay can accommodate a wide range of physicochemical characteristics, low density water insoluble substances do not adequately contact the cornea during treatment (Sina and Gautheron 1998). Also, opaque or colored test substances interfere with opacity and permeability measurements. Appropriate controls must also be included that monitor for positive, negative, solvent, and benchmark activity.

4.4. Decision criteria

Mean corrected opacity values and mean corrected permeability values (OD units ± SD) are calculated for each treatment group. As with the HET-CAM assay, an *in vitro* irritancy score (*IS*) is computed combining both opacity and permeability values. The substance is assigned to categories based on a threshold *in vitro* Irritancy Score of 55.1 and above, or a permeability value greater than 0.600, for the identification of severely irritating chemicals (Sina *et al.* 1995). Substances are assigned to categories according to the classification system outlined in Table 3.

Alternatively, opacity and permeability values are evaluated independently and compared to benchmark materials. Histological observations, if necessary, are used to determine the type and depth of corneal injury, as well as the reversibility of tissue damage. After determination of scores, the cornea is fixed in 10% neutral buffered formalin (NBF-10) at room temperature for at least 24 hours before processing as per paraffin sections. Corneal sections are stained in hematoxylin and eosin and examined for lesions in the epithelium, stroma, and endothelium (Curren *et al.* 2000). Thus, histopathological analysis may be of importance for ocular irritants with questionable observable irritation, for clarification of reversibility, to determine occult changes or to reduce false negative results (Harbell and Curren 2002).

4.5. Validation

E.U. regulatory agencies accept positive outcomes from the BCOP ocular irritation test method for classifying and labeling severe eye irritants. When a negative result is obtained, an *in vivo* test is subsequently required, as BCOP has not been shown to adequately discriminate between eye irritants and non-irritants (EU 2004). The assay is currently used by some U.S. and E.U. companies as an in-house method to assess the ocular irritation potential of a wide range of substances. In addition, the BCOP test method could potentially be used to identify irreversible, corrosive, and severe irritation potential of drugs and chemicals, in a tiered testing strategy (e.g., GHS; U.N. 2003). EPA, GHS, and EU have also evaluated and recommended its possible future use for the identification of mild and moderate ocular irritants based on: histopathological evaluation of corneal tissue; use of a corneal holder that maintains normal

corneal curvature; or, examining the effect of modifying various test method protocol components, such as duration of exposure, on the accuracy and reliability of the test method.

In validation studies conducted by ICCVAM, false negative rates for alcohols and solids range from 42% to 100% depending on the hazard classification system. Coefficient of variation (CV) analysis of BCOP test method intralaboratory repeatability data (*in vitro IS*) from two studies ranged from 11.8% to 14.2% for 16 substances and from 1.1% to 13% for five substances predicted as severe irritants. Intralaboratory reproducibility evaluations indicated mean and median CV values for permeability were 33.4% and 29.0%, respectively, for 25 surfactant-based personal care cleaning formulations. Qualitative assessment of interlaboratory reproducibility categorized 67% to 94% of the substances in the same categories by the participating laboratories. Substances with less than complete agreement in the testing laboratories included chemical classes such as alcohols, ketones, and heterocyclic compounds. Quantitative analysis of interlaboratory reproducibility determined that mean and median CV values ranged between 36% and 17%, in a variety of laboratories. Consequently, based on these results, there was sufficient data to support the use of the BCOP test method, in appropriate circumstances and with certain limitations, as a screening test to identify substances as ocular corrosives and severe irritants (i.e., EPA Category I, U.N. GHS Category 1, EU Category R41) in a tiered-testing strategy (ICCVAM, NIH Publication No. 06-4511, 2006). More recently, however, ICCVAM proposed that the BCOP test method is not recommended to identify substances from all hazard categories as defined by the GHS, EPA, and EU classification systems (EPA 1996; EU 2001; UN 2003), particularly because of the significant lesions associated with 50% of the EPA Category III substances that were false negative in BCOP. In addition, the BCOP test method can be used as a screening test to identify substances not labeled as irritants (i.e., EU Not Labeled, GHS Not Classified), from all other hazard categories (GHS Category 1, 2A, or 2B) (ICCVAM 2009).

