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 (ICCVAM)

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

National Institute of Environmental Health Sciences

National Institutes of Health

U.S. Public Health Service

Department of Health and Human Services

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List of Abbreviations and Acronyms

Cat.	Category
Colipa	European Cosmetic, Toiletry and Perfumery Association
COX	Cyclooxygenase
CPSC	U.S. Consumer Product Safety Commission
DOL	U.S. Department of Labor
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
GHS	United Nations Globally Harmonised System of Classification and Labelling of Chemicals
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IRAG	Interagency Regulatory Alternatives Group
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIOSH	National Institute for Occupational Safety and Health
NRC	National Research Council
NSAID	Nonsteroidal anti-inflammatory drug
OECD	Organisation for Economic Co-operation and Development
OTWG	Ocular Toxicity Working Group
USDA	U.S. Department of Agriculture
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), although not formal policy among all U.S. Federal agencies, is a protocol refinement that could provide a significant reduction of animal pain and distress. Since 1984, the U.S. Consumer Product Safety Commission (CPSC) has recommended preapplication of tetracaine ophthalmic anesthetic for all rabbit eye toxicity studies. However, current EPA and OECD test guidelines for the rabbit eye test state that topical anesthetics can only be used if the user demonstrates that such pretreatments do not interfere with the results of the tests. Therefore, they often are not used because a separate study would likely be necessary to provide such information.

In a 1991 workshop organized by the Interagency Regulatory Alternatives Group (IRAG) 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, since pain is temporarily relieved and the extent of injury can be evaluated (Seabaugh et al. 1993). In 2003 the Environmental Protection Agency (EPA) nominated four areas for evaluation by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), including evaluating ways of alleviating pain and suffering which might arise from administration of mild to moderate irritants in current *in vivo* eye irritation testing. A symposium entitled “Minimizing Pain and Distress in Ocular Toxicity Testing,” was convened in May 2005, and was jointly organized by 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. The workshop was supported by the European Cosmetic, Toiletries and Perfumery Association. Similar to the 1991 IRAG workshop recommendations, the symposium invited experts agreed that topical anesthesia should be routinely provided as a pretreatment to animals used for ocular toxicity testing, but added that combinations of general or topical anesthesia and systemic analgesia should be routinely used to avoid pain, and 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 (i.e., tetracaine or proparacaine) with a systemic analgesic (i.e., 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), but 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). There have also been reports that anesthetics interfere with the irritant response and yield data that are not reliable (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 (GHS Category I [UN 2003], EU Category R41 [EU 2001], or EPA Category I [EPA 1996]) that could be used routinely as humane endpoints to terminate a study.

The purpose of this document is to provide a comprehensive review of 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 in which the effect of pretreatment with the topical anesthetic tetracaine hydrochloride (0.5% w/v) on the ocular irritancy potential of 97 formulations was evaluated.

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Executive Summary

There has been a great deal of clinical experience in both human and veterinary medicine 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 the relative utility of available therapeutic options. This is particularly true of ophthalmic pain. There are only a small number of published studies directly related to the eye, as the majority have focused the relief of post-surgical pain and/or pain resulting from trauma.

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

Use of Topical Anesthetics and Systemic Analgesics

A 1991 Interagency Regulatory Alternatives Group (IRAG) workshop entitled Updating Eye Irritation Methods: Use of Ophthalmic Topical Anesthetics evaluated use of topical ophthalmic anesthetics and/or systemic analgesics during the Draize rabbit eye irritation test. A symposium entitled Minimizing Pain and Distress in Ocular Toxicity Testing re-examined this topic in 2005 (**Appendix A**). 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, which was supported by the European Cosmetic, Toiletry and Perfumery Association (Colipa). Each meeting produced similar recommendations and recognition of the limitations

[1] OECD TG 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, the EPA (1998) 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.:

associated with the use of topical and/or systemic anesthetics. Experts acknowledged that a single treatment with a topical anesthetic to anesthetize the surface of the cornea prior to the application of the test article to the eye could cause slight physiologic changes that could alter the response. However, the predominant view was that such alterations to the response would be slight if any, and any effect on the irritant response would tend to slightly increase. Such topical anesthesia is used in millions of cataract surgeries annually, and during routine eye exams to anesthetize the corneal surface prior to intraocular pressure measurements for glaucoma screening. A recent NICEATM evaluation of the effects of tetracaine hydrochloride (0.5% w/v) pretreatment on the ocular irritancy potential of 97 formulations indicate that such pretreatments had no impact on the hazard classification severity category of observed ocular irritation.

The use of topical anesthetics was considered acceptable by a consensus of those participating in both meetings, since the anesthetics at least avoid the discomfort experienced from installation of the test article on the eye, and temporarily prevent any pain and distress that might result from immediate ocular damage. Participants in both meetings also recommended that combinations of general or topical anesthesia and systemic analgesia be routinely used to avoid pain, and that induced lesions should be treated with continued systemic analgesia. They also recognized that, although many types of systemic analgesics could be considered useful in alleviating pain, opioid analgesics (e.g., buprenorphine) were likely to be most effective in ocular safety testing since others (e.g., nonsteroidal anti-inflammatories) could be expected to adversely affect results based on their affects on the wound healing process.

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

Pretreatment with a systemic analgesic was also recommended to provide for relief of ocular pain that might result from any chemically induced injuries. Pretreatment with pre-emptive analgesia is more effective than waiting to treat after the onset of pain, and is commonly practiced in veterinary medicine. Of systemic analgesics, veterinarians use the lipophilic opioid, buprenorphine, most regularly. Buprenorphine's margin of safety is well characterized in multiple species, and a single dose is recommended for routine pretreatment before a Draize 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 systemic analgesics are recommended to continue until these lesions and/or clinical signs are absent.

The effectiveness of buprenorphine in relieving post-surgical pain in rabbits is well documented. However, there are a limited number of studies that have evaluated the efficacy of buprenorphine in the relief of ocular pain. Trevithick et al. (1989) found that buprenorphine injected at 5-hour intervals maintained a stable degree of analgesia for the 24 hours. In addition, buprenorphine has a long history of managing postoperative pain in humans.

Based on its 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 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 over the 72-hour patch application period with no local irritation with repeated patch application in humans. 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).

Use of Humane Endpoints to Terminate an Ocular Toxicity Study

Public Health Service policy and U.S. Department of Agriculture (USDA) 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, and finally, that 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 (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 (Globally Harmonised System of Classification and Labelling of Chemicals [GHS] Category I [UN 2003], European Union [EU] Category R41 [EU 2001], or EPA Category I [EPA 1996]) that could be used routinely as humane endpoints to terminate a study. Among the invited participants were human and veterinary ophthalmologists and anesthesiologists, scientific experts in ocular hazard testing, research scientists, and industrial toxicologists. After these discussions, the following endpoints were recommended for routine use for early study termination:

- 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 [Organisation for Economic Co-operation and Development 2002])
- Vascularization of the corneal surface (i.e., pannus)
- Destruction of more than 75% of the limbus destroyed

- Lack of diminishment in area of fluorescein staining 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 corneal ulceration extends beyond superficial layers of the stroma or increase in the depth of injury over time

After considering the available relevant data, information, and analyses provided in this background review document, ICCVAM 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 (provided in a separate document, url to be inserted). These recommendations include proposed usefulness and limitations, proposed modifications to the current standardized test method protocol, and proposed future studies and activities.

1.0 Background

Draize et al. developed the rabbit eye test (1944) 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 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 for discomfort resulting from materials with unknown properties 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." 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 (TG) for the rabbit eye test state that topical anesthetics can only be used if the user demonstrates that such pretreatments do not interfere with the results of the tests (EPA 1998; OECD 1987).[2] For this

[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

reason, they are not often 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. The level of injury may be exaggerated by a reduction in ocular defense mechanisms (e.g., neuronal activation of goblet cells for tear fluid secretion), and 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 committee considered the use of anesthetics acceptable because such 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 need for such measures, a symposium entitled Minimizing Pain and Distress in Ocular Toxicity Testing was convened at the National Institutes of Health in Bethesda, Maryland, on May 13, 2005 (**Appendix A**). 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. The European Cosmetic, Toiletry and Perfumery Association (Colipa) 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

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carefully selected to ensure that no significant differences in reaction to the test substance will result from its use" (1998).

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 pre-emptive 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 document is to provide a comprehensive review of 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 in which the effect of pretreatment with the topical anesthetic tetracaine hydrochloride (0.5% w/v) on the ocular irritancy potential of 97 formulations was evaluated.

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 due to the evolutionary process (Wright et al. 1985; Hansen 1997). Animals that are ill or injured 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: increased skeletal muscle tone, blood pressure, and/or heart rate; attraction to the area of pain; pupillary dilation; and altered respiration (Cramlet and Jones 1976; Wright et al. 1985). 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, as well as observations of comfort, appearance, and behavior. 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) that provided 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 with a duration of 20 minutes to two to three 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 pain from superficial sources (e.g., cornea) from pain arising from deeper structures in the eye (Ketring 1980; Bartfield et al. 1994). *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 (**Appendix B**).

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 mechanisms 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 post-operative uses, on which several thorough reviews are available (Flecknell 1984; Flecknell and Liles 1990; Flecknell 1991; Flecknell and Liles 1992; Flecknell 1995). Perhaps

603 the greatest clinical concern regarding the use of these types of agents is the side effects with
604 which they are associated. In humans, opioid administration is commonly associated with
605 respiratory depression. However, this effect is less pronounced in animals, especially when
606 mixed agonist/antagonist opioids (e.g., buprenorphine) are used (Flecknell 1995). In this
607 regard, a wide safety margin for buprenorphine has been demonstrated in rabbits, where
608 doses ranging from 0.0075 to 0.3 mg/kg produce effective analgesia without serious
609 respiratory depression (Flecknell and Liles 1990). Reports of clinical studies in humans
610 describe a low incidence of local and/or systemic adverse effects, a lack of immunotoxicity
611 associated with other opioids (e.g., morphine), and maintenance of cognitive function during
612 long-term therapy (Scott et al. 1980; Budd 2002; Budd and Collett 2003; Sorge and Sittl
613 2004).

