

# RETROSPECTIVE ASSESSMENT OF THE RABBIT ENUCLEATED EYE TEST (REET) AS A SCREEN TO REFINE WORKER SAFETY STUDIES.

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## Abstract

The REET is an *ex vivo* alternative that has been studied as a screen to identify severe eye irritants in the workplace. GSK has incorporated the REET into a tiered protocol designed to reduce the use of the traditional Draize eye protocol. Corneal opacity and thickness, fluorescein dye uptake and the condition of the corneal surface are observed after *in vitro* exposure to test materials. Previously 30 pharmaceutical process materials which had been evaluated with the Draize method were assessed by the REET. An evaluation of the REET data suggested that a score for any one of the following parameters appeared highly predictive of the Draize response: corneal opacity/area  $\geq 3$ ; corneal swelling  $\geq 25$ ; fluorescein dye uptake/area  $\geq 4$  or select gross morphological changes in the corneal epithelium (e.g., pitting, mottling or sloughing). See Figure 1 for an overview of the principle procedures. See Figure 2 for examples of an untreated enucleated eye, a treated non-fluorescein stained eye and a treated fluorescein stained eye.

## Introduction

At GlaxoSmithKline, occupational toxicity tests of active pharmaceuticals, reactive intermediates and raw materials assess the potential for adverse effects when inadvertent exposure occurs in the workplace. Underlying the entire GSK testing program for occupational toxicology is the Russell and Burch<sup>1</sup> concept of the 3Rs (replacement, reduction and refinement) in the use of laboratory animals. GSK utilises a tiered approach in identifying severe eye irritants in order to minimize the use of animals in testing with irritant materials. The GSK approach has been presented previously<sup>2,3</sup>. This approach is based on physical-chemical properties (e.g., pH), chemical similarity to known irritants (literature search), results from animal dermal irritation studies and the Rabbit Enucleated Eye Test (REET). The REET is an *ex vivo* assay that uses enucleated eyes from rabbits euthanized for other tests and can serve as a screen for eye irritancy without the use of living animals. The REET involves the application of test material onto the cornea of a rabbit enucleated eye, which is maintained at a temperature of 32°C  $\pm$  1.5°C, within a superfusion chamber. The direct effect of the test material on the cornea can be assessed by evaluating changes in corneal thickness, corneal opacity, alteration of the corneal epithelium and fluorescein uptake up to 240 minutes following treatment. Previously, GSK has shown that a score for any one of the following parameters appeared highly predictive of the *in vivo* response seen in the Draize<sup>4</sup> eye irritation test: corneal opacity/area  $\geq 3$ ; corneal swelling  $\geq 25$ ; fluorescein dye uptake/area  $\geq 4$  or select gross morphological changes in the corneal epithelium such as pitting, mottling or sloughing<sup>5</sup>. The REET assay has also been shown by other laboratories to reliably differentiate between substances that are negligible or severe ocular irritants<sup>4,5,6</sup>.

In the present study, the previously reported<sup>2</sup> REET data on 30 pharmaceutical process materials (active pharmaceuticals, reactive intermediates and raw materials) has been supplemented with data from 14 more chemical materials to further define the predictivity of the assay. For these additional materials the *in vivo* comparisons were obtained from published data<sup>7</sup>.

