Appendix C

Background Review Document:
Use of Topical Anesthetics, Systemic Analgesics, and Earlier Humane Endpoints to Minimize Pain and Distress in Ocular Safety Testing

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Background Review Document

Use of Topical Anesthetics, Systemic Analgesics, and Earlier Humane Endpoints to Minimize Pain and Distress in Ocular Toxicity Testing

Interagency Coordinating Committee on the Validation of Alternative Methods

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

2010

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List of Abbreviations and Acronyms

BRD	Background Review Document
Colipa	European Cosmetic, Toiletry and Perfumery Association
COX	Cyclooxygenase
CPSC	(U.S.) Consumer Product Safety Commission
ECVAM	European Centre for the Validation of Alternative Methods
EPA	(U.S.) Environmental Protection Agency
EU	European Union
FDA	(U.S.) Food and Drug Administration
FR	*Federal Register*
GHS	United Nations Globally Harmonized System of Classification and Labelling of Chemicals
HCl	Hydrochloric acid
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IRAG	Interagency Regulatory Alternatives Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
kg	Kilogram
mg	Milligram
mM	Millimole
µL	Microliter
µg	Microgram
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NRC	National Research Council
NSAID	Nonsteroidal anti-inflammatory drug
NTP	(U.S.) National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OTWG	Ocular Toxicity Working Group
UN	United Nations
w/v	Weight-to-volume ratio

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Preface

The use of pretreatment analgesia in the Draize rabbit eye test method (Draize et al. 1944), though not formal policy among all U.S. Federal agencies, is a protocol refinement that could provide a significant reduction in animal pain and distress. Since 1984, the U.S. Consumer Product Safety Commission has recommended preapplication of tetracaine ophthalmic anesthetic for all rabbit eye toxicity studies. However, current Environmental Protection Agency (EPA) and Organisation for Economic Co-operation and Development (OECD) test guidelines for the rabbit eye test state that topical anesthetics can be used only if the user demonstrates that such pretreatments do not interfere with the results of the tests. Therefore, topical anesthetics often are not used because a separate study may be necessary to provide such information.

In a 1991 workshop the Interagency Regulatory Alternatives Group (IRAG) organized a workshop entitled "Updating Eye Irritation Methods: Use of Ophthalmic Topical Anesthetics." The consensus among invited experts was that use of anesthesia is acceptable in eye irritation testing because pain is temporarily relieved, and the extent of injury can be evaluated (Seabaugh et al. 1993). In 2003, the EPA nominated four areas for evaluation by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). ICCVAM was asked to evaluate ways of alleviating pain and suffering that might arise from administration of mild to moderate irritants in current *in vivo* eye irritation testing.

ICCVAM, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the European Centre for the Validation of Alternative Methods organized a symposium entitled "Minimizing Pain and Distress in Ocular Toxicity Testing" in May 2005 (**Annex I**). The symposium was supported by the European Cosmetic, Toiletries and Perfumery Association. Similar to the 1991 IRAG workshop, invited experts at agreed that topical anesthesia should be routinely provided as a pretreatment to animals used for ocular toxicity testing. The invited experts added that (1) combinations of general or topical anesthesia and systemic analgesia should be routinely used to avoid pain and (2) induced lesions should be treated with continued systemic analgesia during the observation period. Specifically, the invited experts indicated that sufficient data existed for combining a topical anesthetic (e.g., tetracaine or proparacaine) with a systemic analgesic (e.g., buprenorphine) to minimize or eliminate pain during ocular toxicity testing. In addition, the invited experts indicated that it might be useful to conduct controlled studies in rabbits to confirm the efficacy of this approach. Ideally, data could be collected during routine safety testing and periodically analyzed to determine efficacy for specific lesion types and clinical signs of pain.

A review of studies reported in the literature provides conflicting results on the impact of topical ocular anesthetics on ocular irritation and physiology. Some studies indicate that topical anesthetics do not interfere with the irritation response (Arthur et al. 1986; Heywood and James 1978; Seabaugh et al. 1993; Ulsamer et al. 1977). Others state that there is a trend (although not statistically significant) of increased irritancy in eyes treated with anesthesia (Johnson 1980; Durham et al. 1992). Some have also reported that anesthetics interfere with the irritant response and yield unreliable data (Walberg 1983; Rowan and Goldberg 1985).

Participants at the 2005 symposium "Minimizing Pain and Distress in Ocular Toxicity Testing" also discussed early adverse responses predictive of ocular lesions associated with severe irritant or corrosive substances (EPA Category I [EPA 1998], GHS Category I [UN 2007], EU R41 [EU 2001], or) that could be used routinely as humane endpoints to terminate a study.

The purpose of this document is to comprehensively review all available information on the safety and efficacy (or potential efficacy) of selected anesthetics and analgesics for relieving ocular pain, as well as to identify humane endpoints that could warrant terminating a study. It also describes the

results from a joint study conducted by NICEATM and Product Safety Labs to evaluate the effect of pretreatment with the topical anesthetic tetracaine hydrochloride (0.5% w/v) on the ocular irritancy potential of 97 formulations.

We gratefully acknowledge the organizations and scientists who provided data and information for this document. We also acknowledge the efforts of those individuals who helped prepare this summary review document, including the following staff from the NICEATM support contractor, Integrated Laboratory Systems, Inc.: David Allen, Jon Hamm, Nelson Johnson, Brett Jones, Elizabeth Lipscomb, Linda Litchfield, Gregory Moyer, Catherine Sprankle, and Jim Truax. We thank the members of the ICCVAM Ocular Toxicity Working Group, co-chaired by Karen Hamernik, Ph.D. (EPA), and Jill Merrill, Ph.D. (U.S. Food and Drug Administration), as well as the ICCVAM representatives who reviewed and provided comments throughout the process leading to this draft version. We also thank Valerie Zuang, Ph.D., and Dr. Hajime Kojima, Ph.D., the Ocular Toxicity Working Group liaisons from the European Centre for the Validation of Alternative Methods and the Japanese Centre for the Validation of Alternative Methods, respectively, for their participation.

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Executive Summary

Human and veterinary medicine have provided a great deal of clinical experience with a range of topical anesthetics and systemic analgesics for the relief of ocular pain. However, the subjective nature of identifying and treating pain in animals makes it difficult to establish which therapeutic options are most effective. Few published studies relate directly to the eye. Most studies focus on the relief of pain after surgery and/or pain resulting from trauma.