614 Another concern regarding systemic opioid use is that many of these drugs provide only
615 short-term analgesia, with maintenance of pain relief requiring repeated administration every
616 one to 3 hours. From a practical perspective for a testing laboratory, such a regimen is clearly
617 not feasible. One exception is buprenorphine, which has been shown in humans, pigs,
618 rodents, and rabbits to provide effective pain relief for up to 12 hours (Cowan et al. 1977;
619 Heel et al. 1979; Dum and Herz 1981; Hermanssen et al. 1986; Flecknell and Liles 1990;
620 Flecknell 1996). This may be due to the fact that buprenorphine dissociates very slowly from
621 its receptor relative to other opioids, which has been demonstrated *in vitro* (PDR 2002).
622 Studies in multiple species have also shown that, while the intensity of analgesia induced by
623 buprenorphine does not appear to increase with dose, the duration of analgesia is dose
624 dependent (Cowan et al. 1977; Hermanssen et al. 1986; Hoskin and Hanks 1987; Nolan et al.
625 1987; Flecknell and Liles 1990). However, the onset of action is delayed in rabbits
626 (approximately 30 minutes after treatment), suggesting that buprenorphine treatment prior to
627 testing a potentially irritating/corrosive substance is warranted (Flecknell and Liles 1990).

628 Taken together, these findings likely contribute to the fact that buprenorphine is one of the
629 most commonly used analgesic agents in laboratory and companion animal species, as
630 demonstrated by multiple surveys of its use in veterinary practice (Dohoo and Dohoo 1996;
631 Hubbell and Muir 1996; Watson et al. 1996; Capner et al. 1999; Lascelles et al. 1999; Joubert
632 2001). However, as indicated above, many of the reported veterinary uses of buprenorphine
633 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 indicating 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, excretion of buprenorphine is predominantly via the feces, with only a small amount present in the urine. For this reason, buprenorphine is considered the safest opioid of 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 injectable 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, and 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 one 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 a means of providing 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 also (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 (i.e., maintaining the animal in a supine position during dosing and for at least one 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). Similar to the intranasal route, these properties suggest that rectal gels 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).

3.2.2 Non-steroidal 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, since it inhibits COX activity only in the brain. Acetaminophen may therefore be less likely to interfere with wound healing. 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 threshold level of pain relief of opioids, neither do they elicit the unwanted central nervous system effects such as respiratory depression and physical dependence attributed to many opioids. However, they 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 it is necessary to strike a careful balance between reducing inflammation and retarding wound healing in the use of systemic NSAIDs (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 (USDA) 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 USDA that experience unrelieved pain and distress are justified by regulatory testing requirements.

The Organization of 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 (1) designing studies to minimize any pain, distress, or suffering, consistent with the scientific objective of the study; (2) sacrificing animals at the earliest indication of severe pain, distress, or impending death, and avoiding severe pain, suffering, or death as endpoints, (3) terminating animal studies once study objectives are achieved or when it is realized that these objectives will not be achieved; (4) including knowledge about the test substance in the study design; (5) 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 Environmental Protection Agency (EPA 1996), European Union (EU 2001), and the Globally Harmonised System of Classification and Labelling of Chemicals (GHS; UN 2003) 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 GHS Category I [UN 2003], EU Category R41 [EU 2001], or EPA Category I [EPA 1996]) 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 (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

There has been a great deal of clinical experience in both human and veterinary medicine 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 utility of available therapeutic options. This is particularly true in the case of ophthalmic pain, on which there are only a small number of published studies directly related to the eye, as the majority have focused on relieving post-surgical 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, and thus they are recommended only for use 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 post-surgical pain in rabbits is well documented, a paucity of data supports 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).

Based on this information, it appears that there are sufficient data to suggest that combining a topical anesthetic (e.g., proparacaine) with a systemic analgesic (e.g., buprenorphine or the repeated use of Buprederm™ patches) 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 indicate that they should not be used. In addition, in order to minimize actual 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 (GHS Category I [UN 2003], EU Category R41 [EU 2001], or EPA Category I [EPA 1996]) be used routinely as humane endpoints to terminate a study.

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Appendix A

Minimizing Pain and Distress in Ocular Toxicity Testing: Summary of an ICCVAM/NICEATM/ECVAM Scientific Symposium

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Minimizing Pain and Distress in Ocular Toxicity Testing: Summary of an ICCVAM/NICEATM/ECVAM Scientific Symposium

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

March 2009

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List of Abbreviations and Acronyms

BLS	U.S. Bureau of Labor Statistics
COLIPA	European Cosmetic, Toiletry, and Perfumery Association
CPSC	Consumer Products 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
FHSA	U.S. Federal Hazardous Substances Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GHS	United Nations Globally Harmonized System of Classification and Labeling of Chemicals
ICCVAM	Interagency Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems
IRAG	Interagency Regulatory Alternatives Group
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	U.S. National Institute of Environmental Health Sciences
NSAIDs	Nonsteroidal anti-inflammatory drugs
NTP	U.S. National Toxicology Program
OECD	Organization of Economic Cooperation and Development
OSHA	U.S. Occupational Safety and Health Authority
TSCA	Toxic Substances Control Act
USDA	United States Department of Agriculture

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Overview

The symposium "Minimizing Pain and Distress in Ocular Toxicity Testing" was organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the European Centre for the Validation of Alternative Methods (ECVAM) with support from the European Cosmetic, Toiletries and Perfumery Association (COLIPA). The symposium was held at the National Institutes of Health (NIH), Bethesda, MD on May 13, 2005. The goals of the symposium were to: 1) review current understanding of the sources and mechanisms of pain and distress in chemically induced ocular toxicity testing; 2) identify current best practices for preventing, recognizing, and alleviating ocular pain and distress; and 3) identify additional research, development, and validation studies to support scientifically valid ocular testing procedures that avoid pain and distress. Invited participants included human and veterinary ophthalmologists and anesthesiologists, scientific experts in ocular hazard testing, research scientists, U.S. Federal regulators, and industrial toxicologists. Implementation of recommendations from the symposium should eliminate most of the pain and distress associated with ocular safety testing in the rabbit Draize test.

Introduction

Societal concern for evaluating consumer products for ocular irritation and/or corrosion was heightened in 1933 when a 38-year-old woman went blind after her eyelashes and eyebrows were tinted with a product containing paraphenylenediamine, a chemical with the potential to cause allergic blepharitis, toxic keratoconjunctivitis, and secondary bacterial keratitis (Wilhelmus 2001). In 1938, the U.S. Congress responded to these concerns by enacting the Federal Food, Drug, and Cosmetic Act of 1938, which included extending the regulatory control of the U.S. Food and Drug Administration (FDA) to cosmetics (FDA 1938). This legislation required manufacturers to evaluate product safety before marketing their products (Wilhelmus 2001). Later, several additional legislative statutes were enacted to enable government agencies to regulate a variety of substances that could pose a risk to ocular health. **Table 1** provides a synopsis of current U.S. regulatory laws pertaining to eye irritation and corrosion.

Table 1 Summary of Current U.S. Legislation Related to Ocular Health*

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

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

*Adapted from Wilhelmus (2001)

According to the Bureau of Labor Statistics (BLS), accidental eye injury is the leading cause of visual impairment in the U.S. (BLS 2003). In 2003, eye injuries from chemicals and their products (6,080) accounted for 16% of all eye injuries (36,940) reported as the cause of Days Away From Work for employees. Chemical products in general (e.g., solvents, caustics, soaps/detergents, cleaning/polishing agents, disinfectants) were responsible for approximately half of the injuries, whereas acids and alkalis accounted for 11% of the injuries.

The FDA issued requirements for ocular safety testing in response to the enacted consumer safety laws. The rabbit eye test was developed to identify and classify the ocular hazard potential of new chemicals or chemical products (Draize et al. 1944). The resulting hazard classification is then used to determine labeling requirements that will alert the public to take appropriate precautions in order to prevent ocular injury. Public concern about the use of animals in testing has resulted in significant efforts to develop and validate alternative *in vitro* test methods for ocular hazard assessment. Despite over 25 years of effort, including several large validation studies (e.g., Balls et al. 1995; Gettings et al. 1996), there are still no validated and accepted non-animal ocular safety testing methods. Until valid alternatives are accepted as complete replacements, the animal test will continue to be required by U.S. Federal and European regulatory agencies for ocular hazard evaluation. One of the main concerns with this test method is the pain and distress that may be produced in the test animals.

Previous meetings and workshops have reviewed methods and strategies for reducing pain and distress in ocular safety testing (Seabaugh et al. 1993, Nussenblatt et al. 1988). However, current testing regulations and guidelines only suggest consideration of topical anesthetics after pain and distress is observed in the first animal tested. Routine pre-treatment with topical anesthetics is not recommended, and no mention of how to address post-application pain and distress associated with ocular damage exists. This symposium was organized to review the current understanding of ocular pain mechanisms and physiological pathways, symptoms and signs of the pain response, and methods and strategies that could be used to avoid or alleviate pain and distress, including the incorporation of earlier, more humane endpoints.