5. Isolated Chicken Eye (ICE) test

5.1. Background

The ICE protocol was first developed based on the Isolated Rabbit Eye (IRE) method in order to obviate the need for laboratory animals as the source for test eyes (Prinsen and Koëter, 1993). For this reason it has remained essentially the same as the IRE assay. The ICE test method is an organotypic *in vitro* bioassay where the test substance is applied to the cornea of eyes isolated from chickens that have been processed for human consumption. The model provides short-term maintenance of normal physiological and biochemical function of the chicken eye in an isolated system.

5.2. Overview of the Protocol

Enucleated chicken eyes are immediately dissected and irrigated with isotonic saline. Test substances are applied as single doses for 10 seconds, followed by rinsing with isotonic saline. Corneal reactions are measured at regular intervals up to four hours post-treatment. As with the BCOP assay, damage by the test substance is assessed by determination of corneal indicators: 1. corneal swelling; 2. opacity; and, 3. fluorescein retention (FR). 1. Corneal swelling is determined by calculating the increase in corneal thickness, a quantitative and reliable endpoint of corneal injury, from a baseline measurement (Burton 1972). 2. The degree of corneal damage, as determined by measuring corneal opacity, provides an indicator that is directly correlated to the *in vivo* rabbit eye test. 3. FR provides an assessment of corneal permeability, indicative of damage to the corneal surface. For each substance, a quantitative score is calculated to determine an overall Irritation Index (*II*) or the chemical is qualitatively assigned to an *in vitro* irritancy classification.

5.3. Details of the Typical Protocol

Enucleated chicken eyes are isolated from spring chickens of either sex, approximately seven weeks old and 2.5 to 3.0 kg. The laboratory should be located close to the breeder or slaughterhouse so that transportation to the laboratory and processing is accomplished within two hours. The temperature range during transport is not considered critical. Eyes are immediately dissected and placed in a superfusion apparatus supplied with isotonic saline (32° C ± 1.5°C) through a steel tube attached to a peristaltic pump. Each eye is mounted in a custom-built stainless steel clamp with the cornea positioned vertically and then transferred to a chamber in the superfusion apparatus. Eyes are examined with a slit-lamp microscope for minimum viability requirements and baseline measurements—i.e. corneal thickness should not deviate by more than 10% from the mean value, should not have a FR score of greater than 0.5 (indicating corneal permeability), or should not show corneal opacity or any other signs of damage.

After an equilibration period, the eye is removed from the superfusion apparatus and placed horizontally in the holder. Thirty microliters of a test substance is applied as a single dose for 10 seconds, followed by rinsing with isotonic saline (20 ml). Following exposure to the chemical substance, the extent of damage to the cornea is determined by measuring the corneal indicators. Corneal reactions are measured at regular intervals up to four hours post-treatment, and mean values for each parameter (corneal swelling and opacity) are determined. FR is evaluated at 30 minutes post-treatment. Test eyes and appropriate controls, including positive, negative, solvent, and benchmark controls, as well as control eyes, are examined pre-treatment and at 30, 75, 120, 180, and 240 minutes after treatment.

Quantitative measurements, expressed as a percentage of control, are determined from analysis of corneal swelling only, thus supporting improved precision and reduced interlaboratory variability. Corneal thickness measurements, as indicators of corneal swelling, are performed with an optical pachymeter on a slit-lamp microscope, measured at the corneal apex. Corneal opacity is also determined with slit-lamp examination, by scoring the area of the cornea that is most densely opacified. Based on the highest mean score for corneal opacity observed at any time point, an overall category score is assigned for each test substance. Mean FR values for all test eyes are calculated at 30-minute time points post-treatment only. Substances that adhere to the cornea must be adequately removed before FR is determined. After the final examination at four hours, eyes are typically fixed in 4% NBF for histopathological examination.