Since 1984, the U.S. Consumer Product Safety Commission has recommended applying tetracaine ophthalmic anesthetic before applying test substances in all rabbit eye toxicity studies. However, current test guidelines for the rabbit eye test from the U.S. Environmental Protection Agency (EPA) and Organisation for Economic Co-operation and Development (OECD) state that topical anesthetics can be used only if the user demonstrates that such pretreatment does not interfere with the results of the tests.[1] Therefore, toxicity studies seldom use topical anesthetics because providing the necessary information would likely require a separate study.

Use of Topical Anesthetics and Systemic Analgesics

In 1991, the Interagency Regulatory Alternatives Group organized a workshop titled "Updating Eye Irritation Methods: Use of Ophthalmic Topical Anesthetics." The workshop evaluated use of topical ophthalmic anesthetics and/or systemic analgesics during the Draize rabbit eye test. A symposium titled "Minimizing Pain and Distress in Ocular Toxicity Testing" re-examined this topic in 2005. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the European Centre for the Validation of Alternative Methods organized the symposium, which was supported by the European Cosmetic, Toiletry and Perfumery Association.

Both meetings produced similar recommendations and recognition of the limitations associated with the use of topical anesthetics and systemic analgesics. Experts acknowledged that a single treatment with a topical anesthetic to anesthetize the surface of the cornea before applying the test substance could cause slight physiologic changes that might alter the response. However, most felt that such alterations would be minor, if any. The effect would likely be a slight increase in irritant response. Such topical anesthesia is used in millions of cataract surgeries annually. It is also used during routine eye exams to anesthetize the corneal surface before measuring intraocular pressure for glaucoma screening. NICEATM recently evaluated how pretreatment with tetracaine hydrochloride (0.5% w/v) affected the potential of 97 formulations to irritate the eye. The results indicate that pretreatment did not affect the hazard classification observed during the test.

Most meeting participants considered the use of topical anesthetics acceptable, because the anesthetics at least prevent discomfort caused by applying the test substance on the eye and temporarily prevent any pain and distress that might result from immediate ocular damage. Participants in both meetings recommended that combinations of general or topical anesthesia and systemic analgesia be routinely used to prevent pain. They also recommended that lesions caused by the substances be treated with continued systemic analgesia. Participants also recognized that, although many types of systemic analgesics could help alleviate pain, opioid analgesics (e.g., buprenorphine) were likely to be most effective in ocular safety testing. Because of their effects on the wound healing process, other analgesics (e.g., nonsteroidal anti-inflammatory drugs) could be expected to adversely affect results.

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[1] OECD Test Guideline 405 states, "The type, concentration, and dose of a local anesthetic should be carefully selected to ensure that differences in reaction to the test substance will not result from its use" (OECD 1987). Similarly, the EPA (1998) states, "The type and concentration of the local anesthetic should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use."

The many studies detailing the safety and efficacy of tetracaine and proparacaine suggest that they are among the most widely used topical anesthetics. Proparacaine is relatively harmless to the corneal epithelium and provides extended anesthesia. Thus, it may be more appropriate for treating ophthalmic pain. However, the reported adverse effects of tetracaine and proparacaine on wound healing suggest that their use beyond acute pain relief may be limited. Thus, they are recommended for use only as initial anesthetics in an *in vivo* ocular toxicity test.

Workshop and symposium participants also recommended pretreatment with a systemic analgesic to relieve ocular pain that might result from any chemically induced injuries. Administering preemptive analgesia is more effective than waiting to treat the pain after it begins. Preemptive analgesia is common in veterinary medicine. Among systemic analgesics, veterinarians use the lipophilic opioid, buprenorphine, most frequently. Buprenorphine's margin of safety is well characterized in multiple species. A single dose is recommended for routine pretreatment before a Draize rabbit eye test. If no painful lesions or clinical signs of pain and distress occur, then no further doses are administered. If painful lesions or clinical signs of pain and distress are observed, then continuing systemic analgesia is recommended until these lesions and/or clinical signs are absent.

The effectiveness of buprenorphine in relieving postsurgical pain in rabbits is well documented. However, few studies have evaluated how effectively buprenorphine relieves ocular pain. Trevithick et al. (1989) found that buprenorphine injected at 5-hour intervals maintained a stable degree of analgesia for 24 hours. In addition, buprenorphine has a long history of managing postoperative pain in humans.

Based on its history as an effective analgesic for moderate to severe pain in rabbits, dosing of buprenorphine is typically administered by subcutaneous or intramuscular injection every 12 hours (0.01 to 0.05 mg/kg; Kohn et al. 2007). However, Buprederm™, a new transdermal formulation of buprenorphine, has been shown to provide sustained analgesia during the 72-hour patch application period. No local irritation appeared with repeated patch application in rabbits (Park et al. 2008). This suggests that repeated use of Buprederm™ patches might provide effective pain relief during the observation period required for ocular toxicity testing (i.e., up to 21 days).

Use of Humane Endpoints to Terminate an Ocular Toxicity Study

Public Health Service policy and U.S. Department of Agriculture regulations on pain and distress in laboratory animals state that more than momentary or light pain and distress:

- Should be limited to that which is unavoidable for the conduct of scientifically valuable research or testing
- Should be conducted with appropriate pain-relief medication unless justified in writing by the principal investigator
- Should continue for only the necessary amount of time required to attain the scientific objectives of the study
- These regulations also state that animals suffering severe or chronic pain or distress that cannot be relieved should be humanely killed after or, if appropriate, during the procedure. Finally, the Institutional Animal Care and Use Committees must ensure that the principal investigator complies with the requirements.

A recent report of the National Research Council Committee on Recognition and Alleviation of Pain in Laboratory Animals emphasized the need for increased efforts to identify appropriate humane endpoints (NRC 2009).

Participants at the 2005 symposium "Minimizing Pain and Distress in Ocular Toxicity Testing" also discussed early adverse responses predictive of ocular lesions associated with severe irritant or corrosive substances. Such substances are classified as EPA Category I (1998), Globally Harmonized

System of Classification and Labelling of Chemicals Category 1 (UN 2007), and/or European Union R41 (EU 2001). The adverse responses under discussion could be used routinely as humane endpoints to terminate a study.