Symposium Objectives

The objectives of the symposium were to:

- Identify and better understand mechanisms of pain by reviewing the physiological pathways affected by chemically-induced ocular injury
- Review the known responses to chemical injury in humans (based on accidental exposures) and the levels of pain associated with specific ocular lesions
- Identify available approaches to:
  - Alleviate or avoid ocular pain resulting from initial test article application
    - Can pre-application topical anesthetics be used routinely without interfering with the ocular hazard classification?
  - Alleviate or avoid post-application ocular pain and distress
    - Can pain and distress from induced eye injuries be routinely treated, as with human injuries, without interfering with the hazard classification?
- Identify earlier, more humane endpoints to terminate studies before or at the onset of painful injuries

Overview of 1991 Interagency Regulatory Alternatives Group (IRAG) Workshop

In 1991, an *ad hoc* committee of the 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. 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. The level of injury may be exaggerated by a reduction in ocular defense mechanisms (e.g., reduced tear fluid secretion), and duration of injury may be lengthened by impairment of repair processes (e.g.,

reduced collagen deposition). Despite these issues, and although not official policy of all U.S. Federal agencies, the use of anesthetics was considered acceptable by a consensus of those participating on the committee, since pain is at least temporarily relieved for the animal and the time and extent of injury can still be evaluated.

Symposium Sessions

Following are summaries of the information communicated by the speakers in each session of the symposium.

Recognition and Sources of Pain in Ocular Injuries and Ocular Safety Testing

Presenters for this session included Dr. Marc Feldman of the Cleveland Clinic, Dr. Roger Beuerman of Louisiana State University, and Dr. Kirk Tarlo, of Allergan, Inc.

Human Ocular Injury and Sources of Pain

The human pain response occurs through nociception accompanied by hypersensitivity with central and peripheral sensitization of the injured area. Nociception is an early warning sign, whereas inflammatory pain is present to reduce further injury. Nociceptive pain involves the descending track of the trigeminal nerve. Primary sensory neurons transduce the nociceptive signal, provide peripheral sensitization and produce transcriptional changes in ganglion cells. Numerous physical (e.g., heat, cold, pressure, mechanical) and chemical (e.g., capsaicin, bradykinin, cationic species) agonists are capable of activating nociceptors (e.g., acid sensing ion channels, purinergic receptors). Increased peripheral sensitization occurs from mediators released during the inflammatory process (e.g., bradykinin, prostaglandins) that induce receptor sensitization and activation. Inflammatory pain may lead to either neuropathic pain that is maladaptive and pathologic, or functional pain that limits mobility and perhaps serves as a mechanism to prevent further damage. Central sensitization from secondary hyperalgesia or tactile allodynia[1] has been reported. Disinhibition (e.g., reduced inhibitory transmission, altered modulation from brain) also may result in centrally induced hypersensitivity or late effects (e.g., diffuse pain sensitivity, sickness syndrome).

[1] Allodynia refers to pain from stimuli that are not normally painful. The pain may occur in areas other than those stimulated.

Treatment of a pain response associated with human ocular injury, therefore, should be based on knowledge of the location of its origin and the mechanism(s) involved in its production. Pain therapy should be guided toward the nociception, modulation, and sensitization components.

Mechanisms and Biomarkers of Chemically-Induced Pain in Animals

The sensation of pain is unique and differs depending on the type of stimulation (e.g., thermal, mechanical). Pain intensity also varies with gender, age, and ethnicity, and is affected by stress and other environmental factors. In humans, pain assessment is based on verbal responses from the patient. However, an accurate assessment of chemically induced pain in animals requires an understanding of the mechanisms and biomarkers associated with pain, since the degree of pain cannot be assessed by vocalization. There are sensory nerve terminals located in the corneal epithelium and therefore, chemicals may elicit a pain response without producing noticeable damage. Numerous involuntary reflexes occur in response to painful stimuli in animals (e.g., tearing, blinking, head movement, vascular changes). The corneal pain system is linked to the neurogenic inflammatory response. Disruption of the tear film results in breakdown of the blood-conjunctiva barrier, platelet release mechanism activation, inflammatory cell infiltration, fibronectin deposition, and plasmin production. Disruption of the corneal epithelium results in intracellular calcium modulation, changes in metabolism and pH, inflammatory processes, and wound healing with maturation and repair. Various ion channels (e.g., calcium, sodium, potassium) are involved in the pain response and may be modulated to stimulate or abrogate the pain response.

Prediction of ocular discomfort also may be based on scoring blinking frequency along with the extent of conjunctival hyperemia. Discomfort is scaled using a score of 0 to 4 as normal, minimal (intermittent blinking and/or squinting), mild (blinking and/or squinting with partial eye closure), moderate (repeated blinking and/or squinting; partial to complete eye closure), and severe (prolonged and complete closure of eye; repeated pawing or rubbing). Hyperemia is scored on a scale of 0 to 3 as normal, mild (flushed reddish palpebral conjunctiva with perilimbal dilation), moderate (crimson red palpebral conjunctiva with perilimbal dilation),

and severe (dark beefy red palpebral conjunctiva with congestion of bulbar and palpebral conjunctiva and pronounced perilimbal dilation).

Panel Discussion on Indicators of Pain and Discomfort in Animals

With regard to initial test article application, the panel concluded that if a substance causes ocular pain in humans, pain in an animal should be anticipated. Any eye stimulation, including topical application of a test article, may be sensed as painful or irritating.

It is expected that substances with certain physicochemical properties (e.g., pH less than 6 or above 8, solids, substances that alter normal osmolarity) will cause pain. However, there are no known physicochemical properties that can be used to indicate that a test substance will not cause pain. Application of the test substance at the same temperature as the eye's surface (approximately 32°C) may reduce the pain and discomfort associated with application.

Panelists suggested that, based on human experience, it should be assumed that any chemically induced ocular lesion is associated with pain, regardless of the severity of the injury. They also recommended that a thorough list of lesions that are likely to be indicators of pain and distress should be compiled.

Alleviation and Avoidance of Ocular Injury and Pain

Presenters for this session included Dr. Marc Feldman of the Cleveland Clinic and Dr. Donald Sawyer of MINRAD International.

Options for Alleviating Ocular Pain in Humans

Pain can be a confounding factor that can impact study results. Treatment modalities for ocular pain in humans include local anesthetics (topical or infiltrative), topical or oral nonsteroidal anti-inflammatory drugs (NSAIDs), opiates, and general anesthetics. Topical anesthetics are generally safe, effective, and increasingly used for invasive ocular surgical procedures (e.g., cataract surgeries, glaucoma surgeries, vitrectomies, globe repairs), but are typically cytotoxic under prolonged, repeated use conditions. Side effects of topical anesthetics used preemptively may be reduced by washout. Infiltration local anesthesia requires retrobulbar block, peribulbar block, and sub-Tenon's block, and is associated with a number of risks (e.g., retrobulbar hemorrhage, diplobia, vagal syncope, ocular puncture, central apnea). Furthermore, brainstem anesthesia following a retrobulbar block could induce

such adverse effects as blindness and immobility in the contralateral eye, dyspagia, hearing difficulties, hyper- or hypo-tension, or tachycardia.

NSAIDs provide the advantage of a wide safety index and are effective in preventing sensitization, but do not block nociception. However, NSAIDs at high doses produce gastrointestinal toxicity and renal impairment and some members of this class have been associated with a higher incidence of cardiovascular problems. NSAIDs are useful for pain relief of corneal abrasions and do not appear to adversely effect wound healing. Systemic opiates are commonly used perioperatively and affect modulation systems in nociception and sensitization. Adverse effects associated with opiates include respiratory depression and nausea, and tolerance also may develop during prolonged use. The partial κ-receptor agonist butorphanol and the partial μ-receptor agonist buprenorphine appear to have longer durations of action than morphine. General anesthetics (e.g., isoflurane, ketamine) primarily affect nociception and are used for some ocular surgical procedures, or in patients with dementia, claustrophobia, or movement disorders. Adverse effects include increased intraocular pressure and incidences of nausea. Some are used in combination with anxiolytics (e.g., ketamine and the α-2 receptor agonist xylazine or a combination of morphine, acepromazine, and a topical anesthetic). Competitive depolarizing neuromuscular blocking agents (e.g., d-tubocuarine and pancuronium) should not be used as anesthetics, since they only immobilize the animals without pain relief.

Minimizing Ocular Pain in Animals with Analgesics/Anesthetics

Sensitivity to pain may depend on the level of innervation of the cornea and increases progressively from lowest to highest across species (canines, felines, equines, and humans, respectively). Ocular pain is managed using anesthetics (general and regional), cycloplegics, corticosteroids, NSAIDs, opioids, and alpha agonists. Topical anesthetics decrease the permeability to sodium that results from depolarization of neuronal membranes during injury in which large transient increases in sodium permeability produce the pain sensation. Onset of action is one minute and the duration is 10 to 15 minutes or longer. Proparacaine (0.5% solution) is most widely used as a topical anesthetic, but may delay wound healing, which limits its use to diagnostic procedures. Lidocaine also with an onset of five minutes and duration of 2 to 3 hours is used. Corticosteroids inhibit phospholipase A2 and prevent release

of the proinflammatory mediators of arachidonic acid metabolites. Topical corticosteroids (e.g., dexamethasone acetate, prednisolone acetate) are used for anterior uveitis, but are contraindicated for corneal ulceration because they delay epithelial healing, increase collagenase activity, and depress local immunity. Systemic corticosteroids (e.g., oral prednisone) are used for orbital, posterior segment, and extensive anterior segment pathology at either anti-inflammatory or immunosuppressive dose levels. Subconjunctival triamcinolone may provide long-lasting relief (2 to 3 weeks) and is used for episcleritis, scleritis, uveitis, or noninfectious keratoconjunctivitis, but granulomas can occur at the injection site. NSAIDs (e.g., diclofenac, indomethacin, flurbiprofen, ketorolac) reduce corneal sensitivity. For surgical pain management, acepromazine or butorphanol are used as premedicaments. Parasympatholytics (e.g., reversibly bind to acetylcholine receptors) prevent ciliary spasm and are used to relieve pain of anterior uveitis and corneal ulceration. Ketoprofen is used for postoperative analgesia. Propofol is used for induction, and isoflurane for general anesthesia. Postsurgical pain is managed using the longer lasting opiate partial μ-receptor agonist buprenorphine (intravenous, subcutaneous, or bucchal) and the anxiolytics diazepam or midazolam.