5.4. Decision Criteria

For the ICE assay method, the irritation potential of the test substance is based on the maximum mean values of the measurements and is defined within categories ranging from non-irritant to severely irritant. The four irritancy categories, therefore, are interpreted from corneal thickness, corneal opacity, and fluorescein retention measurements, monitored alone or in combination, according to the classifications listed in Table 4.

5.5. Validation

In validation studies conducted by ICCVAM, the accuracy of the ICE test method for identifying ocular corrosives and severe irritants, as defined by the EPA (1996), EU (2001), and GHS (UN 2003), was evaluated and assessed in separate *in vitro-in vivo* comparative studies and found to be comparable among the three hazard classification systems (83% to 87%). Sensitivity and specificity calculations ranged from 50% to 59% and 92% to 94%, respectively. False positive rates ranged from 6% to 8%, while the false negative rates ranged from 41% to 50%, although the latter improved to 29 to 33% when outlier substances within these chemical and physical classes were excluded from the database. CV values for corneal

thickness measurements ranged from 0.9% to 6.1%. Alcohols demonstrated the highest false positive rate in all three classification systems, ranging from 27% to 50%.

The validation study concluded that the method evaluates only corneal effects. It does not take into account the influence on the iris and the conjunctiva and does not measure reversibility of corneal effects. Systemic effects following ocular instillation are not observed and the assay will not identify slow-acting irritants. However, there are sufficient data to support the use of the ICE test method as a screening test to identify substances as ocular corrosives and severe irritants (i.e., EPA Category I, UN GHS Category 1, EU R41) in a tiered-testing strategy, as part of a WoE approach. Furthermore, the study recommended that histopathological evaluation of the corneal tissue, using a standardized scoring scheme, should be included to characterize and improve the usefulness of the method (ICCVAM, NIH publication No. 06-4511, 2006).

6. Isolated Rabbit Eye (IRE) Test

6.1. Background

The IRE test was developed as an *in vitro* alternative to the *in vivo* Draize rabbit eye test method for the assessment of eye irritation (Rabbit Enucleated Eye Test, REET; Burton *et al.* 1981). As with the ICE, the IRE is an organotypic test method that eliminates the use of live animals for ocular irritancy testing and thus obviates the pain and suffering potentially associated with the *in vivo* Draize rabbit eye test. The method is also purported to incorporate rabbit eyes isolated from euthanized animals used for other purposes, such as a food source.

6.2. Overview of the Protocol and Validation Summary

In the IRE test method, candidate substances are applied over the corneas of enucleated rabbit eyes in a manner similar to the ICE test. As with the BCOP, the effects of the test substance on the cornea of the isolated eye are measured quantitatively as an increase in thickness (swelling), opacity and fluorescein penetration, as well as for morphological changes to the corneal epithelium. The lack of a widely accepted standardized protocol for detecting ocular corrosives and severe irritants, however, has hampered the acceptance of this test method as a replacement for the Draize rabbit eye test. In addition, based on the accuracy, false negative and false positive rates (35% to 40%), ICCVAM did not recommend the method for screening and identifying ocular corrosives, severe irritants, or for distinguishing non-irritants from mild irritants (ICCVAM 06-4511, 2006; ICCVAM 2009). There also are insufficient data using all four recommended IRE endpoints (corneal opacity, corneal swelling, fluorescein penetration, and histological observations of significant effect on corneal epithelium). Thus, any further information about the model and the protocols are found in other reviews (Balls *et al.* 1995; Burton *et al.* 1981; Gettings *et al.* 1996; Guerriero *et al.* 2004).