Symposium invitees included human and veterinary ophthalmologists and anesthesiologists, scientific experts in ocular hazard testing, research scientists, and industrial toxicologists. After discussion, they recommended the following endpoints for routine use for early study termination:

- Endpoints currently accepted for study termination (OECD 2002):
  - Draize corneal opacity score of 4 that persists for 48 hours
  - Corneal perforation or significant corneal ulceration, including staphyloma
  - Blood in the anterior chamber of the eye
  - Absence of light reflex that persists for 72 hours
  - Ulceration of the conjunctival membrane
  - Necrosis of the conjunctiva or nictitating membrane
  - Sloughing
- Vascularization of the corneal surface (i.e., pannus)
- Destruction of more than 75% of the limbus
- Area of fluorescein staining not diminishing over time based on daily assessment
- Lack of re-epithelialization 5 days after application of the test substance
- Depth of injury to the cornea (routinely using slit-lamp and fluorescein staining), where ulceration extends beyond superficial layers of the stroma, or increase in the depth of injury over time

ICCVAM has considered the relevant data, information, and analyses provided in this background review document and developed draft recommendations on the use of topical anesthetics, systemic analgesics, and humane endpoints to avoid or minimize pain and distress in ocular toxicity testing. These recommendations are provided in a separate document. The recommendations include proposed usefulness and limitations, proposed changes to the current standardized test method protocol, and proposed future studies and activities.

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1.0 Background

Draize et al. (1944) developed the rabbit eye test to test the ocular hazard potential of new chemicals or chemical products. Substances identified as potential ocular hazards could then be appropriately labeled and handled to protect humans from potential exposure. Sensitivity to animal use and concerns about the reliability of this test method have led to a search for alternative *in vitro* test methods for ocular hazard assessment (e.g., cell-based models, organotypic models, hemodynamic models). Several of these *in vitro* test systems have been evaluated in large validation studies (e.g., Balls et al. 1995; Gettings et al. 1996). However, until validated alternatives are accepted as complete replacements, the Draize rabbit eye test will continue to be required for ocular hazard evaluation by U.S. Federal and European regulatory agencies.

One of the main concerns with this test method is the possibility that pain and/or discomfort may be produced in the test animals. In spite of efforts designed to screen substances for suspected corrosive or severe ocular irritant properties (e.g., eliminating pH extremes and dermal corrosives from testing), the potential remains for discomfort from materials with unknown remains. However, it should be noted that the Public Health Service Policy on Humane Care and Use of Laboratory Animals states that "Procedures that may cause more than momentary or slight pain or distress to the animals will be performed with appropriate sedation, analgesia, or anesthesia unless the procedure is justified for scientific reasons in writing by the investigator" (PHS 2002). This implies that such measures should be regularly considered.

Since 1984, the U.S. Consumer Product Safety Commission (CPSC) has recommended preapplication of tetracaine ophthalmic anesthetic for all rabbit eye toxicity studies (CPSC 1984). However, current U.S. Environmental Protection Agency (EPA) and Organisation for Economic Co-operation and Development (OECD) test guidelines for the rabbit eye test state that topical anesthetics can be used only if the user demonstrates that such pretreatments do not interfere with the results of the tests (EPA 1998; OECD 1987).[2] For this reason, anesthetics are seldom used because a separate study to provide such information would often be necessary.

In 1991, an *ad hoc* committee of the Interagency Regulatory Alternatives Group (IRAG) organized the workshop, "Updating Eye Irritation Methods: Use of Ophthalmic Topical Anesthetics" (Seabaugh et al. 1993) to evaluate the use of anesthetics in eye irritation testing. Two commonly used anesthetics, tetracaine (0.5%–5%) and proparacaine (0.1%–0.5%), produce an almost immediate effect lasting up to 20 minutes. These anesthetics eliminate local pain and touch sensation but also increase ocular permeability, reduce tear volume, reduce blink frequency, and delay wound healing.

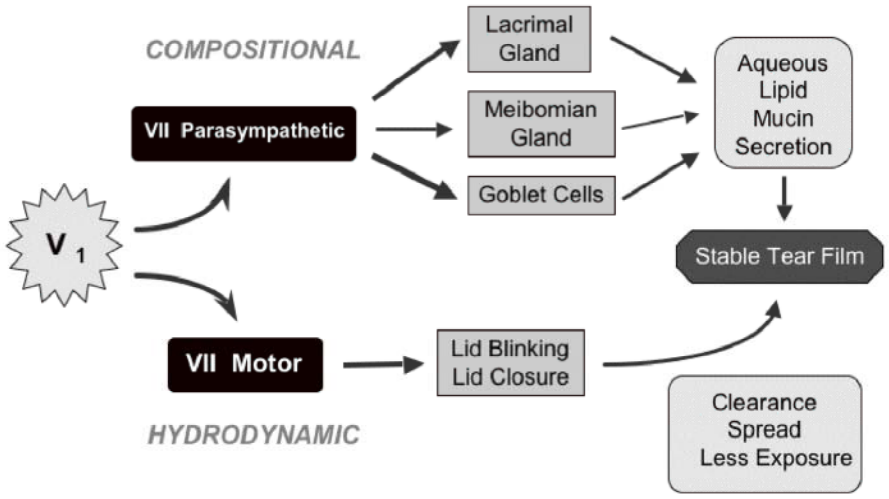
Briefly, the ocular defense is controlled by two neural reflexes via sensory input from V1 (i.e., the first branch of the trigeminal nerve) and via two separate (i.e., motor and parasympathetic) branches of the VII facial nerve. The VII facial nerve dictates the hydrodynamic and compositional elements of the external adnexae, lids and ocular surface epithelia for maintaining a stable tear film (**Figure 1-1**) (Tseng and Tsubota 1997). Therefore, the level of ocular injury may be exaggerated following topical anesthetic administration due to reduction in ocular defense mechanisms (e.g., neuronal activation of goblet cells for tear fluid secretion). Duration of injury may be lengthened by impairment of repair processes (e.g., decreased release of chemokines or reduction in level of collagen deposition). Despite these issues, and although it was not formal policy among U.S. Federal agencies, a consensus of those participating on the IRAG committee considered the use of anesthetics acceptable because such

[2] OECD Test Guideline 405 states that "The type, concentration, and dose of a local anesthetic should be carefully selected to ensure that differences in reaction to the test substance will not result from its use." Similarly, EPA states that "The type and concentration of the local anesthetic should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use" (1998).

measures provide at least temporary pain relief for the animal, and the time and extent of injury can still be evaluated.

Despite these recommendations, there is little evidence to suggest that measures to prevent or reduce pain during the rabbit eye test are regularly employed. In order to re-examine the need for such measures, a symposium entitled "Minimizing Pain and Distress in Ocular Toxicity Testing" met at the National Institutes of Health in Bethesda, Maryland, on May 13, 2005 (**Annex I**). The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the European Centre for the Validation of Alternative Methods (ECVAM) organized the symposium.