Topical ocular anesthetics may be divided into those with either 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 with a duration of effect of 20 minutes to 2 to 3 hours. Application frequency of these topical anesthetics increases duration, but not depth of anesthesia. As previously discussed, topical anesthetics are 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 test substances (e.g., increase permeability of corneal epithelium, breakdown barriers that shield toxicity) and thus confound test results. Topical anesthetics should be used for ocular pain relief in animal testing, but observations for corneal damage, decreased tearing, or increased penetration of test materials should be closely monitored for impact on test results.

Panel Discussion on Avoiding and Minimizing Ocular Pain and Distress

Optimal pretreatment analgesics to be considered to reduce pain on initial test article application include combinations of general or topical anesthesia with pre-emptive systemic analgesia for maximal efficacy in treating study-related pain. Local topical anesthetics such as proparacaine (0.5%) are recommended for short term use with the understanding that wound healing might be delayed on long term administration, which could increase the hazard classification of a test substance. As noted with local topical anesthetics, pretreatment analgesics could increase the hazard classification of test substances by inhibition of wound healing. However, the efficacy of pretreatment with topical anesthetics for pain resolution and the known complications of their use are sufficiently understood to warrant their continued use for pain relief.

General anesthetics may be administered by injection or inhalation, and systemic analgesics (e.g., buprenorphine) may be delivered via a topical patch system. Analgesia or anesthesia depends on the specific drug used and may vary considerably within a single class.

Since 1984, the CPSC has recommended preapplication of tetracaine ophthalmic anesthetic for all rabbit eye toxicity studies. Topical anesthetics can exaggerate chemically induced ocular injury by decreasing ocular defenses (e.g., increased epithelial permeability, reduced tearing, reduced blinking) and impairing wound healing. However, documented effects of delayed wound healing are more pronounced with repeated exposure, rather than single use.

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 chemically induced lesions by mechanisms previously mentioned, but pain relief should be obligatory in animals with eye lesions.

Perhaps a more appropriate approach would be to administer pre-emptive analgesics before the ocular insult, because these drugs are most effective at preventing pain, rather than as therapeutic agents after the development of a lesion. Potentially useful agents include narcotic analgesics (e.g., buprenorphine), NSAIDs (e.g., indomethacin, diclofenac, flurbiprofen, ketorolac), and anxiolytics (e.g., acepromazine). New research should focus on the evaluation of systemic analgesic agents, doses, and dose intervals to provide effective

391 analgesia. The effects of analgesics/anesthetics on hazard category classification should be
392 documented.

393 **Biomarkers for Severe/Irreversible Ocular Effects as Earlier Humane Endpoints**

394 Presenters for this session included Dr. William Stokes of the National Institute of
395 Environmental Health Sciences and Dr. Norbert Schrage of the Aachen Center of
396 Technology Transfer in Ophthalmology.

397 Public Health Service policy and U.S. Department of Agriculture (USDA) regulations on
398 pain and distress in laboratory animals state that more than momentary or light pain and
399 distress: 1) must be limited to that which is unavoidable for the conduct of scientifically
400 valuable research or testing; 2) must be conducted with appropriate pain relief medication
401 unless justified in writing by the principal investigator; and 3) will continue for only a
402 necessary amount of time. These regulations also state that animals suffering severe or
403 chronic pain or distress that cannot be relieved should be humanely killed after or, if
404 appropriate, during the procedure, and finally, that Institutional Animal Care and Use
405 Committees must ensure that the principal investigator complies with the requirements. The
406 majority of animals reported to the USDA that experience unrelieved pain and distress are
407 justified by regulatory testing requirements. Use of analgesics and tranquilizers for regulatory
408 purposes requires a determination that these agents do not interfere with a study. For this
409 reason, they are rarely used (EPA 1998, OECD 1987). Most regulatory agencies recommend
410 euthanasia for severe pain and distress or moribund conditions.

411 The Organization of Economic Co-operation and Development (OECD) has published a
412 guidance document on the recognition, assessment, and use of clinical signs as humane
413 endpoints for experimental animals used in safety assessment (OECD 2000). According to
414 this document, guiding principles for humane endpoints include: 1) designing studies to
415 minimize any pain, distress, or suffering, consistent with the scientific objective of the study,
416 2) sacrifice of animals at the earliest indication of severe pain and distress or impending
417 death, and severe pain, suffering, or death are to be avoided as endpoints, 3) termination of
418 animal studies once study objectives are achieved or when it is realized that these objectives
419 will not be achieved, 4) including knowledge about the test substance in the study design, 5)
420 defining in the protocol or standard operating procedure, conditions under which

interventions to alleviate pain and distress by humane killing should be made by authorized personnel. Accordingly, humane endpoints recognized and accepted by current Environmental Protection Agency (EPA 1996), European Union (EU) (EU 2001), and the Globally Harmonized System (UN 2003) regulatory guidelines for ocular hazard assessment include severe and enduring signs of pain or distress, or eye lesions considered to be irreversible.

Panel Discussion on Biomarkers for Severe/Irreversible Ocular Effects

In an attempt to identify additional biomarkers to serve as humane endpoints, panelists discussed early adverse responses predictive of ocular injury outcome in humans. Signs of minor irritation that were cited included tearing, pain, conjunctival redness, fluorescein stippling, loss of superficial wing cells (cells in the corneal epithelium with convex anterior surfaces and concave posterior surfaces) observed using confocal microscopy, and epithelial edema. Early predictive reactions include chemosis of the conjunctiva, blood vessel occlusion, epithelial erosion (cornea and conjunctiva), necrosis demarcation, limbal necrosis, or corneal edema. Intermediate reactions that are predictive of pain include conjunctival necrosis, hyperemic revascularization, persistent epithelial erosion, ulceration, limbal degeneration, conjunctival overgrowth, and corneal vascularization.

Currently, empirical ocular lesions predictive of maximal severity (severe irritant or corrosive with irreversible effects including GHS Category I [UN 2003], EU Category R41 [EU 2001], or EPA Category I [EPA 1996]) that could be used routinely as humane endpoints to terminate a study are: 1) endpoints currently accepted for study termination (e.g., Draize corneal opacity score of 4); 2) vascularization of the corneal surface (i.e., pannus); 3) greater than 75% of the limbus destroyed; 4) area of fluorescein staining not diminished over time and/or depth of injury increased over time; 5) lack of re-epithelialization five days after application of the test substance; 6) extent of depth of injury to the cornea (routinely using slit-lamp and fluorescein staining) where corneal ulceration extends beyond superficial layers of the stroma.

The panel discussion suggested that additional endpoints 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 (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.

Potential biomarkers suggesting that lesions would fully reverse were also discussed. Panelists suggested that conjunctival redness present at day 7 would typically be expected to fully reverse by day 21, and that a test could be terminated if the cornea is clear and no inflammation is present at 48 hours using a slit-lamp examination.

Methods also were identified that were recommended for additional study to determine their utility in producing humane endpoints. These included: 1) photodocumentation of ocular injuries (gross and slit-lamp), 2) slit-lamp biomicroscopy with fluorescein or other vital dye staining, 3) pachymetry measurements, 4) depth of injury measurements, 5) postmortem observations (e.g., histopathology, live/dead cell assays using fresh excised tissue), 6) extent and destruction of the limbus and relationship to re-epithelialization of the cornea, and 7) altered tear production and lesion persistence. The Panelists noted that standardized procedures with these methods are needed to facilitate the collection of data in a systematic fashion.

Conclusion and Recommendations

This symposium provided a forum for the presentation and discussion of: 1) known and putative mechanisms of ocular pain and distress in humans and animals; 2) treatment and prevention of pain and distress; 3) impact of these treatments on regulatory testing requirements; and 4) areas for future research. Ophthalmologists, academic scientists, federal regulators, industrial toxicologists, and experts in the development and use of alternative toxicological methods provided various perspectives on current use of specific treatments. Importantly, specific treatments to alleviate pain and distress in animal models of ocular toxicity required for the optimization and validation of alternative toxicological methods and their impact on regulatory requirements were considered.

The primary conclusions of the experts who participated in this symposium were:

- Pain relief in animals used for ocular toxicity testing should be provided as a pretreatment when there is reason to believe a painful response will be

produced (e.g., test substance produces pain in humans, solution is not iso-osmotic or isotonic, pH is less than 6 or greater than 8, etc.).

- Clinical signs of pain in animals should be carefully observed (examples of some of these signs are provided in **Table 2**) and the study terminated if significant pain or distress is evident.
- Combinations of general or topical anesthesia with pre-emptive systemic analgesia should be used for maximal efficacy in treating study-related pain on initial test article application.
- Adverse responses likely to induce painful responses include minor reversible effects (e.g., conjunctival redness and chemosis, hyperemic revascularization), intermediate predictive effects (e.g., blood vessel occlusion, epithelial erosion or ulceration, limbal degeneration), and severe irreversible effects (e.g., pannus, significant depth of injury, corneal opacity score of 4, etc.).
- Additional biomarkers and techniques should be incorporated into *in vivo* ocular testing to improve the prediction of the humane endpoints (e.g., lack of re-epithelialization)

Table 2 Clinical Signs and Biomarkers Indicative of Pain

Sign/Biomarker
• Intermittent to repeated blinking and/or squinting[1]
• Partial to complete eye closure
• Repeated pawing or eye rubbing
• Vocalization[2]
• Conjunctival hyperemia and chemosis
• Increased blood pressure, respiration, or heart rate
• Electrophysiological responses measured in trigeminal ganglia

[1] Under normal conditions, rabbits do not blink often (Wilhelmus 2001)

[2] Rarely occurs

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Appendix B: Draft Report

Effect of Topical Anesthetic Pretreatment on *In Vivo* Ocular Irritation Hazard Classification

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

February 2009

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Executive Summary

Background

Accidental eye injury is the leading cause of visual impairment in the United States (U.S. Dept. of Labor Statistics [DOL] 2004). In 2002, injuries from chemicals and their products accounted for 16% of all eye injuries reported as the cause of days away from work f (DOL 2004). Because not all employers are required to report such injuries, these numbers may underestimate the actual number of eye injuries. Based on emergency department reports for work-related eye injuries, the National Institute of Occupational Safety and Health (NIOSH) estimated that approximately 39,200 chemical-related eye injuries occurred in 1998 (NIOSH Work-related Injury Statistics, 2004).