7. Epithelial Models

7.1. EpiOcular™ (MatTek Corporation, Ashland, MA, USA)

EpiOcular™ is a an *in vitro* human corneal model, designed to replace the traditional animal Draize eye test. It is a three dimensional *in vitro* human corneal epithelium composed of normal human-derived epidermal keratinocytes cultured on a permeable polycarbonate membrane that forms a stratified, squamous multi-layered epithelium similar to that of the eye cornea. The tissue construct has an air-liquid interface and exhibits morphological and growth characteristics that mimic *in vivo* conditions. Cellular viability is measured with the MTT colorimetric assay, as well as other *in vitro* indicators, using an ET_{50} (effective time) and IC_{50} endpoint for comparison among different potential chemical substances and to the *in vivo* Draize test. The model provides data that resolves degrees of cellular cytotoxicology as

a reflection of ocular irritation, from moderate-to-very mild irritancy range through responses induced by high moderate-to-severe irritation (Barile 2007; Eskes *et al.*, 2005).

Currently, ECVAM is conducting formal validation studies with EpiOcular™ in two phases using coded and non-coded test materials (Blazka *et al.*, 2003; Eskes *et al.*, 2005). Preliminary evaluation suggests that the assay is applicable to both hydrophilic and hydrophobic test materials in either liquid or solid state, and can differentiate mild to moderate irritants while identifying severe irritants. In combination with an organotypic model, the *in vitro* battery could possibly be useful to resolve the full range of irritation potentials (Eskes *et al.*, 2005).

7.2. Human corneal epithelium (HCE™, SkinEthic Laboratories, Nice, France)

Similar to the EpiOcular™ model, the HCE™ model consists of immortalized human corneal epithelial cells cultured at the air–liquid interface on a polycarbonate substrate membrane. The air–epithelial tissue lacks a stratum corneum and thus morphologically resembles the corneal mucosa of the human eye. Cellular viability is measured with the MTT colorimetric assay, as well as other *in vitro* indicators, particularly LDH release, histology, quantification of cytokine release, and gene expression. ECVAM sponsored *in vivo/in vitro* comparisons in formal validation studies currently indicate usefulness of the model as a pre-screen for eye irritation of test ingredients and raw materials. Although the method has not satisfied a complete formal validation study, the HCE model is also suitable to detect corneal repair and recovery *in vitro* (De Wever *et al.*, 2002; Nguyen *et al.*, 2003).

8. Conclusions

In the U.S., two major systems are currently used for *in vivo* ocular irritation classification—the FHSA guideline (Federal Hazardous Substances Act; U.S. CPSC 1995), and the EPA guideline (U.S. EPA 1996). The FHSA guideline states that a test substance is considered an eye irritant if four or more of six rabbits have positive ocular scores in non-irrigated eyes within 72 hours after instillation of the test substance. The EPA classification guideline considers the kinds of ocular effects produced in the *in vivo* rabbit eye test, as well as the reversibility and the severity of the effects. However, unlike the FSHA system, incidence is not considered, as classification is based on the rabbit that exhibits the most severe response in a group of three or more rabbits.

Comparisons of the various organotypic methods presents a variety of advantages and disadvantages unique to the particular systems. For instance, the limitations of the bovine cornea in the BCOP isolated eye test are its dimensions and the thickness of the cornea, which creates difficulty in screening mild irritants. The same applies for the pig cornea. In addition, due to the slaughtering process, obtaining eyes from processed cows and pigs, as well as the labor required to remove the eye, is more difficult than in chickens (Prinsen and Koëter 1993). The structure of the chicken cornea is comparable to the rabbit cornea and, additionally, has well-developed features as the human. Finally, because of the dark black background provided by the iris of a chicken eye, changes in opacity are more easily discriminated than in the rabbit.