Figure 1-1. A Stable Tear Film is Maintained by a Sound Ocular Surface Defense Governed by Neuroanatomic Integration (Tseng and Tsubota 1997)



The European Cosmetic, Toiletry and Perfumery Association provided additional funding. Invited experts included ophthalmologists, scientific experts in ocular hazard testing and method development, research scientists, U.S. Federal regulators, and industry toxicologists. This symposium was organized to better understand the mechanisms and physiological pathways of the pain response, to recognize symptoms and signs of the pain response, and to identify effective means to alleviate or prevent pain while preserving the ocular injury responses used to identify hazard potential. The experts who participated in this symposium concluded that pain relief in animals used for ocular toxicity testing should routinely be provided as a pretreatment. In addition, they recommended that combinations of general or topical anesthesia and preemptive systemic analgesia be routinely used to avoid pain on initial test article application. They also recommended the use of continued systemic analgesia treatment of any persistent lesions.

The purpose of this background review document is to comprehensively review available information on the safety and efficacy (or potential efficacy) of selected anesthetics and analgesics for relieving ocular pain, as well as to identify humane endpoints that could warrant terminating a study. It also describes the results from a joint study conducted by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods and Product Safety Labs, which

evaluated the effect of pretreatment with the topical anesthetic tetracaine hydrochloride (0.5% w/v) on the ocular irritancy potential of 97 formulations (**Annex II**).

2.0 Clinical Identification of Ocular Pain in Animals

There is no direct measure for the experience of pain, and the recognition of pain in animals has been further confounded in part by the evolutionary process (Wright et al. 1985; Hansen 1997). Ill or injured animals are typically abandoned by their companions because they may become targets for predators. In this regard, abnormal behavior is avoided at all costs to ensure survival. While domestic and laboratory animal species have largely been removed from such survival pressures, these inherited behaviors may still hinder the interpretation of animal pain (Wright et al. 1985). With that said, an animal in pain, regardless of the species in question, will likely display one or more of the following symptoms (Cramlet and Jones 1976; Wright et al. 1985):

- Increased skeletal muscle tone, blood pressure, and/or heart rate
- Attraction to the area of pain
- Pupillary dilation
- Altered respiration

Furthermore, it has been proposed that signs such as reluctance to move, scratching, and rubbing indicate ophthalmic pain specifically (Wright et al. 1985).

Pain scoring systems in humans rely on an interactive dialogue between the patient and clinician to assign a subjective approximation of intensity (e.g., Scott and Huskisson 1976). Although such an interaction with animals is not feasible, subjective pain scoring systems have been developed for companion animal species (e.g., Smith et al. 2004) that grade the extent of movement and vocalization. Comfort, appearance, and behavior are also observed and graded. These scores are then combined into a total subjective pain score that may be used to define thresholds for severe pain. Such scoring systems may not be applicable to laboratory animal species because of their behavioral differences. However, trauma eventually produces some degree of pain, and the presence of pain should be assumed following tissue injury. Therefore, it may be more important to establish whether an animal would benefit from analgesic therapy, rather than whether or not the animal is experiencing pain (Hansen 1997). Most recently, an American College of Laboratory Animal Medicine Task Force published *Guidelines for the Assessment and Management of Pain in Rodents and Rabbits* (Kohn et al. 2007), which provides methods for assessing pain and recommendations for pain management.

3.0 Options for Pain Relief in Animals

3.1 Topical Anesthetics

Local anesthesia refers to the loss of sensation in a limited area of the body (Wright et al. 1985). Topical anesthetics reduce pain by blocking sodium channels in excitable neurons, thus inhibiting the action potential generated by membrane depolarization when large, transient increases in sodium permeability are produced in response to an irritant (Catterall and Mackie 2001). However, topical anesthetics are also associated with a series of local adverse effects (e.g., delayed wound healing, production of corneal erosions and epithelial sloughing, decreased lacrimation, and tear film disruption). Furthermore, increased frequency and longer use may result in epithelial defects with corneal stromal ring infiltrates. Topical anesthetics may also interfere with the toxicokinetics of test substances (e.g., increase permeability of corneal epithelium, break down barriers that shield toxicity) and thus confound test results.

Topical ocular anesthetics may be divided into those with ester (e.g., cocaine, procaine, tetracaine, proparacaine), amide (e.g., lidocaine, bupivacaine, mepivacaine), or other linkages (e.g., benzocaine, dibucaine). These topical agents act on the inner surface of the axonal membrane sodium channels and must penetrate lipid barriers for access. Onset of action ranges from 0.5 to 3 minutes after administration with a duration of 20 minutes to 2 to 3 hours. Application frequency of these topical anesthetics increases duration but not depth of anesthesia.

The two most commonly used topical ocular anesthetics are proparacaine and tetracaine (Wilson 1990, Bartfield et al. 1994). Lidocaine is also commonly used. These drugs are intended for short-term use only, because chronic use is associated with toxicity to ocular tissues that subsequently delays corneal wound healing (Zagelbaum et al. 1994; Moreira et al. 1999). They are also contraindicated in the treatment of corneal ulcers because they disrupt the tear film and retard the initial phase of re-epithelialization (Ketring 1980). Chronic use of topical anesthetics has even been associated with permanent corneal scarring and decreased vision (Rapuano 1990). However, these agents rapidly reduce the subjective signs of corneal pain, and thus can quickly differentiate between pain from superficial sources (e.g., cornea) from pain arising from deeper structures in the eye (Ketring 1980; Bartfield et al. 1994).

The presence of preservatives (e.g., benzalkonium chloride, chlorobutanol) in topical anesthetic ophthalmic formulations and their potential effect on ocular irritation classification schemes cannot be discounted either. For example, benzalkonium chloride, a Category I irritant, may cause surface epithelial damage and a complete breakdown of transcorneal electrical resistance linked to a breakdown in barrier function (Chetoni et al. 2003).

In vitro studies suggest that tetracaine is more damaging to the corneal epithelium than proparacaine (Grant and Acosta 1994; Moreira et al. 1999). In addition, clinical studies indicate that instillation of proparacaine eye drops is less painful than instillation of tetracaine (Bartfield et al. 1994). These findings suggest that proparacaine may be considered the preferred topical anesthetic for ocular studies. However, a recent evaluation by NICEATM of the effects of topical pretreatment with tetracaine hydrochloride (0.5% w/v) on the ocular irritancy potential of 97 formulations indicated that such pretreatments had no impact on (1) the hazard classification severity category of observed ocular irritation, (2) the variability in rabbit ocular irritation responses, or (3) the number of days required for an ocular lesion to clear (**Annex II**). A comparison of the relevant properties of proparacaine and tetracaine with regard to their impacts on corneal wound healing and irritant hazard classification is detailed in **Annex III.**

The rabbit has a low blink rate relative to humans and several authors have directly or indirectly studied the effect of topical anesthetics on blink rate. Maurice (1995) used fluorophores and a

noninvasive fluorometer and found that the low blink rate in rabbits would be expected to increase 3-fold the area under the curve for drug penetration in the corneal tear film relative to humans. Thus, the penetration of a drug could be underestimated on the basis of blink rate alone. However, for most drugs, the epithelial permeability is sufficiently high to permit drug penetration from the tear film into the epithelium within minutes, in which case contact time becomes irrelevant.