The ocular irritation or corrosion potential of substances to which humans may be exposed has been evaluated since 1944 using the Draize rabbit eye test (Draize et al. 1944). Due to the potential pain and distress that may occur in rabbits after application of a severely irritating or corrosive test substance, several approaches have been undertaken to revise the current *in vivo* test method protocol and testing scheme to decrease the likelihood of causing pain and distress. For example, a weight-of-evidence approach based on all available information (e.g., pH values, dermal corrosivity information, structure-activity relationship data) has been used to classify substances as severely irritating or corrosive prior to *in vivo* testing. However, despite these efforts, some substances that are tested in rabbits may cause pain and distress. Therefore, additional refinements to the *in vivo* test method have been proposed, which include the use of a topical ocular anesthetic prior to test substance administration in the rabbit eye test. This report focuses on results of an evaluation of the effects of pretreatment with the topical anesthetic tetracaine hydrochloride (0.5% w/v) on the ocular irritancy potential of 97 formulations.

Database Used for the Evaluation

Product Safety Laboratories provided *in vivo* rabbit eye test scores for all observation days for 97 formulations, together with information about testing conditions (e.g., concentration of formulation tested, amount tested). Due to confidentiality requirements, the compositions of the tested formulations were unknown for the purposes of this evaluation.

Test Method Protocol

The formulations were tested in either 3 or 6 rabbits. Sixteen substances were tested in 6 rabbit studies (n=96 rabbits), and 81 substances were tested in three rabbit studies (n=243 rabbits). *In vivo* testing was conducted in accordance with the U.S. Environmental Protection Agency (EPA) guideline on acute eye irritation testing (EPA 1998). Rabbits were tested sequentially, with the first tested rabbit not receiving anesthesia. If any of the subsequently tested rabbits displayed signs of pain or distress after test article application (e.g., vocalization, pawing at the treated eye), the remaining rabbits were pretreated with 0.5% (w/v) tetracaine hydrochloride ophthalmic solution. Two drops of the anesthetic were placed directly on the cornea in each rabbit eye between 30 seconds and approximately 2 minutes prior to instillation of test substance. The conduct of the remainder of the test method protocol was identical to the protocol described in the EPA guideline on acute eye irritation testing (EPA 1998).

Eyes were evaluated at predetermined intervals (e.g., 1 hour and 1, 2, 3, 7, 14, and 21 days after test substance instillation) for development of irritation and/or corrosion. If eye irritation was considered irreversible (e.g., corneal opacity and/or conjunctival irritation was considered severe), the study was terminated. The degree of irritation was scored using the Draize irritation scale. The observation period was at least 72 hours and not longer than 21 days to allow for evaluation of reversal of observed effects.

Results: Impact of Topical Anesthetic Pretreatment on Regulatory Irritancy Classification

Each formulation tested was assessed to determine if the average irritancy response for the rabbits pretreated with topical anesthesia was more severe or less severe than that observed for the rabbits not pretreated with topical anesthesia. Rabbits pretreated with topical anesthesia tended to produce more severe responses than rabbits that were not pretreated with topical anesthesia for all three regulatory hazard classification schemes. However, none of the observed differences were statistically significant.

An additional analysis was conducted to evaluate the variability among rabbit responses, within a given formulation, when topical anesthesia pretreatment was used as a criterion. For most of the formulations, there was no difference in rabbit irritancy classifications between

rabbits pretreated with topical anesthesia and those that were not pretreated. For all the evaluated regulatory hazard classifications, there appeared to be better agreement in rabbit responses when rabbits that were not pretreated with anesthesia were compared to those that were pretreated with anesthesia. However, none of the observed differences were statistically significant.

Results: Impact of Topical Anesthetic on the Number of Days Required for an Ocular Lesion to Clear

Each formulation tested was assessed to determine if the number of days required for a lesion to reverse for animals pretreated with topical anesthesia was different than animals that were not pretreated with topical anesthesia. None of the differences observed in the day-to-clearing evaluation (when topically anesthetized rabbits were compared to nonanesthetized rabbits) were statistically significant. The largest observed difference was for opacity clearing day, which tended to be slightly greater in the rabbits pretreated with topical anesthesia when compared to those that were not pretreated. However, this difference (33 vs. 22) was not statistically significant. Corneal opacity was the endpoint with the largest difference in number of days until clearing. Although not statistically significant either, the time to clear for corneal lesions in rabbits pretreated with topical anesthesia was slightly longer than in rabbits that were not pretreated.

Summary

For most of the formulations tested, topical anesthetic pretreatment 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. When a difference in ocular irritation was observed, the rabbits pretreated with topical anesthesia more frequently exhibited a more severe response than was observed for rabbits that were not pretreated. However, none of the observed differences were statistically significant. The observed differences occurred in both directions (increasing and decreasing the level of irritancy), which suggests a relation to the inherent variability of the rabbit response rather than to topical anesthetic pretreatment.

These results indicate that topical pretreatment with 0.5% (w/v) tetracaine hydrochloride ophthalmic solution had no significant impact on the variability in rabbit responses to

formulations or the number of days required for an ocular lesion to clear. The topical anesthesia pretreatment also did not significantly affect the irritancy classification for the United Nations Globally Harmonised System of Classification and Labelling, EPA, and European Union classification systems.

1.0 Introduction

Accidental eye injury is the leading cause of visual impairment in the United States (U.S. Dept. of Labor [DOL] 2004). In 2002, injuries from chemicals and their products accounted for 16% of all eye injuries reported as the cause of days away from work for employees (DOL 2004). Because not all employers are required to report such injuries, these numbers may underestimate the actual number of eye injuries. Based on emergency department reports for work related eye injuries, the National Institute of Occupational Safety and Health (NIOSH) estimated that approximately 39,200 chemical-related eye injuries occurred in 1998 (NIOSH, 2004).

The ocular irritation or corrosion potential of substances to which humans may be exposed has been evaluated since 1944 using the Draize rabbit eye test (Draize et al. 1944). Several approaches have been undertaken to revise the current *in vivo* test method protocol and testing scheme to decrease the likelihood of potential pain and distress in rabbits during instillation of an irritating test substance. For example, a weight-of-evidence approach has been used to eliminate severely irritating or corrosive substances prior to *in vivo* testing. Criteria that may be used to identify and classify substances as ocular corrosives or severe irritants prior to *in vivo* testing include high or low pH values ($2 < pH < 11.5$), dermal corrosivity, and structure-activity relationship studies that indicate corrosive properties. However, despite these efforts, some substances that are tested *in vivo* are likely to cause pain and distress in the rabbit. Therefore, additional refinements to the *in vivo* test method have been proposed, including the use of a topical ocular anesthetic prior to test substance administration.

Previous studies have shown that the efficacy of topical ocular anesthetics can be dependent upon a variety of a factors including, but not limited to, the anesthetic used, the anesthetic dose used, the application procedure, and the species tested (Ulsamer et al. 1977; Heywood et al 1978; Johnson, 1980; Anonymous, 1981; Walberg, 1983; Rowan and Goldberg, 1985; Arthur et al. 1986; Durham et al. 1992; Seabaugh et al. 1993). Commonly evaluated topical anesthetics include proparacaine, tetracaine, butacaine, and amethocaine.

In 1986, the Modified Ocular Safety Testing Task Force of the Pharmacology and Toxicology Committee of the Cosmetic, Toiletry, and Fragrance Association, Inc., evaluated

proparacaine and tetracaine (both tested at 0.5% (w/v)) for their potential to increase or decrease the irritancy of four test substances. Results showed that neither topical anesthetic had a significant effect on the observed irritancy of substances tested but noted a trend of increased irritancy in anesthetized eyes (Arthur et al. 1986). Heywood and James stated that 0.5% proparacaine produced no statistically significant difference between the anesthetized and nonanesthetized corneas when 10% sodium lauryl sulfate was used as the irritant.

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 to evaluate the use of anesthetics in eye irritation testing. The workshop indicated that the commonly used anesthetics tetracaine (0.5-5%) and proparacaine (0.1-0.5%) produced an almost immediate anesthetic effect lasting up to 20 minutes. These anesthetics eliminated local pain and touch sensation but increased ocular permeability, reduced tear volume, reduced blink frequency, and delayed wound healing (Seabaugh et al. 1993).

Studies by Walberg (Walberg 1983; Rowan and Goldberg 1985) suggested that use of tetracaine hydrochloride (0.5%, two drops on the eye 30 seconds before test substance application) interfered with the irritant response and yielded data that were not reliable. Comparatively, other studies indicated that two doses of tetracaine (10 minutes apart) were effective in abolishing pain and did not interfere with the irritant response (Walberg 1983; Anonymous 1981).

Ulsamer and colleagues reported that when one eye was pretreated with 0.1 mL of 2% butacaine sulfate and the other eye was not, the mean corneal opacity scores significantly differed in 14% (4/29) of the comparisons made between eyes. In all cases, the anesthetized eye had a higher mean corneal opacity score (Ulsamer et al.1977). Johnson described an *in vivo* evaluation of 31 unidentified substances in which, if the first tested rabbit showed evidence of pain (e.g., eye closure), then the remaining rabbits were pretreated with a topical anesthetic (amethocaine hydrochloride) prior to test substance application (Johnson 1980). The results showed that the level of eye irritation for 14 substances was equivalent between anesthetized and nonanaesthetized rabbits. Of the remaining 17 test substances, the level of eye irritation was greater in anesthetized rabbits in all cases.

