Effect of Topical Anesthetic Pre-treatment on *In Vivo* Ocular Irritation Hazard Classification

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Abstract

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. Due to the potential for pain and distress that may occur in rabbits after application of a severely irritating or corrosive substance, alternative approaches have been proposed and developed to reduce the number of such substances that require animal testing (e.g., a weight-of-evidence approach, use of topical ocular anesthetics prior to test substance administration). This evaluation focuses on the effect of topical application of 0.5% (w/v) tetracaine hydrochloride on the irritancy potential of 97 proprietary formulations, tested in 339 rabbits, evaluated using a sequential testing scheme. In this testing scheme, the first rabbit did not receive topical anesthetic pre-treatment. If a rabbit appeared to exhibit pain or suffering after formulation administration, subsequent rabbits were pre-treated with the topical anesthetic. For all formulations, the final rabbit tested was pre-treated with the topical anesthetic. Irritancy classifications were assigned to each rabbit according to three regulatory hazard classification systems (i.e., EU, EPA, GHS). Although none of the observed differences were statistically significant, rabbits pre-treated with anesthesia appeared to produce slightly more severe responses for all three hazard classification systems than rabbits that were not pre-treated. Further, studies indicated that anesthetic pre-treatment had no impact on the variability of rabbit irritancy classifications for the same formulation. Finally, analyses indicated that anesthetic pre-treatment did not significantly increase the number of days needed for opacity, iris, or conjunctival lesions to fully reverse. Combined, these findings support the routine use of 0.5% (w/v) tetracaine hydrochloride as a pre-treatment in the *in vivo* Draize rabbit eye test.

Introduction

Accidental eye injury is the leading cause of visual impairment in the United States (1). 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 (1). Based on emergency department reports for work related eye injuries, the National Institute of Occupational Safety and Health estimated that approximately 39,200 chemical-related eye injuries occurred in 1998 (2).

Since 1944, ocular corrosion or irritation potential of substances has been evaluated using the *in vivo* Draize rabbit eye test (3). 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 has been used to classify substances as being 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, including the use of a topical ocular anesthetic prior to test substance administration.

Previous studies indicate that the efficacy of topical ocular anesthetics is 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 (4-12). Studies also have shown that topical anesthetics can alter ocular physiology (e.g., increase permeability of the corneal epithelium), which may impact the irritancy classification of the tested substance (4, 6).

The present evaluation focuses on the effect of topical application of 0.5% (w/v) tetracaine hydrochloride on the irritancy potential of 97 proprietary formulations. The impact of the topical anesthetic on (a) irritancy classification category, (b) agreement in irritancy classifications between pre-treated and untreated rabbits tested with the same formulation, and (c) the days-to-clearing of lesions were evaluated. Irritancy classifications were assigned according to three regulatory hazard classification schemes which are used or proposed to be used for regulatory classification and labeling; the United Nations Globally Harmonized System for Classification and Labelling (GHS) (13), the U.S. Environmental Protection Agency (EPA) classification scheme (14), and the European Union (EU) classification scheme (15).

Materials And Methods

Database: Eurofins Product Safety Labs (PSL; Dayton, NJ 08810) conducted these studies on behalf of their clients to comply with regulatory requirements of governmental agencies. PSL is AAALAC accredited. It should be noted that these studies were not conducted solely to evaluate the effects of anesthetic on the outcome of ocular irritation studies. PSL provided to NICEATM *in vivo* rabbit eye test scores for all observation days for 97 proprietary formulations in tabular form, 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. The analysis of the data for this publication was secondary to the primary regulatory objectives.

***In Vivo* Test Method Protocol:** The formulations were tested in either three (81 formulations) or six (16 formulations) rabbits. *In vivo* testing was conducted in accordance with the EPA guideline on acute eye irritation testing (16). Briefly, formulations were applied in a single dose to one eye of a rabbit; the other eye served as a control. Eyes were evaluated at pre-determined intervals (i.e., at 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 severe conjunctival irritation), the study was terminated. The degree of irritation was scored using the Draize irritation scale (3). The observation period was at least 72 hours and, to allow for evaluation of reversal of observed effects, up to but not longer than 21 days.

Topical anesthetic pre-treatment was provided to rabbits in a protocol similar to the one described by Johnson (11). Rabbits were tested sequentially, with the first tested rabbit not receiving topical anesthesia. If a rabbit displayed signs of pain or distress (e.g., vocalization, pawing at the treated eye), the remaining rabbits were pre-treated 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 in each rabbit eye between approximately 30 seconds and two minutes prior to instillation of a test substance. The conduct of the remainder of the test method protocol is identical to the protocol described in the EPA guideline on acute eye irritation testing (16).