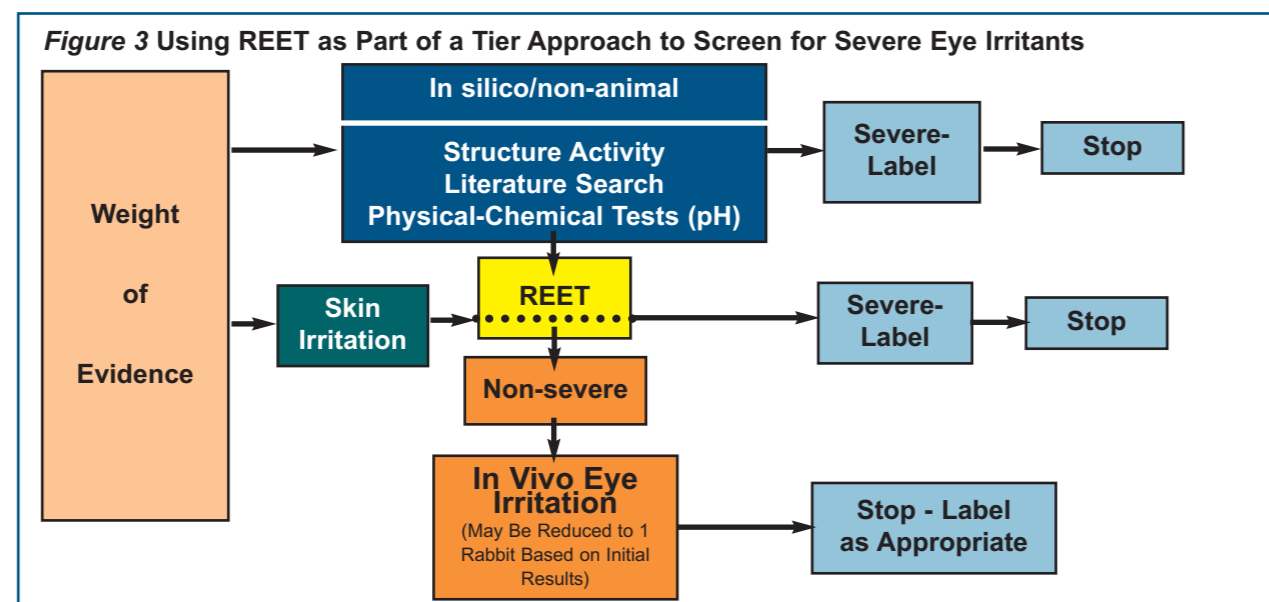
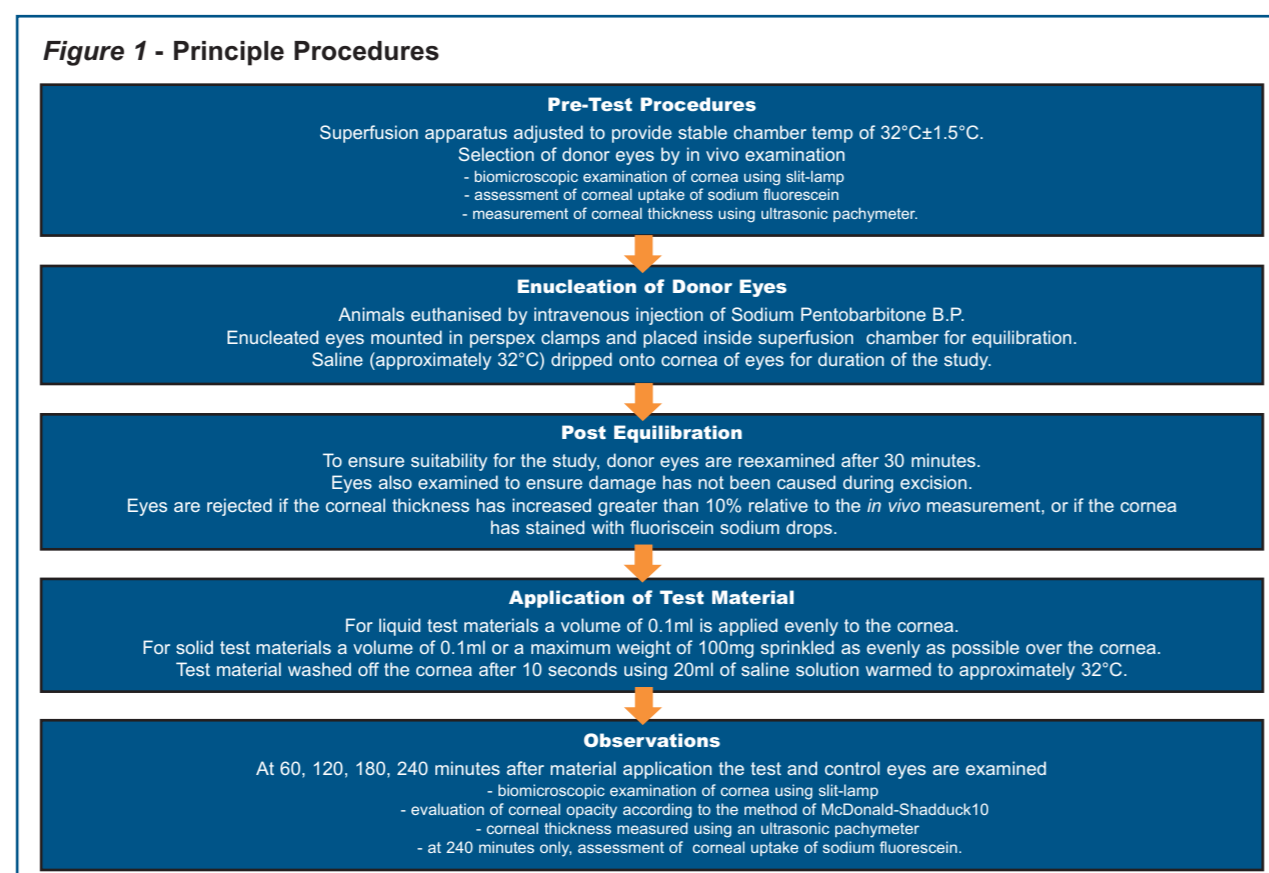
## Methods and Materials