Studies also have shown that topical anesthetics can alter ocular physiology (Seabaugh et al. 1993; Rowan and Goldberg, 1985; Durham et al. 1992). Local effects of topical anesthetics include but are not limited to increased permeability of the corneal epithelium, corneal epithelial cell sloughing, decreased lacrimation, and alteration of tear film production. Alone or in combination, these effects may influence the irritancy classification of the tested substance.

The present evaluation focuses on the effect of topical application of 0.5% (w/v) tetracaine hydrochloride on the irritancy potential of 97 formulations. The impact of the anesthetic on irritancy scores, agreement in irritancy classifications between pretreated and untreated rabbits tested with the same formulation, and on the days-to-clearing of ocular lesions were evaluated. Irritancy classifications were assigned according to three hazard classification schemes that are used or proposed for future use in the future for regulatory hazard classification and labeling; the United Nations Globally Harmonized System for Classification and Labelling (GHS) (UN 2003), the U.S. Environmental Protection Agency (EPA 1996) classification scheme, and the European Union (EU 2001) classification scheme.

2.0 Materials and Methods

2.1 Database

Product Safety Laboratories (Dayton, NJ) provided *in vivo* rabbit eye test scores in tabular form for all observation days for 97 formulations, together with information about testing conditions (e.g., concentration of formulation tested, amount tested). Due to confidentiality requirements, the compositions of the tested formulations were unknown during this evaluation.

2.2 In Vivo Test Method Protocol

The formulations were tested in either 3 or 6 rabbits. Sixteen substances were tested in six rabbit studies (n=96 rabbits), and 81 substances were tested in three rabbit studies (n=243 rabbits). *In vivo* testing was conducted in accordance with the EPA guideline on acute eye irritation testing (EPA 1998). Briefly, formulations were applied in a single dose to one eye of a rabbit with the other eye serving as a control. Eyes were evaluated for development of irritation and/or corrosion at predetermined intervals (e.g., 1 hour and 1, 2, 3, 7, 14, and 21 days after test substance instillation). If eye irritation was considered

irreversible (e.g., corneal opacity and/or conjunctival irritation is considered severe), the study was terminated. The degree of irritation was scored using the Draize irritation scale (Draize et al. 1944). The observation period was at least 72 hours and not longer than 21 days to allow for evaluation of reversal of observed effects.

Anesthetic pretreatment was provided to rabbits in a protocol similar to the one described by Johnson (Durham et al. 1992). Rabbits were tested sequentially, with the first tested rabbit not receiving anesthesia. If any of the subsequently tested rabbits displayed signs of pain or distress after test article application (e.g., vocalization, pawing at the treated eye), the remaining rabbits were pretreated with 0.5% (w/v) tetracaine hydrochloride ophthalmic solution (Bausch & Lomb, Tampa, FL; stored at ambient laboratory temperature and humidity). Two drops of the anesthetic were placed directly on the cornea in each rabbit eye between 30 seconds and approximately 2 minutes before instillation of test substance. The remainder of the test method protocol was conducted exactly as described in the protocol described in the EPA guideline on acute eye irritation testing (EPA 1998).

All studies were conducted in accordance with Good Laboratory Practice guidelines (EPA 2005a, 2005b; FDA 2006).

2.3 Irritancy Classification of Test Substances

As noted above, the *in vivo* rabbit eye database used to conduct this analysis included studies that were conducted in 3 or 6 rabbits. However, some of the *in vivo* classification systems used in this analysis (see below) were intended for studies using 3 or fewer rabbits. Thus, to maximize the amount of data available for the evaluation, the decision criteria for each classification system were expanded to include studies that used more than 3 rabbits.

All regulatory systems require eye lesions to be scored using the Draize scoring system (Draize et al. 1944). In order for a formulation to be included in this evaluation, the following criteria must have been fulfilled:

- A volume of 0.1 mL for liquids, solids, pastes, or particulates (with a weight of not more than 0.1 g) was tested in each rabbit.
- Observations of the eye were recorded at least 24, 48, and 72 hours after test substance application if no severe effect was observed.

- Observations of the eye were made until reversibility was assessed (i.e., lesions were cleared, as defined by the hazard classification definition) or until 21 days had passed. Results from a study terminated early were included if the rationale for the early termination was documented.

If any of the above criteria were not fulfilled, the data were not used for the analysis.

2.4 Hazard Classification Systems

Three regulatory hazard classification systems were used for evaluation of the data. The criteria for ocular irritancy classification required by each of these systems is provided below.

2.4.1 United Nations Globally Harmonized System for Classification and Labelling

The classification of substances according to the GHS classification system was conducted sequentially. Initially each rabbit tested was classified i one of four categories (Category 1, Category 2A, Category 2B, and Not Classified) based on the criteria outlined in **Table 2-1**.

Table 2-1 Criteria for Classification of Rabbits According to the GHS Classification System

GHS Category	Rabbit Criteria Used for Classification
Category 1	Group A[1]: - Effects in the cornea, iris, or conjunctiva that were not expected to reverse or did not fully reverse[2] within the observation period of 21 days, or - A corneal opacity score of 4 on the Draize scoring scale (Draize et al. 1944) at any time during the test Group B[1]: - Rabbit with mean scores (average of the scores on Days 1, 2, and 3) for opacity ≥3 and/or iritis ≥1.5
Category 2A	- Rabbit with mean scores (rabbit values are averaged across observation Days 1, 2, and 3) for one of more of the following: Iritis ≥1 but <1.5 Corneal opacity ≥1 but <3 Redness ≥2 Chemosis ≥2 and the effects fully reverse within 21 days
Category 2B	- Rabbit with mean scores (rabbit values are averaged across observation Days 1, 2, and 3) for one of more of the following: Iritis ≥1 but <1.5 Corneal opacity ≥1 but <3 Redness ≥2 Chemosis ≥2 and the effect fully reversed within 7 days
Not Classified	Rabbit mean scores fall below threshold values for Category 1, 2A, and 2B

Abbreviation: GHS = United Nations Globally Harmonized System

[1]"Group A" and "Group B" designations are internal designations used for classification purposes; they are not GHS-defined designations.

[2]Full reversal of the effects was defined as corneal opacity, iritis, redness, and chemosis =0.

After each result was categorized, the ocular irritancy hazard classification was determined for each substance. As shown in **Table 2-2**, substance classification depended on the proportion of tests that produced the same response. If a substance was tested in more than 3 rabbits, decision criteria were modified so that the proportionality needed for classification was maintained (e.g., 1 out of 3 or 2 out 6 rabbits were required for classification for most categories). However, in some cases, additional classification rules were necessary to include the available data (which are distinguished by italicized text in **Table 2-2**).

Table 2-2 Criteria for Classification of Substances According to the GHS Classification System, Listed in Order of Decreasing Severity

GHS Category	Criteria Necessary for Substance Classification
Category 1	At least 1 of 3 rabbits or 2 of 6 rabbits classified as Category 1, Group A[1] *One of 6 rabbits classified as Category 1, Group A and at least 1 of 6 rabbits classified as Category 1, Group B*[1] At least 2 of 3 rabbits or 4 of 6 rabbits classified as Category 1, Group B[1]
Category 2A	1. At least 2 of 3 rabbits or 4 of 6 rabbits classified as Category 2A 2. *One of 3 (2 of 6) rabbits classified as Category 2A and 1 of 3 (2 of 6) rabbits classified as Category 2B*
Category 2B	At least 2 of 3 rabbits or 4 of 6 rabbits classified as Category 2B
Not Classified	At least 2 of 3 rabbits or 4 of 6 rabbits classified as Not Classified

Abbreviations: GHS = United Nations Globally Harmonized System
Italicized text indicates rules that were developed to include additional data.
[1]"Group A" and "Group B" designations are internal designations used for classification purposes; they are not GHS-defined designations.

If an unequivocal substance classification could not be made due to the response pattern of the tested rabbits for a substance (e.g., 1 rabbit classified as Category 1, Group B; 2 rabbits classified as Category 2B; 3 rabbits classified as Not Classified), the data were excluded.

2.4.2 U.S. Environmental Protection Agency

The classification of substances according to the EPA classification system was conducted sequentially. Initially each rabbit was classified in one of four categories (Category I, II, III, or IV) (**Table 2-3**).

Table 2-3 Criteria for Ocular Hazard Classification of Rabbits According to the EPA Classification System, Listed in Order of Decreasing Severity

EPA Category	Criteria for Rabbit Classification
Category I	- Corrosive, corneal involvement or irritation (iris or cornea score ≥1 or redness or chemosis ≥2) persisting more than 21 days or - Corneal effects that are not expected to reverse by 21 days
Category II	- Corneal involvement or irritation clearing[1] in 8 to 21 days
Category III	- Corneal involvement or irritation clearing in 7 days or less
Category IV	- Minimal or no effects clearing in less than 24 hours

Abbreviation: EPA = U.S. Environmental Protection Agency
[1]For the purposes of this analysis, clearing was defined as iritis or cornea score <1 and redness or chemosis score <2.

Substance classification depended upon the most severe category observed among the tested rabbits.

2.4.3 European Union

Substance classification according to the EU classification system (**Table 2-4**) was conducted sequentially. Average Draize scores were used for classification of substances in the EU system; calculations depended on the number of rabbits tested in a study. For studies

1277 therein which 3 rabbits were tested, the average Draize scores (over observation Days 1, 2,
1278 and 3) for each endpoint were calculated for each rabbit. For studies in which more than
1279 3 rabbits were tested, the average Draize scores (over observation Days 1, 2, and 3) for each
1280 endpoint was calculated for all tested rabbits. The criteria used for substance classification
1281 are provided in Table 2-4.