More recently, ICCVAM has reviewed the validation status of these *in vitro* test methods for identifying non-severe ocular irritants (i.e., those that induce reversible ocular damage) and substances not labeled as irritants. For instance, the original ICCVAM recommendation for the use of the BCOP and ICE test methods to identify substances as ocular corrosives or severe irritants remains unchanged (ICCVAM, 2009). Although these two methods are recommended for screening and identifying ocular corrosives and severe irritants as described above, in a tiered-testing strategy as part of a WoE approach, based on an evaluation of available data and corresponding performance, the Committee did not recommended the models for identifying

substances from all hazard categories as defined by the GHS, EPA, and EU classification systems. Similarly, the recommendation for the HET-CAM and IRE test methods has not changed since the original ICCVAM evaluation (ICCVAM, NIH Publication No. 06-4511, 2006)—that is, they are not recommended for screening ocular corrosives or severe irritants. Moreover, these methods are not recommended to identify substances from all hazard categories, including substances not labeled as irritants (ICCVAM 2009). As previously noted, while none of the four *in vitro* test methods are currently considered as replacements for the *in vivo* rabbit eye test, further optimization and validation of these methods, and other *in vitro* techniques, is necessary to reduce and refine animal use for ocular safety testing.

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Abbreviations

BCOP	bovine corneal opacity & permeability
CAMVA	chorioallantoic membrane vascular assay
CAM-TB	chorioallantoic membrane trypan blue staining test
CEC	Commission of the European Communities
CEET	chicken enucleated eye test
EPA	Environmental Protection Agency
FL	fluorescein leakage
GHS	Globally Harmonized System
HCE	human corneal epithelium
HET-CAM	hen's egg test-chorioallantoic membrane
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICE	isolated chicken eye
IRE	isolated rabbit eye
MTT	methylthiazol tetrazolium test
NRU/NRR	neutral red uptake/release
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NTP	National Toxicology Program
RBC	red blood cell
SM	silicon microphysiometer

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Table 1

Summary of Current Organotypic Methods for Ocular Toxicology Testing

Name*	Validation Status	Test Method Indicator	Objective of Test
HET-CAM	Evaluated; limited usefulness	Hemorrhage, coagulation, cytolysis	Ocular sensitivity & corrosion
BCOP	Validated by ICCVAM for ocular corrosives and severe irritants	Increase in corneal opacity, thickness and permeability	Ocular sensitivity & corrosion
ICE	Validated by ICCVAM for ocular corrosives and severe irritants	Increase in corneal opacity, thickness and permeability	Ocular sensitivity & corrosion
IRE	Evaluated; limited usefulness	Increase in corneal opacity and thickness	Ocular sensitivity & corrosion
EpiOcular™ (MatTek)	In validation studies (ECVAM)	MTT cell viability	Ocular sensitivity & corrosion
HCE™ (SkinEthic)	In validation studies (ECVAM)	MTT cell viability	Ocular sensitivity & corrosion

* Abbreviations: BCOP = bovine corneal; HCE = human corneal epithelium; HET-CAM = hen's egg test-chorioallantoic membrane; ICE = isolated chicken eye; IRE = isolated rabbit eye; MTT = methylthiazol tetrazolium test.

Table 2

HET-CAM Irritation Score Classification System

Substance score	Category of IS classification
0 - 0.9	Nonirritant or practically none
1 - 4.9	Weak or slight irritation
5 - 8.9 or 5 - 9.9	Moderate irritation
9 - 21 or 10 – 21	Strong or severe irritation

Abbreviations: IS = irritation score

Table 3

BCOP In Vitro Irritancy Score Classification System

In Vitro IS	Category of IS classification
0 – 25	Mild irritant
25.1 - 55	Moderate irritant
>55.1	Severe irritant

Abbreviations: IS = irritancy score

Table 4

ICE Irritancy Classification System

Parameter	Measurement	Score	Category*
Corneal thickness	Mean corneal swelling	Percentage	I to IV
Corneal Opacity	Mean maximum score	0 to 4.0	I to IV
FR	Mean FR score	0 to 3.0	I to IV

Abbreviations: ICE = isolated chicken eye test method; FR = fluorescein retention.

*Category assignments for test substances include: I, non-irritant; II, slight irritant; III, moderate irritant; or, IV, severe irritant.