Schwartz et al. (1998) studied tetrodotoxin for its potential to produce long-lasting topical anesthesia in the eye of the rabbit. Anesthesia produced by topical administration of 10 mM tetrodotoxin solution produced anesthesia that lasted 8 hours compared to 1 hour or slightly longer for 0.5% proparacaine. The blink rate was reduced 67% by 10 mM tetrodotoxin compared to approximately 13% for proparacaine. Lower concentrations of tetrodotoxin, 0.1 and 1 mM, produced no anesthesia or anesthesia of shorter duration, respectively, compared to the 10 mM concentration. It should be noted that while no signs of overt systemic toxicity were observed in the study, the LD_{50} of tetrodotoxin in the rabbit is less than 10 µg/kg by intramuscular or subcutaneous routes of administration. Naase et al. (2005) studied the spontaneous eyeblink rates of human volunteers without exogenous stimuli by using the topical anesthetic, benoxinate (0.4%). The authors reported a 63% decrease in the spontaneous eyeblink rate after anesthetic treatment, but found that the patterns of the blink rates (i.e., symmetrical, J- and I-type) were unaffected by anesthetic treatment.

3.2 Systemic Analgesics

Analgesia refers to relief of pain. Post-treatment modalities include the use of systemic analgesics for relief of pain associated with chemically induced lesions. Repeated use of topical anesthetics could exaggerate or prolong chemically induced lesions by causing a reduction in ocular defense mechanisms (e.g., neuronal activation of goblet cells for tear fluid secretion), as previously mentioned. For this reason, administering systemic analgesics during the post-treatment observation period may be a more useful approach to relieving pain from ocular lesions.

3.2.1 Opioid Analgesics

Much of the available data on the efficacy of systemic opioid analgesics focus on peri- or postoperative uses, on which several thorough reviews are available (Flecknell 1984; Flecknell and Liles 1990; Flecknell 1991; Flecknell and Liles 1992; Flecknell 1995). Perhaps the greatest clinical concern regarding the use of these types of agents is the side effects with which they are associated. In humans, opioid administration is commonly associated with respiratory depression. However, this effect is less pronounced in animals, especially when mixed agonist/antagonist opioids (e.g., buprenorphine) are used (Flecknell 1995). In this regard, a wide safety margin for buprenorphine has been demonstrated in rabbits, where doses ranging from 0.0075 to 0.3 mg/kg produce effective analgesia without serious respiratory depression (Flecknell and Liles 1990). Reports of clinical studies in humans describe a low incidence of local and/or systemic adverse effects, a lack of immunotoxicity associated with other opioids (e.g., morphine), and maintenance of cognitive function during long-term therapy (Scott et al. 1980; Budd 2002; Budd and Collett 2003; Sorge and Sittl 2004).

Another concern regarding systemic opioid use is that many of these drugs provide only short-term analgesia, with maintenance of pain relief requiring repeated administration every 1 to 3 hours. From a practical perspective for a testing laboratory, such a regimen is clearly not feasible. One exception is buprenorphine, which has been shown in humans, pigs, rodents, and rabbits to provide effective pain relief for up to 12 hours (Cowan et al. 1977; Heel et al. 1979; Dum and Herz 1981; Hermanssen et al. 1986; Flecknell and Liles 1990; Flecknell 1996). This may be due to the fact that buprenorphine dissociates very slowly from its receptor relative to other opioids, which has been demonstrated *in vitro* (PDR 2004). Studies in multiple species have also shown that, while the intensity of analgesia induced by buprenorphine does not appear to increase with dose, the duration of analgesia is dose

dependent (Cowan et al. 1977; Hermanssen et al. 1986; Hoskin and Hanks 1987; Nolan et al. 1987; Flecknell and Liles 1990). However, the onset of action is delayed in rabbits (approximately 30 minutes after treatment), suggesting that buprenorphine treatment prior to testing a potentially irritating/corrosive substance is warranted (Flecknell and Liles 1990).

Taken together, these findings likely contribute to the fact that buprenorphine is one of the most commonly used analgesic agents in laboratory and companion animal species, as demonstrated by multiple surveys of its use in veterinary practice (Dohoo and Dohoo 1996; Hubbell and Muir 1996; Watson et al. 1996; Capner et al. 1999; Lascelles et al. 1999; Joubert 2001). However, as indicated above, many of the reported veterinary uses of buprenorphine have focused on relief of surgical pain. Based on its long history of successful veterinary use as an analgesic for moderate to severe pain in rabbits, dosing of buprenorphine is typically provided by subcutaneous or intramuscular injections every 12 hours (0.01 to 0.05 mg/kg; Kohn et al. 2007).

A limited number of studies have evaluated the efficacy of buprenorphine in the relief of ocular pain. Trevithick et al. (1989) used esthesiometry to evaluate prolonged corneal analgesia produced in rabbits by repeated intramuscular injections of buprenorphine or meperidine in the presence of short-term anesthesia induced by ketamine and xylazine. Analgesia was established based on esthesiometric measurements of the intensity of surface pressure to the cornea required to induce a blink reflex. The authors found that buprenorphine injections at 5-hour intervals were sufficient to maintain a stable degree of analgesia for the entire study period (24 hours). The dosing regimen was based on previous studies in which the maximum period of analgesia obtained was 5 hours (Trevithick et al. 1989).

3.2.1.1 Alternative Dosing Routes for Buprenorphine

Regardless of the route of administration, buprenorphine is primarily excreted in the feces, with only a small amount present in the urine. For this reason, buprenorphine is considered the safest opioid for use in cases of renal impairment (Budd and Collett 2003). Buprenorphine undergoes significant first-pass metabolism in the gastrointestinal mucosa and liver following oral administration and is therefore typically administered by intravenous, intramuscular, or subcutaneous injection. However, in an effort to reduce the pain and distress associated with parenteral delivery, alternative dosing strategies might be worthy of consideration. Because buprenorphine hydrochloride is lipophilic and has a low molecular weight, it has been recognized as an excellent candidate for sublingual and/or transdermal delivery, both of which bypass first-pass metabolism. However, sublingual delivery successfully bypasses first-pass metabolism only when the drug is not swallowed, and at least 50% of a sublingual dose may be recovered in the saliva (Mendelson et al. 1997; Hand et al. 1990; Lindhardt et al. 2001). This caveat makes the veterinary utility of such a route questionable.