1282 ***2.5 Analysis***

1283 For each of the 97 formulations evaluated, the impact of the anesthesia was assessed based
1284 on (1) the severity of the irritancy and (2) the number of days necessary for the lesion to
1285 clear,. The formulations were then classified into one of three categories: (1) anesthesia
1286 increased or worsened the observed variable, (2) anesthesia decreased or lessened the
1287 observed variable, or (3) anesthesia did not affect the observed variable. These relative
1288 frequencies of observed variables that increased/worsened and those that decreased/lessened
1289 were then compared by a sign test (Siegel and Castellan, 1956) to assess statistical
1290 significance of the anesthesia effect.

Table 2-4 Criteria for Classification of Substances According to the EU Classification System, Listed in Order of Decreasing Severity

EU Category	Three Rabbits Tested	Greater than Three Rabbits Tested
R41	1. Two or more rabbits with the following average Draize scores over Days 1, 2, and 3: Opacity ≥3 Iritis =2 2. At least 1 rabbit (on Day 21) in which the effect has not reversed[1] 3. At least 1 rabbit (when study is terminated after Day 14 and before Day 21) with Opacity ≥3 or Iritis =2 4. At least 1 rabbit with any of the following noted effects: (a) Corneal perforation or ulceration (b) Blood in the anterior chamber of the eye (c) Opacity = 4 for 48 hours (d) Absence of light reflex for 72 hours (e) Ulceration of the conjunctival membrane (f) Necrosis of the conjunctivae or nictitating membrane (g) Sloughing	1. The following overall mean rabbit Draize scores over Days 1, 2, and 3: Opacity ≥3 or Iritis >1.5 2. At least 2 rabbits (on Day 21) in which the effect has not reversed 3. At least 2 rabbits (when study is terminated after Day 14 and before Day 21) with Opacity ≥3 or Iritis =2 4. At least 1 rabbit with any of the following noted effects: (a) Corneal perforation or ulceration (b) Blood in the anterior chamber of the eye (c) Opacity = 4 for 48 hours (d) Absence of light reflex for 72 hours (e) Ulceration of the conjunctival membrane (f) Necrosis of the conjunctivae or nictitating membrane (g) Sloughing
R36	Two or more rabbits with the following average Draize scores over Days 1, 2, and 3: 2 ≤ Opacity <3 1 ≤ Iritis <2 Redness ≥2.5 Chemosis ≥2	The following overall mean rabbit Draize scores over Days 1, 2, and 3: 2 ≤ Opacity <3 1 ≤ Iritis <1.5 Redness ≥2.5 Chemosis ≥2
Not Labeled	Substance cannot be classified as R41 or R36	Substance cannot be classified as R41 or R36

Abbreviation: EU = European Union

[1]Full reversal of the effects was defined as corneal opacity, chemosis, redness, or iritis =0.

3.0 Results

3.1 Classification of Formulations

A subset of the rabbits could not be classified based on the GHS, EPA, or EU systems because the criteria described in the Materials and Methods section were not fulfilled. Based on these criteria, 25 rabbits (8 not pretreated and 17 pretreated with anesthesia) could not be classified using the GHS classification system. For the EU and EPA classification systems, 27 rabbits (9 not pretreated and 18 pretreated with anesthesia) and 23 rabbits (6 not pretreated and 17 pretreated with anesthesia) could not be classified, respectively.

Based on the above results, a subset of formulations could not be used to compare the effects of anesthesia on irritancy classification due to insufficient animal response data (i.e., irritancy data for anesthetized and nonanesthetized rabbits treated with the same formulation were unavailable). In the present database, nine formulations were excluded from the GHS and EU classification system evaluations, and seven formulations were excluded from the EPA classification system evaluation (**see Table 3-1**).

3.2 Effect on Irritancy Classification

Each formulation tested was assessed to determine if the average irritancy response for the animals pretreated with tetracaine hydrochloride was different (i.e., more or less severe) than for the animals not pretreated with tetracaine hydrochloride.

As shown in **Table 3-1**, for all three hazard classification schemes, rabbits pretreated with anesthesia tended to produce more severe responses than rabbits that were not pretreated with anesthesia. However, none of the observed differences were statistically significant. The greatest difference was observed in the GHS classification scheme, in which 20 formulations produced a more severe average response in the pretreated rabbits, while 13 formulations produced a more severe average response in the rabbits that were not pretreated with tetracaine hydrochloride.

Table 3-1 Effect of Anesthesia Pretreatment on Irritancy Classification Response

Direction of Response	GHS	EU	EPA
More severe average response in anesthetized animals	20[1]	17	22
Less severe average response in anesthetized animals	13	11	16
No difference in average response between anesthetized and nonanesthetized animals	55	60	52
Number of formulations that could not be used because there was insufficient data[2]	9	9	7
Total Number of Formulations	**97**	**97**	**97**

Abbreviations: EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = United Nations Globally Harmonized System

[1]*Number* represents the number of formulations identified with the noted criteria.

[2]Some formulations and the animals tested with that formulation could not be used for this evaluation because there was insufficient animal data with which to compare anesthetized and nonanesthetized animals.

Of the substances that elicited a more or less severe response in rabbits pretreated with tetracaine hydrochloride, only five formulations where shown differ by more than two ocular hazard classification categories for at least one of the hazard classification systems evaluated

(**Table 3-2**). There was no consistent pattern regarding whether the anesthesia played a role in this variability of response. In some cases, the animals with anesthesia clearly produced a more severe response than those animals without anesthesia, while for other chemicals an opposite trend was seen (**Table 3-2**).

Table 3-3 shows the distributions of individual rabbit responses for different severity classifications used for each regulatory hazard classification system. The results collapse data over different formulations and, therefore, preclude a formal statistical analysis. However, the data in this table support the results presented in **Table 3-1** (i.e., rabbits pretreated with anesthesia tend to produce more severe responses than rabbits that were not pretreated with anesthesia).

1339 **Table 3-2 Animal Classifications for Substances with Differences of at Least Two Hazard Classification Categories**

Substance Code	Animal Number	Pretreated	Animal GHS Classification	Overall GHS Classification	Animal EU Classification	Overall EU Classification	Animal EPA Classification	Overall EPA Classification
10640	1	NO	Cat2A	Category 2A	R36	R36	Category II	Category I
10640	2	NO	Cat2A		R36		Category II	
10640	3	NO	Cat 1, Group A[1]		R41		Category I	
10640	4	YES	Cat2A		R36		Category III	
10640	5	YES	Cat2B		R36		Category III	
10640	6	YES	Not Classified		Not Labeled		Category III	
12422	1	NO	Cat2B	Category 1	R36	R41	Category III	Category I
12422	2	YES	Cat2B		R36		Category III	
12422	3	YES	Cat 1, Group A		R41		Category I	
12483	1	NO	Cat2A	Category 1	R36	R41	Category II	Category I
12483	2	NO	Cat 1, Group A		R41		Category I	
12483	3	YES	Cat2B		Not Labeled		Category III	
13375	1	NO	Cat2B	Category 1	Not Labeled	R41	Category III	Category I
13375	2	YES	Cat 1, Group A		R41		Category I	
13375	3	YES	Cat 1, Group A		R41		Category I	
13381	1	NO	Cat 1, Group A	Category 1	R41	R41	Category I	Category I
13381	2	YES	Cat2A		R36		Category II	
13381	3	YES	Cat2A		R36		Category III	

1340 Abbreviations: Cat = category; EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = United Nations Globally Harmonized System
1341 [1]"Group A" is an internal designation used for classification purposes; it is not a GHS-defined designation (see **Table 2-4** for additional details).

1342

Table 3-3 Distribution of Rabbits Among Hazard Classification Irritancy Categories

GHS				EU				EPA			
Classification Category	Number of Rabbits	Anesthesia Pretreatment		Classification Category	Number of Rabbits	Anesthesia Pretreatment		Classification Category	Number of Rabbits	Anesthesia Pretreatment	
		No	Yes			No	Yes			No	Yes
Category 1	36	13[1] (10.9%)	27 (13.8%)	R41	40	13 (11.0%)	27 (13.9%)	Category I	36	12 (9.9%)	24 (12.3%)
Category 2A	72	27 (22.7%)	45 (23.1%)	R36	101	35 (29.7%)	66 (34.0%)	Category II	63	23 (19.0%)	40 (20.5%)
Category 2B	79	31 (26.1%)	48 (24.6%)	NL	171	70 (59.3%)	101 (52.1%)	Category III	161	67 (55.4%)	94 (48.2%)
Not Classified	123	48 (40.3%)	75 (38.5%)					Category IV	56	19 (15.7%)	37 (19.0%)
Total	314	119	195	Total	312	118	194	Total	316	121	195
SCNM	25	8	17	SCNM	27	9	18	SCNM	23	6	17
Overall Total	339	127	212	Overall Total	339	127	212	Overall Total	339	127	212

Abbreviations: EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = United Nations Globally Harmonized System; NL = Not labeled; SCNM = Study criteria not met

[1]*Number* represents the number of rabbits identified with the noted severity classification. The number in parentheses represents the percentage of rabbits based on the total number of classifiable rabbits ("Total" row).

An additional analysis used anesthesia pretreatment as a criterion to evaluate the variability among animals within a given formulation. For most of the formulations, irritancy classifications for rabbits pretreated with tetracaine hydrochloride did not differ from those of rabbits not pretreated (**Table 3-4**). Interestingly, for all these classification systems (especially the EU system), the agreement in irritancy response between rabbits was better when the anesthesia pretreatments were different (EU = 18 substances) than in those in which the anesthesia pretreatments were the same, regardless of whether or not an anesthetic was used (EU =10 substances). However, none of the observed differences were statistically significant.