All studies were conducted in accordance with Good Laboratory Practices guidelines (17, 18, 19).

Irritancy Classification of Test Animals and Substances: 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 three rabbits.

All regulatory systems require eye lesions to be scored using the Draize scoring system (3). In order for a formulation to be included in this evaluation, all of the following criteria must have been fulfilled:

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

Hazard Classification Systems: Three regulatory hazard classification systems were evaluated. The criteria for ocular irritancy classification required by each of these systems are provided in the following tables.

United Nations Globally Harmonized System for Classification and Labelling: The classification of substances according to the GHS classification system (13) was conducted sequentially. Initially, each rabbit tested was classified into one of four categories (Category 1, Category 2A, Category 2B, and nonirritant) based on the criteria outlined in **Table 1**.

Table 1 Criteria for Classification of Rabbits According to the GHS Classification System (13)

GHS Category	Rabbit Criteria Necessary 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 at any time during the test Group B[1]: – Rabbit with mean scores (average of the scores on day 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
Nonirritant	Rabbit mean scores fall below threshold values for Category 1, 2A, and 2B

Abbreviations: GHS = United Nations Globally Harmonized System.

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

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

After each rabbit was categorized, the ocular irritancy potential of the substance was determined. As shown in **Table 2**, substance classification depended on the proportion of rabbits that produced the same response. In some cases, additional classification rules were developed to include the available data (distinguished by italicized text in **Table 2**). If an unequivocal substance classification could not be made due to the response pattern of the tested rabbits for a substance (e.g., one rabbit classified as Category 1, Group B; two rabbits classified as Category 2B; three rabbits classified as nonirritant), the data were excluded from these analyses.

Table 2 Criteria for Classification of Substances According to the GHS Classification System, Listed in Order of Decreasing Severity (13)

GHS Category	Criteria Necessary for Substance Classification
Category 1	1. At least 1 of 3 rabbits or 2 of 6 rabbits classified as Category 1, Group A[1] 2. *One of 6 rabbits classified as Category 1, Group A and at least 1 of 6 rabbits classified as Category 1, Group B*[1] 3. 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
Nonirritant	At least 2 of 3 rabbits or 4 of 6 rabbits classified as nonirritant

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 and not GHS defined designations.

U.S. Environmental Protection Agency: The classification of substances according to the EPA classification system (14) was conducted sequentially. Initially, each rabbit was classified into one of four categories (Category I, II, III, or IV) (**Table 3**). Substance classification was dependent upon the most severe irritation category observed among the tested rabbits.

Table 3 Criteria for Classification of Rabbits According to the EPA Classification System, Listed in Order of Decreasing Severity (14)

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.

***European Union*:** Substance classification according to the EU classification system was conducted sequentially (15). Average Draize scores were used for classification of substances in the EU system; calculations were dependent on the number of rabbits tested in a study. The criteria used for substance classification are provided in **Table 4**.

Table 4 Criteria for Classification of Substances According to the EU Classification System, Listed in Order of Decreasing Severity (15)

EU Category	Three Rabbits Tested	Greater than Three Rabbits Tested
R41	1. Two or more rabbits where the average rabbit Draize scores over Days 1, 2, and 3 were: Opacity ≥3 Iritis = 2 2. At least one rabbit (on Day 21) where the effect has not reversed[1] 3. At least one rabbit (when study is terminated after Day 14 and before Day 21) where Opacity ≥3 or Iritis = 2 4. At least one rabbit where any of the following effects are noted: 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 nicitating membrane g) sloughing	1. Overall mean rabbit Draize scores over Days 1, 2, and 3 were: Opacity ≥3 or Iritis >1.5 2. At least two rabbits (on Day 21) where the effect has not reversed 3. At least two rabbits (when study is terminated after Day 14 and before Day 21) where Opacity ≥3 or Iritis = 2 4. At least one rabbit where any of the following effects are noted: 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 nicitating membrane g) sloughing
R36	Two or more rabbits where the average rabbit Draize scores over Days 1, 2, and 3 were: 2 ≤ Opacity <3 1 ≤ Iritis <2 Redness ≥2.5 Chemosis ≥2	Overall mean rabbit Draize scores over Days 1, 2, and 3 were: 2 ≤ Opacity <3 1 ≤ Iritis <1.5 Redness ≥2.5 Chemosis ≥2
Nonirritant	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.