Detailed methods and materials have already been summarised in our previous presentation along with photographs of the equipment and how the REET assay is conducted<sup>2</sup>. To briefly summarise, the REET uses enucleated eyes from rabbits euthanized in other tests with a standardised protocol developed at SafePharm Laboratories in conjunction with GSK. The test material is applied for 10 seconds onto the cornea of the enucleated eye, which is maintained at a temperature of 32°C  $\pm$  1.5°C within a superfusion chamber. A direct effect of the test material on the cornea is determined by evaluating changes in corneal thickness, corneal opacity, alteration of the corneal epithelium and fluorescein

uptake for up to 240 minutes following treatment. A score for any one of the following parameters appeared highly predictive of the Draize response: corneal opacity/area  $\geq 3$ ; corneal swelling  $\geq 25$ ; fluorescein dye uptake/area  $\geq 4$  or gross morphological changes in the corneal epithelium (e.g., pitting, mottling or sloughing).

See Figure 1 for an overview of the principle procedures.

See Figure 2 for examples of an untreated enucleated eye, a treated non-fluorescein stained eye and a treated fluorescein stained eye.



## Results and Discussion

Previously, 30 pharmaceutical process materials that were evaluated with the Draize method (except where precluded by extremes in pH or dermal results) were concurrently assessed by the REET. The outcome of that study, based on a visual inspection of the data, was a proposed series of criteria consisting of multiple endpoints in which to identify compounds as potentially severe eye irritants (Table 1). Compounds which did not meet the criteria were considered to be non-severe (non-irritants, mild or moderate eye irritants). Overall, this approach appeared highly predictive of the Draize response (Tables 2, 3) for these first 30 compounds. The REET-positive criteria was met by 17/30 materials and included 13 labeled severe (R41) with 1 moderate and 3 mild by *in vivo* evaluation. Thirteen chemicals did not meet the REET criteria: 9 were minimal/mild eye irritants and 4 were moderate. Eighty-seven percent (87%) of the time the REET correctly predicted both severe and non-severe eye irritants.

The data from the next 14 chemical materials were not concurrently evaluated using both the REET and Draize methods. These were commonly available chemicals in which the severity of eye effects were previously published. The REET-positive criteria

was met by 8/14 materials and included 2 labeled severe (R41) with 5 moderate and 1 mild. Six chemicals did not meet the REET criteria: 5 were non/minimal/mild eye irritants and 1 was a moderate. Fifty-seven percent (57%) of the time the REET correctly predicted both severe and non-severe eye irritants. The reason why the REET did not predict as well for the group of 14 raw materials is unclear. In the first group of 30 pharmaceutical process materials, most of the comparative *in vivo* and REET work was done about the same time and utilised the same lot/batch number of compound and was conducted in the same laboratory. Potential confounding factors in the 14 additional chemical materials that resulted in lower predictivity may be due to:

- the *in vivo* eye irritation data was taken from a publication which pooled multiple sources of data which came from different laboratories;
- the *in vivo* eye irritation and the REET assay data were generated at different times;
- the *in vivo* eye irritation and the REET assay used different lot/batch numbers of compound.