Table 3-4 Effect of Anesthesia Pretreatment on Agreement of Irritancy Classification Response

Agreement of Response	GHS	EU	EPA
Better agreement in irritancy response among rabbits with matching pretreatment (either anesthesia or no anesthesia)	16[1]	10	17
Better agreement in irritancy response among rabbits without matching pretreatment	17	18	20
No difference between matched and unmatched pretreatment	55	60	53
Number of formulations that could not be used because there was insufficient data[2]	9	9	7
Total Number of Formulations	**97**	**97**	**97**

Abbreviations: EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = United Nations Globally Harmonized System

[1]*Number* represents the number of formulations identified with the noted criteria.

[2]Some formulations, and the animals tested with that formulation, could not be used for this evaluation because there was insufficient animal data with which to compare anesthetized and nonanesthetized animals.

3.3 Effect on Day of Lesion Clearing

Since regulatory classifications rely in part on the day all ocular lesions reverse, we evaluated whether pretreatment with tetracaine hydrochloride lengthened or shortened the number of days required for lesion clearing. Based on the available data, when anesthetized rabbits were compared to nonanesthetized rabbits, none of the differences observed in the day-to-clearing evaluation were statistically significant (**Table 3-5**). The largest difference observed was for opacity clearing time, which tended to be slightly greater in the rabbits pretreated with tetracaine hydrochloride than in those that were not pretreated. However, this difference (33 vs. 22) is not significant by a sign test (p <0.10).

Table 3-5 Effect of Anesthesia Pretreatment on Day of Clearing of Ocular Lesions

	Opacity Clearing	Iris Clearing	Redness Clearing (EPA)[1]	Redness Clearing (EU/GHS)[1]	Chemosis Clearing (EPA)[1]	Chemosis Clearing (EU/EPA)[1]
Longer clearing time, on average, for anesthetized animals versus nonanesthetized animals	33[2]	28	30	33	24	22
Shorter clearing time, on average, for anesthetized animals versus nonanesthetized animals	22	22	30	29	25	29
No difference in clearing time on average between anesthetized and nonanesthetized animals	27	37	32	24	43	39
Number of formulations that could not be used because there was insufficient data[3]	15	10	5	11	5	7
Total Number of Formulations	**97**	**97**	**97**	**97**	**97**	**97**

Abbreviations: EPA = U.S. Environmental Protection Agency; EU = European Union; GHS = United Nations Globally Harmonized System

[1]Different analyses were conducted for the EPA classification system than for the EU and GHS classification system because the day of clearing is defined differently. Clearing for the EPA is defined as a score of 0 or 1, while clearing for the GHS and EU classification systems is defined as a score of 0.

[2]*Number* represents the number of formulations identified with the noted criteria.

[3]Some formulations, and the animals tested with that formulation, could not be used for this evaluation because there was insufficient animal data with which to compare anesthetized and nonanesthetized animals.

Table 3-6 provides a comparison of the number of animals for each clearing day evaluated for the corneal opacity endpoint. The data show that, overall, the time for corneal lesions in rabbits pretreated with tetracaine hydrochloride was slightly longer than in rabbits that were not pretreated with tetracaine hydrochloride.

Table 3-6 Distribution of Rabbits (With and Without Anesthesia Pretreatment), Based on Clearing Day for Corneal Opacity Lesions

Clearing Day for Opacity Lesion	Number of Rabbits Not Pretreated with Anesthesia	Number of Rabbits Pretreated with Anesthesia
>21[1]	11 (9.2%)	19 (9.9%)[2]
21	6 (5.0%)	5 (2.6%)
14	4 (3.3%)	19 (9.9%)
10	12 (10.0%)	18 (9.4%)
7	15 (12.5%)	25 (13.0%)
4	9 (7.5%)	13 (6.8%)
3	11 (9.2%)	22 (11.5%)
2	4 (3.3%)	9 (4.7%)
1	0 (0.0%)	2 (1.0%)
0[3]	48 (40.0%)	60 (31.3%)
No Clearing[4]	7	20
Total Number of Rabbits	**127**	**212**

[1]Lesion was present on last day of observation period (21 days).
[2]*Percentage* represents the number of animals for the noted clearing day per the total number of usable animals (192 for the number of animals pretreated with anesthesia, and 120 for the number of animals not pretreated with anesthesia).
[3]No lesions were observed at any time points evaluated.
[4]These experiments were terminated prior to clearing of lesions; therefore, the data could not be used in the evaluation.

4.0 Discussion

Efforts increasingly have focused on refining the current *in vivo* Draize rabbit eye test method protocol to reduce the level of pain and distress experienced by rabbits when test substances are placed in the eye. One area that has been reviewed extensively has been the use of topical anesthetics prior to administration of a test substance. While it is generally agreed that the application of a topical anesthetic will likely decrease the pain perceived by a rabbit in the early stages of the *in vivo* eye irritation test, there are competing concerns that topical anesthetics may alter ocular physiology and thus modify the irritation response observed.

Overall, previous studies provide conflicting results on the impact of topical ocular anesthetics on ocular irritation and physiology. While some studies indicate that topical anesthetics do not interfere with the irritation response (Ulsamer et al. 1977; Heywood and James 1978; Anonymous 1981; Arthur et al. 1986; Seabaugh et al. 1993), others state that there is a trend (although not statistically significant) of increased irritancy in anesthetized eyes (Johnson 1980; Durham et al. 1992). Still others note that anesthetics interfere with the irritant response and yielded data that were not reliable (Walberg 1983; Rowan and Goldberg 1985). Differences in efficacy of the topical ocular anesthetics evaluated in these studies could depend on a variety of a factors including but not limited to the anesthetic used, the

anesthetic dose used, the application procedure, and the species tested (Ulsamer et al. 1977; Heywood et al. 1978; Johnson 1980; Anonymous 1981; Walberg 1983; Rowan and Goldberg 1985; Arthur et al. 1986; Durham et al. 1992; Seabaugh et al. 1993). Due to the limited data available, however, an in-depth assessment on the impact of these different factors on the overall results has yet to be conducted.

Despite these conflicting issues and although not formal policy among all U.S. Federal agencies, the use of anesthetics was considered acceptable by a consensus of those participating in a 1991 IRAG workshop (Seabaugh et al. 1993). It was noted that because pain is relieved at least temporarily and the time and extent of injury can still be evaluated, anesthetic use should be considered on a case-by-case basis. It is noteworthy that in 1984 the U.S. Consumer Products Safety Commission (CPSC) stated that two applications of tetracaine, 10 to 15 minutes apart, should be administered prior to test substance administration during ocular irritation testing (CPSC 1984).

The present study further studied topical anesthetics to assess the impact of using two drops of tetracaine hydrochloride (0.5% (w/v)), 30 to 120 seconds prior to test article application, on ocular irritancy. For a majority of the formulations evaluated no difference was observed in the severity of irritancy observed in rabbits pretreated with tetracaine and in those that were not pretreated (i.e., the irritancy classifications between treated and untreated rabbits were the same). When a difference in irritancy classifications was observed, the rabbits pretreated with anesthesia tended to produce a slightly more severe response than those without anesthesia. This is similar to results seen in previous studies (Durham et al. 1992). This trend, which was not statistically significant, was observed for all hazard classification systems evaluated. Since the formulation compositions were unknown, an assessment of whether there were similarities among formulations that were comparably affected by the anesthetic pretreatment could not be conducted.

A lack of association between severity of classification and anesthesia pretreatment also was observed when the distribution of rabbits among irritancy classification categories was evaluated. Similar to the results described above, the distribution of rabbits indicated that pretreatment with anesthesia did not increase the likelihood of producing a more severe response than those without anesthesia.

The argument could be made that, although 0.5% (w/v) tetracaine hydrochloride did not appear to affect the responses of the pretreated rabbits and those not pretreated, it could have altered the variability in the individual rabbit responses for each tested formulation. Therefore, we examined the variability among rabbit irritancy responses when anesthesia pretreatment was used as a defining criterion. The results show that anesthesia pretreatment had no significant effect on the observed variability among rabbit responses.

Of the five formulations with which rabbit responses differed by more than two classification categories (e.g., GHS Category 2B classification for one test rabbit and GHS Category 1, Group A for another test rabbit), there was no consistent pattern in the pretreatment effect. In some cases, the rabbits pretreated with tetracaine hydrochloride produced a more severe response than those animals not pretreated with tetracaine hydrochloride, while for other formulations the opposite trend was observed. Because the observed variability occurs in both directions (increasing and decreasing the level of irritancy), the observed variability in rabbit response may be unrelated to the anesthesia but instead related to the inherent variability of the rabbit response to the tested formulations.

Because all three evaluated hazard classification systems use for irritancy classification the day of clearing of all lesions, the impact of anesthesia pretreatment on this criterion was evaluated also. Similar to the results of the previous analyses, none of the observed differences in the days-to-clearing were statistically significant. Interestingly, while pretreatment with tetracaine tended to increase the length of time needed for ocular and iridal lesions to clear, anesthesia pretreatment tended to decrease the length of time needed for conjunctival chemosis lesions to clear. The significance and the mechanisms for this observed effect are currently unknown.

Due to the lack of available comparative data, further evaluations comparing the efficacy of tetracaine versus other topical anesthetics and the optimal dosing regimen (e.g., number of drops to be administered, location of anesthetic application) could not be assessed. Thus additional studies are recommended to further evaluate these areas.

In conclusion, these results indicate that pretreatment with 0.5% (w/v) tetracaine hydrochloride ophthalmic solution had no significant impact on the irritancy classification of rabbits according to the GHS, EPA, and EU classification systems. The anesthesia

pretreatment did not affect the variability in rabbit response either. Furthermore, anesthetic pretreatment had no statistically significant effect on the number of days until ocular lesions cleared. Therefore, this evaluation combined with previous studies supports the routine use of 0.5% tetracaine hydrochloride prior to testing rabbits in the *in vivo* Draize rabbit eye test.

5.0 References

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