In vitro skin penetration studies have demonstrated that transdermal delivery of buprenorphine can achieve a systemic analgesic effect (Roy et al. 1994). In fact, transdermal buprenophine is presently being prescribed clinically in Europe and Australia for the treatment of chronic severe disabling pain. It is also being studied in the United States for its safety and efficacy for similar indications. For transdermal delivery, buprenorphine is incorporated within an adhesive polymer matrix that provides slow, consistent release into the circulation at a predetermined rate, maintaining a relatively constant serum drug concentration over at least 72 hours (Sittl 2005).

A new transdermal formulation of buprenorphine currently under development using a proprietary hydrogel matrix technology (Buprederm™) has shown faster absorption and sustained analgesia throughout a 72-hour period. Maximum analgesic effect was obtained between 3 and 6 hours and was maintained for 24 hours after patch application (Park et al. 2008). In a multiple-dose study in which patches were applied to rabbits every 4 days (3 days attachment and 1 day detachment) for 28 days, Buprederm™ was found to provide maximum plasma buprenorphine concentration by 3 hours after administration, with this concentration being maintained for 72 hours. Over the 28 days, there was no

accumulation of buprenorphine systemically or in the local skin, and analgesia was maintained without measurable skin irritation (Park et al. 2008). Buprederm™ may therefore provide both fast-acting and long-lasting analgesia suitable for use in the rabbit eye irritation test. Investigations will be necessary to determine the impact of Buprederm™ on test results.

Intranasal delivery of buprenorphine has been studied in humans, rabbits, and sheep (Eriksen et al. 1989; Lindhardt et al. 2000; Lindhardt et al. 2001). A reported advantage of the intranasal route is the reduced mean time to maximal serum concentration (i.e., T_{max}) relative to the sublingual and transdermal routes (Lindhardt et al. 2001). This property may make intranasal buprenorphine delivery more amenable to the treatment of acute pain. However, it should be noted that this method requires specific manipulation of the animal to maximize drug delivery. The animal must be maintained in a supine position during dosing and for at least 1 minute after dosing.

Rectal gels containing buprenorphine have also been formulated with water-soluble dietary fibers, xanthan, and locust bean gums. Using these gels, rapid absorption and bioavailability of buprenorphine was achieved in rabbits without adversely affecting the rectal mucosa (Watanabe et al. 1996). These properties suggest that rectal gels, like the intranasal route, may be preferable to transdermal or sublingual buprenorphine delivery systems for the treatment of acute pain. This method also requires specific manipulation of the test animals because they must be restrained during the dosing procedure with the gel tube adhered to the anus and fastened with a clip to prevent rejection (Watanabe et al. 1996).

Hanson et al. (2001) reported that buprenorphine administered twice daily at an analgesic dose of 0.05 mg/kg had no effect on immunological evaluation of Shigella vaccine candidates in the Sereny test, a model of keratoconjunctivitis in the guinea pig. It did, however, result in a significant increase in mucopurulent discharge that required frequent cleaning of the affected eyes. The authors indicated that this effect did not appear to affect the outcome of the test results. The authors also reported significant weight loss of 5.5% to 5.8% in buprenorphine-treated animals relative to the saline control group, which gained 4% to 5% in body weight over the 5-day course of study.

3.2.2 Nonsteroidal Anti-inflammatory Drugs (NSAIDs)

NSAIDs inhibit fever, pain, and inflammation by inhibiting the two isoforms of the enzyme fatty acid cyclooxygenase (COX; the constitutive COX-1 and the cytokine and inflammatory mediator-inducible COX-2) with varying degrees of selectivity (Vane et al. 1998). Inhibition of COX decreases arachidonic acid metabolism and the resulting prostaglandin and leukotriene products that induce pain, fever, and other inflammatory processes. One NSAID, acetaminophen, is an effective analgesic and antipyretic agent but is less effective as an anti-inflammatory agent because it inhibits COX activity only in the brain. Acetaminophen may therefore be less likely to interfere with wound healing.

Several published reports have examined the effect of NSAIDs on the eye wound healing process in rabbits, particularly following excimer laser keratectomy surgery (Loya et al. 1994; Nassaralla et al. 1995; Park and Kim 1996; Kaji et al. 2000). The results have been varied. Kaji et al. (2000) reported that topical administration of diclofenac significantly decreased early-phase conjunctival inflammation in rabbits but did not inhibit corneal haze formation. Similar studies have also reported that topical diclofenac administration influenced corneal and stromal wound healing in rabbits following excimer laser surgery (Nassaralla et al. 1995; Park and Kim 1996). In contrast, Loya et al. (1994) reported that diclofenac did not significantly affect corneal wound healing or epithelial migration rate when used up to eight times daily. Similarly, Hersh et al. (1990) observed that diclofenac decreased early epithelialization but had no apparent effect on corneal stromal healing. Finally, it was reported that suprofen and flurbiprofen, two alternative topical ophthalmic NSAIDs,

did not significantly inhibit corneal wound healing in rabbits either (Miller et al. 1981; Lee et al. 1985).

When employed as analgesics, NSAIDs are efficacious for pain of low to moderate intensity, such as dental pain. While they do not produce the maximal pain relief threshold of opioids, neither do they elicit the unwanted central nervous system effects such as respiratory depression and physical dependence attributed to many opioids. However, NSAIDs are associated with certain adverse effects. Common side effects of nonselective COX inhibitors include gastric ulceration and intolerance, inhibition of platelet function, alterations in renal and hepatic function, and hypersensitivity reactions. In contrast, selective COX-2 inhibitors produce less gastric irritation, do not inhibit platelet function, and are less likely to produce hypersensitivity reactions (Roberts and Morrow 2001).

With respect to ocular use, systemic Banamine® (flunixin megulamine) has been used with some success in combination with topical antibiotics to treat corneal stromal abscesses in horses (Hendrix et al. 1995). However, the authors noted that, similar to topical NSAIDs, Banamine's inhibition of the COX pathway provided by systemic NSAIDs likely delayed corneal vascularization, which in turn delayed resolution of the lesion. This implies that the use of systemic NSAIDs must strike a careful balance between reducing inflammation and retarding wound healing (Hendrix et al. 1995).

