Topical Anesthetic Pre-treatment in the Draize Eye Test: Impact on Hazard Classification

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Introduction

Accidental eye injury is the leading cause of visual impairment in the United States (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. However, despite these efforts, some substances that are tested in rabbits may cause pain and distress. Therefore additional refinements to the method have been proposed, including the use of a topical ocular anesthetic prior to test substance administration (4-12).

This study evaluates the effect of topical application of 0.5% (w/v) tetracaine hydrochloride on the resulting irritancy classification of 97 proprietary formulations. Hazard classifications were assigned according to three regulatory hazard classification schemes, 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) 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). PSL conducted these studies on behalf of their clients to comply with EPA regulatory requirements. Studies were not conducted solely to evaluate the effects of anesthetic on the outcome of ocular irritation studies. The analysis of the data for this publication was secondary to the primary regulatory objectives of the original studies (i.e., hazard classification).

In Vivo Test Method Protocol

The formulations were tested in either three (81 formulations) or six (16 formulations) rabbits. Topical anesthetic pre-treatment was provided to rabbits as 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). Two drops of the anesthetic were placed in each rabbit eye approximately 2.5 minutes prior to instillation of a test substance. The remainder of the test method protocol followed EPA guidelines on acute eye irritation testing (16).

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

Hazard 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.

In order for a formulation to be included in this evaluation, all of the following criteria must have been fulfilled:

- A volume of 0.1 mL for liquids, solids, pastes, or particulates (weighing ≤ 0.1 g) was tested in each rabbit.
- Gross observations recorded, at minimum, at 24, 48, and 72 hours following test substance application (unless a corrosive effect was observed, at which time the study was terminated).
- Gross observations recorded until reversibility could be 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

The criteria for ocular hazard classification required by each of the three hazard classification systems evaluated are provided in the following tables.

United Nations Globally Harmonized System for Classification and Labelling

The classification of substances was conducted sequentially. Each rabbit tested was classified into one of four categories (Category 1, 2A, 2B, or 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 (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(s) fully reversed within 21 days
Category 2B	– Rabbit with mean scores (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(s) fully reversed within 7 days
Nonirritant	Rabbit with mean scores below the 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 for this analysis and are not GHS defined designations.
[2]Full reversal of the effects was defined as corneal opacity, iritis, redness, and chemosis = 0.

As shown in **Table 2**, the final substance classification depended on the proportion of rabbits that produced the same response. Additional classification rules (italicized text in **Table 2**) were developed to include all available data. Substances for which an unequivocal classification could not be made 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 for this analysis and are not GHS defined designations.

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

Materials and Methods

U.S. Environmental Protection Agency

Each rabbit was classified into one of four categories (Category I, II, III, or IV) (**Table 3**). The final substance classification depended 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

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; 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 formulations, the impact of topical anesthesia pre-treatment on the evaluated variable (e.g., severity of the hazard classification observed) was assessed. The impact of the topical anesthesia was determined based on assessing the average hazard classification response in a rabbit(s) not treated with topical anesthesia versus the average hazard classification response in a rabbit(s) pre-treated with topical anesthesia. In studies where only a single rabbit was either untreated or pre-treated, the average hazard classification response was defined as the response in that rabbit.

The formulations were classified into one of three categories: topical anesthesia (a) increased the severity of the observed variable, (b) decreased the severity of the observed variable, or (c) did not affect the observed variable. The relative frequencies of studies 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 Ocular Hazard Classification

As shown in **Table 5**, rabbits pre-treated with topical anesthesia produced more severe hazard classification responses than rabbits that were not pre-treated, although none of the observed differences were statistically significant.

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

Direction of Response	GHS	EU	EPA
More severe average ocular hazard classification response in topically anesthetized rabbits	20[1]	17	22
Less severe average ocular hazard classification response in topically anesthetized rabbits	13	11	16
No difference in average ocular hazard 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 hazard 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 hazard classifications when anesthesia pre-treatment was used as a criterion (**Table 6**).

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

Agreement of Response	GHS	EU	EPA
More agreement in hazard classification response among rabbits with the same topical anesthetic pre-treatment regimen[1]	16[2]	10	17
More agreement in hazard 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.

Results

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

None of the differences observed in the day-to-clearing evaluation 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	33	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 day-of-clearing of corneal lesions in rabbits pre-treated with topical anesthesia was slightly later 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)	9.9% (19)
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.

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 hazard classification responses
  - The number of days required for an ocular lesion to clear
- When a difference was observed, the pre-treated rabbits more frequently exhibited a more severe hazard classification than observed for rabbits that were not pre-treated. However, none of the differences were statistically significant.
- Since the observed variability occurs in both directions (increasing and decreasing the level of hazard classification), any observed differences are likely related to the inherent variability of the rabbit response.
- The largest difference (although not statistically significant) for the number of days required for an ocular lesion to clear was for opacity
- 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 hazard classification 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.

References

(1) US Dept. of Labor, Bureau of Labor Statistics http://www.bls.gov/iif/oshcdnew.htm.
(2) Work-Related Injury Statistics Query System. NIOSH. http://www2a.cdc.gov/risqs/.
(3) Draize et al. 1944. J Pharm Exp Ther. 82:377-390.
(4) Arthur et al. 1986. J Toxicol – Cut & Ocular Toxicol. 5:215-227.
(5) Heywood and James. 1978. J Soc Cosmet Chem. 29:25-29.
(6) Seabaugh et al. 1993. Food Chem Toxicol. 31:95-98.
(7) Walberg J. 1983. Toxicol Lett. 18:49-55.
(8) Rowan and Goldberg. 1984. Ann Rev Pharmacol Toxicol. 25:225-247.
(9) Anonymous. 1981. Int J Stud Anim Prob. 2:174.
(10) Ulsamer et al. 1977. 16th Annual Meeting. Society of Toxicology Abstracts; Abstract 143.
(11) Johnson AW. 1980. Proceeding of the CTFA ocular safety testing workshop: In vivo and in vitro approaches. Cosmetic, Toiletries, and Fragrance Association. October 6-7, 1980.
(12) Durham et al. 1992. Lab Anim Sci. 42:535-541.
(13) UN. 2003. http://www.unece.org/trans/danger/publi/ghs/ghs_rev00/00files_e.html.
(14) EPA. 1996. EPA737-B-96-001.
(15) EU. Official J European Communities 2001; L255:1-333.
(16) EPA. 1998. EPA 712-C-98-195.
(17) EPA. 2005. 40 CFR 792.
(18) EPA. 2005. 40 CFR 160.
(19) FDA. 2006. 21 CFR 58.
(20) Siegel SN and Castellan Jr. NJ. 1956. Nonparametric statistics for the behavioral sciences. 1st ed.; New York City, NY: McGraw Hill.

NIEHS National Institute of Environmental Health Sciences
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