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

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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 (Dayton, NJ) 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 Harmonized 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 2007), the U.S. Environmental Protection Agency (EPA 2003) 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 required by each of these systems for ocular irritancy classification 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 in 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 of 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**). Substance classification depended upon the most severe category observed among the tested rabbits.

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.

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

2.5 Analysis

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

continued

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

EU Category	Three Rabbits Tested	Greater than Three Rabbits Tested
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

Abbreviations: 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 less severe average response in the rabbits that were 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).

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	

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

[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).

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 was 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 Harmonised 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) was not significant using 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 type and dose of anesthetic 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 examined 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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