Further analysis of the original publications in pooled reference will be undertaken in an attempt to further refine analysis of the 14 supplemental materials.

Pooling all data resulted in REET positive criteria being met by 25/44 materials and included 15 labeled severe (R41) with 6 moderate and 4 mild. Nineteen chemicals did not meet the REET criteria: 14 were non/minimal/mild eye irritants and 5 were moderate. Seventy-seven percent (77%) of the time the REET correctly predicted both severe and non-severe eye irritants when all the data was combined. However, based on the way GSK is utilising the REET assay at present, it is not sensitive enough to differentiate a mild/moderate eye irritant from a non-irritant. There were no false negatives in either dataset. Overall, the REET assay overpredicted the severity of a result 23% of the time.

GSK has used the REET as part of an integrated approach to hazard evaluation for worker safety assessment of pharmaceutical materials to minimize animal use (Figure 3). This approach incorporates the REET, in conjunction with assessing a material's physical-chemical properties, chemical similarity to known irritants and the results from animal dermal irritation studies, to identify severe eye irritants and exclude them from further *in vivo* testing. Recently, based on our accumulated experience with the REET assay, negatives still do go on to the Draize eye irritation test but we have taken this approach one step further. The Draize test is initially conducted with just one rabbit and at the end of the 3-day observation period the data is evaluated. If the material appears to be either a severe eye irritant or a non/minimal eye irritant then the study is terminated and appropriately labeled. Anything in between then continues with 2 more rabbits. As an example, a recent project in 2003 involved looking at 27 materials in various pharmaceutical processes for eye irritation. Before the REET assay and our current approach, this could have potentially involved 81 rabbits if each compound used the 3 rabbit Draize eye irritation test. Using our current approach only 21 rabbits were actually used and resulted in saving 60 rabbits without compromising worker

Table 1 - Summary of REET Criteria to Detect Severe Eye Irritants

| REET Parameter*                 | REET Cut-Off Value To Detect Severe Eye Irritants |
|---------------------------------|---|
| Maximum Corneal Opacity         | $\geq 3$  |
| Cloudy                          |   |
| Area                            |   |
| Maximum Fluorescein Uptake      | $\geq 4$  |
| Intensity                       |   |
| Area                            |   |
| Mean Corneal Swelling (mins.)   | $\geq 25$   |
| 60                              |   |
| 120                             |   |
| 180                             |   |
| 240                             |   |
| Corneal Epithelium Observations | Any with Pitting, Mottling or Sloughing           |

\*Any parameter that meets or exceeds the cut-off indicates a severe eye irritant.

Table 2 - Summary of REET Data

| REET Prediction                            | First 30 Pharmaceutical Process Materials* | Additional 14 Chemical Materials** | Combined Compound Data |
|--|--|------------------------------------|------------------------|
|  | No. (%)                                    | No. (%)                            | No. (%)                |
| Severe Eye Irritant Met REET Criteria      | 17   | 8                                  | 25                     |
| Actually Identified as R41***              | 13   | 2                                  | 15                     |
| Non-Severe Did Not Meet REET Criteria      | 13   | 6                                  | 19                     |
| Actually Identified as Non-Severe****      | 13   | 6                                  | 19                     |
| REET Over Predict Severe (False Positive)  | 4 (13)                                     | 6 (43)                             | 10 (23)                |
| REET Under Predict Severe (False Negative) | 0 (0)                                      | 0 (0)                              | 0 (0)                  |
| REET Correct (R41 + Non Severe)            | 26 (87)                                    | 8 (57)                             | 34 (77)                |
| <b>Total Number Of Compounds Tested</b>    | <b>30</b>                                  | <b>14</b>                          | <b>44</b>              |

\*Most of the *in vivo* and REET work was done about the same time, utilised the same lot/batch numbers and was conducted in the same laboratory.  
 \*\*The *in vivo* eye irritation data was taken from a publication which pooled multiple sources of data, came from different laboratories, data was generated at a different time from the REET assay used different lot/batch numbers of compound.  
 \*\*\*Mostly based on *in vivo* data, but some comparisons came from physical chemical properties and information from published literature. See Table 3.  
 \*\*\*\*Based on *in vivo* data only.