Analysis: For each of the 97 proprietary formulations, the impact of topical anesthesia pre-treatment on the variable (i.e., severity of the irritancy classification observed, agreement in irritancy classifications between pre-treated and untreated rabbits tested with the same formulation, days-to-clearing of the observed lesions) being evaluated was assessed. The impact of the topical anesthesia was determined based on assessing the average irritancy classification response in rabbit(s) not treated with topical anesthesia versus the average irritancy classification response in rabbit(s) pre-treated with topical anesthesia. In studies where only a single rabbit was either untreated or pre-treated with topical anesthesia, the average irritancy classification response was defined as the response in that single rabbit.

The formulations were classified into one of three categories: (a) topical anesthesia increased the severity of the observed variable (e.g., severity of the irritancy classification, number of days required for a lesion to clear), (b) topical anesthesia decreased the severity of the observed variable, or (c) topical anesthesia did not affect the observed variable.

These relative frequencies of experiments in which the severity of the observed variable was increased or decreased were compared by a sign test (20) to assess the statistical significance of the topical anesthesia effect.

RESULTS: Impact of Topical Anesthetic Pre-Treatment on Regulatory Irritancy Classification

Each formulation tested was assessed to determine if the average irritancy classification response for the rabbits pre-treated with topical anesthesia was more severe or less severe than that observed for the rabbits not pre-treated with topical anesthesia.

As shown in **Table 5**, rabbits pre-treated with topical anesthesia tended to produce more severe irritancy classification responses than rabbits that were not pre-treated with topical anesthesia for all three regulatory hazard classification schemes. However, none of the observed differences were statistically significant.

Table 5 Effect of Topical Anesthesia Pre-treatment on Irritancy Classification Response Category

Direction of Response	GHS	EU	EPA
More severe average ocular irritation classification response in topically anesthetized rabbits	20[1]	17	22
Less severe average ocular irritation classification response in topically anesthetized rabbits	13	11	16
No difference in average ocular irritation classification response between topically anesthetized and non-anesthetized rabbits	55	60	52
Formulations with 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.

[2]Some formulations, and the animals tested with that formulation, could not be used for this evaluation because there was not sufficient animal data to conduct a comparison between anesthetized and non-anesthetized rabbits.

An additional analysis was conducted to evaluate the variability among rabbit irritation classification responses, within a given formulation, when topical anesthesia pre-treatment was used as a criterion. For most of the formulations, there was no significant difference in rabbit irritancy classifications between rabbits pre-treated with topical anesthesia and those that were not pre-treated (**Table 6**). Interestingly, for all the evaluated regulatory hazard classifications, there appeared to be better agreement in rabbit responses when rabbits that were not pre-treated with anesthesia were compared to those that were pre-treated with anesthesia (**Table 6**, second row). None of the observed differences were statistically significant.

Table 6 Effect of Topical Anesthesia Pre-treatment on Agreement of Irritancy Classification Response Category

Agreement of Response	GHS	EU	EPA
More agreement in irritancy classification response among rabbits with the same topical anesthetic pre-treatment regimen[1]	16[2]	10	17
More agreement in irritancy classification response among rabbits with different topical anesthetic pre-treatment regimen[1]	17	18	20
No difference between rabbits with different topical anesthetic pre-treatment regimen	55	60	53
Number of formulations with insufficient data[3]	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]"Same anesthetic pre-treatment regimen" indicates that the rabbits that were evaluated were either all pre-treated or all not pre-treated with anesthesia. "Different anesthetic pre-treatment regimen" indicates that one rabbit was pre-treated with anesthesia while the other was not.

[2]Number represents the number of formulations.

[3]Some formulations, and the animals tested with that formulation, could not be used for this evaluation because there was not sufficient animal data to conduct a comparison between anesthetized and non-anesthetized animals.

NIEHS National Institute of Environmental Health Sciences

U.S. Department of Health and Human Services, National Institutes of Health

ICCVAM The Interagency Coordinating Committee on the Validation of Alternative Methods

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

More information on ICCVAM and NICEATM can be accessed at http://iccvam.niehs.nih.gov/

RESULTS: Impact of Topical Anesthetic on Day-of-Lesion-Clearing

Each formulation tested was assessed to determine if the number of days required for a lesion to reverse for animals pre-treated with topical anesthesia was different than animals that were not pre-treated with topical anesthesia.