4.0 Biomarkers for Severe/Irreversible Ocular Effects as Earlier Humane Endpoints

Public Health Service policy and U.S. Department of Agriculture regulations on pain and distress in laboratory animals state that more than momentary or light pain and distress: (1) must be limited to that which is unavoidable for the conduct of scientifically valuable research or testing, (2) must be conducted with appropriate pain relief medication unless justified in writing by the principal investigator, and (3) will continue for only a necessary amount of time. These regulations also state that animals suffering severe or chronic pain or distress that cannot be relieved should be humanely killed after or, if appropriate, during the procedure, and, finally, that Institutional Animal Care and Use Committees must ensure that the principal investigator complies with the requirements. The majority of animals reported to the Department of Agriculture that experience unrelieved pain and distress are justified by regulatory testing requirements.

The Organisation for Economic Co-operation and Development (OECD) published a guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety assessment (OECD 2000). According to this document, guiding principles for humane endpoints include the following:

- designing studies to minimize any pain, distress, or suffering, consistent with the scientific objective of the study
- sacrificing animals at the earliest indication of severe pain, distress, or impending death, and avoiding severe pain, suffering, or death as endpoints
- terminating animal studies once study objectives are achieved or when it is realized that these objectives will not be achieved
- including knowledge about the test substance in the study design
- defining in the protocol or standard operating procedure the conditions under which authorized personnel should intervene to alleviate pain and distress by humane killing.

Accordingly, humane endpoints recognized and accepted by current EPA (2003), Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN 2007) and European Union (EU 2001) regulatory guidelines for ocular hazard assessment include severe and enduring signs of pain or distress or eye lesions considered to be irreversible.

A recent report of the National Research Council Committee on Recognition and Alleviation of Pain in Laboratory Animals emphasized the need for increased efforts to identify appropriate humane endpoints (NRC 2009).

During the 2005 symposium “Minimizing Pain and Distress in Ocular Toxicity Testing,” panelists discussed early adverse responses predictive of ocular injury outcome in humans. Following are ocular lesions considered predictive of maximal severity (severe irritant or corrosive with irreversible effects, including EPA Category I [EPA 2003], GHS Category I [UN 2007], and EU R41 [EU 2001]) that could be used routinely as humane endpoints to terminate a study:

- Endpoints currently accepted for study termination (i.e., Draize corneal opacity score of 4 that persists for 48 hours, corneal perforation or significant corneal ulceration including staphyloma, blood in the anterior chamber of the eye, absence of light reflex that persists for 72 hours, ulceration of the conjunctival membrane, necrosis of the conjunctiva or nictitating membrane, or sloughing [OECD 2002])
- Vascularization of the corneal surface (i.e., pannus)
- Destruction of more than 75% of the limbus
- No diminishment in area of fluorescein staining and/or increase in depth of injury increased over time

- Lack of re-epithelialization 5 days after application of the test substance
- Depth of injury to the cornea (routinely using slit-lamp and fluorescein staining) in which corneal ulceration extends beyond superficial layers of the stroma

The panel discussion also led to a discussion of other endpoints that might allow for early termination of a study. These include destruction of the limbus and the relationship to re-epithelialization of the cornea, and positive results in Shirmer's test, which measures moisture content of the corneal tear film. A positive result in Shirmer's test would suggest that conjunctival redness is likely to return to normal within 21 days.

5.0 Summary

Both human and veterinary medicine have provided a great deal of clinical experience with a range of topical anesthetics and systemic analgesics for the relief of pain. However, the subjective nature of identifying and treating pain in animals makes it difficult to establish the relative usefulness of available therapeutic options. This is particularly true in the case of ophthalmic pain. Few published studies relate directly to the eye, as the majority have focused on the relief of postsurgical pain and/or pain resulting from trauma.

Based on the large volume of studies detailing the safety and efficacy of tetracaine and proparacaine, these topical anesthetics appear to be among the most widely used in practice. Proparacaine may be considered more appropriate for treating ophthalmic pain given its relative innocuousness to the corneal epithelium and the extended duration of anesthesia it affords. However, their reported adverse effects on wound healing suggest that the utility of these agents beyond acute pain relief may be limited. Thus they are recommended for use only as initial analgesic therapy in an *in vivo* ocular toxicity test.

The most commonly used systemic analgesic among veterinarians is the lipophilic opioid buprenorphine, which has a well-characterized margin of safety in multiple species. While its usefulness in relieving postsurgical pain in rabbits is well documented, little data support its use for ophthalmic pain. However, Buprederm™, a new transdermal formulation of buprenorphine currently under development, provides sustained analgesia over the 72-hour patch application period, with no local irritation with repeated patch application. This suggests that repeated use of Buprederm™ patches may provide effective pain relief over the observation period required during ocular toxicity testing (i.e., up to 21 days).

Sufficient data suggest that combining a topical anesthetic (e.g., proparacaine) with a systemic analgesic (e.g., buprenorphine or Buprederm™ patches used repeatedly) may provide an effective therapeutic approach to minimizing or eliminating ocular pain during ocular toxicity testing. For this reason, ICCVAM proposes that topical anesthetics be routinely used prior to instillation of a test substance unless adequate scientific rationale indicates that they should not be used. In addition, in order to minimize pain and distress from ocular damage caused by corrosive or severely irritating substances, a single dose of a systemic analgesic should be used routinely before instillation of a test substance. Treatment with a systemic analgesic should continue as long as a test animal displays clinical signs of more than momentary or slight pain or distress (e.g., vocalization, pawing at the treated eye).

As an additional measure to minimize pain and distress, ICCVAM recommends that ocular lesions considered predictive of severe irritant or corrosive substances (EPA Category I [EPA 2003], GHS Category 1 [UN 2007], and EU R41 [EU 2001]) be used routinely as humane endpoints to terminate a study.

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7.0 Glossary[3]

Adnexa: Adjacent anatomical parts.

Analgesia: A deadening or absence of the sense of pain without loss of consciousness.

Anesthesia: The loss of sensation or of the response to pain stimuli that results from inhibition of nerve excitation or conduction.

Anesthetic: A drug that induces anesthesia by inhibiting nerve excitation or conduction when applied or injected locally at the site of injury or topically (e.g., on the skin, mucous membrane, or surface of the cornea).

Assay:[4] The experimental system used. Often used interchangeably with *test* and *test method*

Chemokines: Any of various cytokines produced in acute and chronic inflammation that mobilize and activate white blood cells.

Chemosis: A form of eye irritation in which the membranes that line the eyelids and surface of the eye (*conjunctiva*) become swollen.

Classification system: An arrangement of quantified results or data into groups or categories according to previously established criteria.