safety. Our experience using the tier approach has shown that very infrequently a compound classified as a non-severe eye irritant based on the REET may act with a delayed effect in the Draize. A day or two after the initial compound application in the Draize study a persistent corneal effect may occur and this results in a classification as a severe eye irritant. This type of severe eye irritation may not be picked up by the REET, which is why a REET non-severe goes on to a limited Draize test. The tiered screening approach at GSK greatly reduces the number of animals used in the Draize eye irritation assay, reduces the extent of reactions experienced by animals in the Draize test and apparently predicts correctly all the severe eye irritants.

## Conclusion

- A series of criteria consisting of multiple endpoints in the REET assay was able to correctly identify 77% of the materials tested as either severe or non-severe eye irritants.
- The application of the REET assay supports the concepts of the 3Rs (replacement, reduction and refinement) in the use of laboratory animals for occupational toxicity testing in identifying severe eye irritants.
- The REET is a valuable and practical screening tool when added to a tiered and weight of evidence approach to identifying materials that are severe eye irritants in the work place.

Table 3 - Individual REET Data

| Test Material                       | Physical Form | EU Class <sup>7</sup> | Key & Calandra Classification <sup>8</sup> | REET Results                         |      |   |      |  |       |       |       |   |                     |                |  | Corneal Epithelium Observations | REET Prediction | Correlation with EU Class |
|-------------------------------------|---------------|-----------------------|--|--------------------------------------|------|---|------|--|-------|-------|-------|---|---------------------|----------------|--|---------------------------------|-----------------|---------------------------|
|                                     |               |                       |  | Maximum Corneal Opacity <sup>9</sup> |      | Maximum Fluorescein Uptake <sup>9</sup> |      | Mean Corneal Swelling <sup>9</sup> (mins.) |       |       |       |   |                     |                |  |                                 |                 |                           |
|                                     |               |                       |  | Cloudy                               | Area | Intensity                               | Area | 60   | 120   | 180   | 240   |   |                     |                |  |                                 |                 |                           |
| 1. RM - Aromatic sulphonyl chloride | S             | R41                   | Predicted - based on literature            | 3                                    | 4    | 2                                       | 3    | 21.8                                       | 30.4  | 39.5  | 45.4  | Residual test material present                            | Severe irritant     | Correct        |  |                                 |                 |                           |
| 2. RM - Piperazine                  | S             | R41                   | Predicted - based on pH < 2                | 1                                    | 4    | 1                                       | 4    | 9.3  | 14.1  | 15.7  | 20.0  | Normal  | Severe irritant     | Correct        |  |                                 |                 |                           |
| 3. RM - Cyanamide                   | S             | R41                   | Extremely severe (8)                       | 2                                    | 3    | 2                                       | 2    | 40.7                                       | 58.7  | 64.3  | 71.0  | Sloughing   | Severe irritant     | Correct        |  |                                 |                 |                           |
| 4. API - Aromatic sulphinate        | S             | R41                   | At least very severe (7)                   | 2                                    | 3    | 2                                       | 1    | 3.2  | 4.8   | 4.0   | 7.6   | Residual test material present                            | Severe irritant     | Correct        |  |                                 |                 |                           |
| 5. API - Substituted ethylamine     | S             | R41                   | At least very severe (7)                   | 2                                    | 4    | 3                                       | 4    | 58.8                                       | 79.6  | 99.2  | 113.0 | Mottled Appearance  | Severe irritant     | Correct        |  |                                 |                 |                           |
| 6. API - Guanidine                  | S             | R41                   | At least severe (6)                        | 2                                    | 3    | 4                                       | 2    | 13.2                                       | 21.7  | 27.0  | 27.7  | Sloughing   | Severe irritant     | Correct        |  |                                 |                 |                           |
| 7. INT - Substituted aniline        | S             | R41                   | At least severe (6)                        | 0                                    | 0    | 2                                       | 2    | 6.6  | 6.1   | 9.3   | 7.4   | Residual test material adhered to cornea, pitting         | Severe irritant     | Correct        |  |                                 |                 |                           |