None of the differences observed in the day-to-clearing evaluation (when topically anesthetized rabbits were compared to non-anesthetized rabbits) were statistically significant (**Table 7**). The largest observed difference was for opacity clearing day, which tended to be slightly greater in the rabbits pre-treated with topical anesthesia when compared to those that were not pre-treated. However, this difference (33 vs. 22) was not statistically significant by a sign test.

Table 7 Effect of Topical Anesthesia Pre-treatment on Day-of-Clearing of Ocular Lesion

	Opacity Clearing	Iris Clearing	Redness Clearing (EPA)[1]	Redness Clearing (EU/GHS)[1]	Chemosis Clearing (EPA)[1]	Chemosis Clearing (EU/GHS)[1]
Longer clearing time, on average, for topically anesthetized vs. non-anesthetized rabbits	33[2]	28	30	30	24	22
Shorter clearing time, on average, for topically anesthetized vs. non-anesthetized rabbits	22	22	30	29	25	29
No difference in clearing time between topically anesthetized and non-anesthetized rabbits	27	37	32	24	43	39
Number of formulations with insufficient data[3]	15	10	5	11	5	7
Total 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 compared to the EU and GHS classification system, since 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.

[3]Some formulations, and the animals tested with that formulation, could not be used for this evaluation because there was not sufficient animal data to conduct a comparison between anesthetized and non-anesthetized animals.

For the endpoint with the largest difference in day-to-clearing (corneal opacity), **Table 8** provides a comparison of the number of rabbits for each clearing day evaluated. As noted above, the data show that the time to clear corneal lesions in rabbits pre-treated with topical anesthesia was slightly longer than in rabbits that were not pre-treated. However, this difference was not statistically significant.

Table 8 Distribution of Rabbits (With and Without Topical Anesthesia Pre-treatment), Based on Clearing Day for Corneal Opacity Lesion

Clearing Day for Opacity Lesion	Percentage of Rabbits Not Pre-treated with Topical Anesthesia	Percentage of Rabbits Pre-treated with Topical Anesthesia
>21[1]	9.2% (11)[2]	9.9% (19)
21	5.0% (6)	2.6% (5)
14	3.3% (4)	19.9% (9)
10	10.0% (12)	9.4% (18)
7	12.5% (15)	13.0% (25)
4	7.5% (9)	6.8% (13)
3	9.2% (11)	11.5% (22)
2	3.3% (4)	4.7% (9)
1	0.0% (0)	1.0% (2)
0[3]	40.0% (48)	31.3% (60)
No Clearing[4]	7	20
Total Rabbits	**127**	**212**

[1]Lesion present on last day of observation period (21 days).

[2]Number of rabbits in parentheses. Percentage represents the number of animals for the noted clearing day per the total number of usable animals (120 for the number of animals not pre-treated with topical anesthesia and 192 for the number of animals pre-treated with topical anesthesia)

[3]No lesions observed at any time points evaluated

[4]Rabbits terminated *prior* to clearing of lesion; therefore could not be used in evaluation.

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Summary and Conclusions

- For the majority of the formulations tested, topical anesthetic pre-treatment had no statistically significant impact on:
  - The hazard classification severity category of observed ocular irritation
  - The variability in rabbit ocular irritation classification responses
  - The number of days required for an ocular lesion to clear
- When a difference in ocular irritation was observed, the rabbits pre-treated with topical anesthesia more frequently exhibited a more severe hazard classification than observed for rabbits that were not pre-treated. However, none of the observed differences were statistically significant.
- Since the observed variability occurs in both directions (increasing and decreasing the level of irritancy classification), any observed differences in ocular irritation classification are likely related to the inherent variability of the rabbit response to the tested formulation rather than topical anesthetic pre-treatment.
- The largest observed difference for the number of days required for an ocular lesion to clear was for opacity, which was greater in the rabbits pre-treated with topical anesthesia when compared to those that were not pre-treated. However, the difference was not statistically significant.
- An assessment of whether there were similarities between formulations that were comparably affected by topical anesthetic pre-treatment could not be conducted, since their compositions were unknown.
- Evaluations comparing the efficacy of tetracaine hydrochloride versus other topical anesthetics and the optimal dosing regimen (e.g., number of drops to be administered, location of anesthetic application, etc) could not be assessed due to lack of available data.
- The results indicate that topical pre-treatment with 0.5% (w/v) tetracaine hydrochloride ophthalmic solution had no significant impact on the irritancy classification of rabbits, for the GHS, EPA, and EU classification systems.
- Combined with previous studies, these results support the routine use of 0.5% (w/v) tetracaine hydrochloride as a topical pre-treatment in the *in vivo* Draize ocular irritation test.

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