Conjunctiva: The mucous membrane that lines the inner surfaces of the eyelids and folds back to cover the front surface of the eyeball, except for the central clear portion of the outer eye (the cornea). The conjunctiva is composed of three sections: palpebral conjunctiva, bulbar conjunctiva, and fornix.

Cornea: The transparent part of the coat of the eyeball that covers the iris and pupil and admits light to the interior.

Corneal opacity: A subjective measurement of the extent of opaqueness of the cornea following exposure to a test substance. Increased corneal opacity is indicative of damage to the cornea.

Corrosion: Destruction of tissue at the site of contact with a substance.

Corrosive: A substance that causes irreversible tissue damage at the site of contact.

Cyclooxygenase: Either of two related enzymes (i.e., COX-1 and COX-2) that control the production of prostaglandins and are blocked by aspirin

Cytokines: Any of several regulatory proteins, such as the interleukins and lymphokines, that are released by cells of the immune system and act as intercellular mediators in the generation of an immune response.

Depth-of-injury: The level of penetration to which injury to various tissue layers of the corneal epithelium produced by a test substance (e.g., epithelium, stroma, endothelium).

Distress: To cause pain, or stress, or suffering to.

Endpoint:[4] The biological process, response, or effect assessed by a test method.

Esthesiometry: The measurement of the degree of tactile or other sensibility.

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[3] The definitions in this Glossary are restricted to their uses with respect to the Draize rabbit eye test method and in the assessment or treatment of pain and distress.

[4] Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM 2003)

Fluorescein staining: A subjective measurement of the extent of fluorescein sodium that is retained by epithelial cells in the cornea following exposure to a test substance. Increased fluorescein retention is indicative of damage to the corneal epithelium.

Globally Harmonized System (GHS): A classification system presented by the United Nations that provides (a) a harmonized criteria for classifying substances and mixtures according to their health, environmental and physical hazards, and (b) harmonized hazard communication elements, including requirements for labeling and safety data sheets.

Hazard:[4] The potential for an adverse health or ecological effect. A hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

Humane endpoints: Predetermined criteria (e.g., severe opacity, perforation, ulceration, or necrosis of the cornea) used to evaluate whether a study should be discontinued early for humane or ethical reasons.

Intramuscular injection: An injection into the substance of a muscle.

Intravenous injection: An injection into a vein.

In vitro: In glass. Refers to assays that are carried out in an artificial system (e.g., in a test tube or petri dish) and typically use single-cell organisms, cultured cells, cell-free extracts, or purified cellular components.

In vivo: In the living organism. Refers to assays performed in multicellular organisms.

Iris: The contractile diaphragm perforated by the pupil and forming the colored portion of the eye.

Lacrimation: Secretion and discharge of tears.

Light reflex: Contraction of the pupil when light falls on the eye.

Limbus: The edge of the cornea where it joins the sclera.

Necrosis: Death of cells or tissues through injury or disease, especially in a localized area of the body.

NSAID: A nonsteroidal anti-inflammatory drug such as aspirin or ibuprofen.

Ocular: Of or relating to the eye.

Ocular corrosive: A substance that causes irreversible tissue damage in the eye following application to the anterior surface of the eye.

Ocular irritant: A substance that produces a reversible change in the eye following application to the anterior surface of the eye.

Ophthalmic: Of or relating to the eye; ocular.

Opioid: Any of various sedative narcotics containing opium or one or more of its natural or synthetic derivatives or a drug, hormone, or other chemical substance having sedative or narcotic effects similar to those containing opium or its derivatives: a natural brain opiate.

Organotypic: An alternative test method that uses an organ harvested from animals that have been killed for food or for other purposes (e.g. isolated chicken eye).

Pain: An unpleasant sensation occurring in varying degrees of severity as a consequence of injury, disease, or emotional disorder; suffering or distress.

Pannus: A specific type of corneal inflammation that begins within the conjunctiva, and with time spreads to the cornea. Also referred to as *chronic superficial keratitis.*

Parenteral injection: Taken into the body or administered in a manner other than through the digestive tract; intravenous or intramuscular.

pH: A measure of the acidity or alkalinity of a solution. A pH of 7.0 is neutral; higher pHs are alkaline, lower pHs are acidic.

Protocol:[4] The precise, step-by-step description of a test, including the listing of all necessary reagents, criteria and procedures for the evaluation of the test data.

Re-epithelialization: The mechanism of reparation of the epithelium involving formation of new cells in the limbus and their growth and migration to replace those cells lost in an area of tissue damage.

Refinement alternative:[4] A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being.

Reliability:[4] A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability.

Replacement alternative:[4] A new or modified 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).

Sereny test: A model of keratoconjunctivitis produced within 24 hours after inoculation of the conjunctival sac with bacteria such as *Escherichia coli* or *Listeria monocytogenes*.

Severe irritant: (a) A substance that causes tissue damage in the eye following application to the anterior surface of the eye that is not reversible within 21 days of application or causes serious physical decay of vision. (b) Substances that are classified as GHS Category 1, EPA Category I, or EU R41 ocular irritants.

Shirmer's test: A test for tear production performed by measuring the area of moisture on a piece of filter paper inserted over the conjunctival sac of the lower lid, with the end of the paper hanging down on the outside.

Slit-lamp microscope: An instrument used to directly examine the eye under the magnification of a binocular microscope by creating a stereoscopic, erect image; may also be used with a depth-measuring device to objectively measure corneal thickness.

Sloughing: To shed or cast off epithelial cells; necrotic tissue in the process of separating from viable portions of the body.

Staphyloma: Protrusion of the sclera or cornea, usually lined with uveal tissue, due to inflammation.

Subcutaneous injection: An injection into the subcutaneous layer of the skin.

Tear film: The field covering the anterior surface of the cornea composed of three layers (i.e., mucous, aqueous, lipid) produced by lacrimal fluid and secretions of the meibomian and conjunctival glands.

Test:[4] The experimental system used; used interchangeably with *test method* and *assay*.

Test method[4]**:** A process or procedure used to obtain information on the characteristics of a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified conditions. Used interchangeably with *test* and *assay*. See also *validated test method* and *reference test*.

Ulceration: The process of forming a lesion (e.g., erosion) of the corneal epithelium that over time may be accompanied by formation of pus and necrosis of surrounding tissue, usually resulting from inflammation or ischemia.

Validated test method:[4] An accepted test method for which validation studies have been completed to determine the relevance and reliability of this method for a specific proposed use.

Validation:[4] The process by which the reliability and relevance of a procedure are established for a specific purpose.

Vascularization: The process of becoming vascular; angiogenesis.

Weight of evidence (process): The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

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