| 8. RM - Quaternary ammonium         | S             | R41                   | At least severe (6)                        | 2                                    | 4    | 4                                       | 4    | 22.2                                       | 74.7  | 83.9  | 109.9 | Pitting/Sloughing   | Severe irritant     | Correct        |  |                                 |                 |                           |
| 9. INT - Pyridine                   | S             | R41                   | At least severe (6)                        | 3                                    | 4    | 3                                       | 4    | 31.8                                       | 43.6  | 53.0  | 62.2  | Mottled, sloughing  | Severe irritant     | Correct        |  |                                 |                 |                           |
| 10. RM - Pyridine complex           | S             | R41                   | At least severe (7)                        | 2                                    | 2    | 2                                       | 3    | 16.8                                       | 23.6  | 28.4  | 25.4  | Normal  | Severe irritant     | Correct        |  |                                 |                 |                           |
| 11. INT - Chlorinated aniline       | S             | R41                   | Predicted from literature                  | 4                                    | 4    | 3                                       | 4    | 51.4                                       | 98.0  | 112.6 | 118.2 | Mottled, residual test material, smooth cloudy appearance | Severe irritant     | Correct        |  |                                 |                 |                           |
| 12. RM - Substituted piperidine     | S             | R41                   | Predicted - corrosive to skin              | 4                                    | 4    | 4                                       | 3    | 111.5                                      | 117.5 | 83.9  | 101.1 | Sloughing/mottled   | Severe irritant     | Correct        |  |                                 |                 |                           |
| 13. RM - Benzylamine                | L             | R41                   | Predicted - corrosive to skin              | 2                                    | 4    | 2                                       | 4    | 20.3                                       | 75.4  | 80.5  | 98.2  | Cloudy, pitted  | Severe irritant     | Correct        |  |                                 |                 |                           |
| 14. API - Carbamoyl                 | S             | R36                   | Moderate (5)                               | 0                                    | 0    | 0                                       | 0    | 7.3  | 4.8   | 5.2   | 5.9   | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 15. RM - Aromatic acetamide         | S             | R36                   | Moderate (5)                               | 0                                    | 0    | 0                                       | 0    | 6.3  | 6.9   | 6.6   | 7.6   | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 16. INT - Substituted pyridine      | S             | NC                    | Moderate (5)                               | 0                                    | 0    | 0                                       | 0    | 4.6  | 4.9   | 4.7   | 2.7   | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 17. INT - Substituted pyridine      | S             | NC                    | Moderate (5)                               | 1                                    | 4    | 0                                       | 0    | 4.5  | 4.9   | 4.3   | 2.5   | Normal  | Severe irritant     | Over predicted |  |                                 |                 |                           |
| 18. RM - Sulfonyl chloride          | S             | NC                    | Moderate (5)                               | 1                                    | 2    | 1                                       | 2    | 8.6  | 12.9  | 14.2  | 14.9  | Residual test material present                            | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 19. RM - Aromatic aldehyde          | S             | NC                    | Mild (4)                                   | 0                                    | 0    | 0                                       | 0    | 7.2  | 13.0  | 15.5  | 17.4  | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 20. INT - Piperazine                | S             | NC                    | Mild (4)                                   | 2                                    | 2    | 2                                       | 2    | 12.4                                       | 12.0  | 19.1  | 22.4  | Mottled Appearance  | Severe irritant     | Over predicted |  |                                 |                 |                           |
| 21. INT - Substituted aniline       | L             | NC                    | Mild (4)                                   | 0                                    | 0    | 1                                       | 4    | 17.6                                       | 22.5  | 28.3  | 31.5  | Normal  | Severe irritant     | Over predicted |  |                                 |                 |                           |
| 22. RM - Acetanilide                | S             | NC                    | Minimal (3)                                | 0                                    | 0    | 0                                       | 0    | 3.2  | 1.4   | 1.5   | 2.6   | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 23. INT - Substituted aniline       | S             | NC                    | Mild (4)                                   | 0                                    | 0    | 1                                       | 3    | 4.8  | 5.6   | 3.4   | 6.8   | Residual test material adhered to cornea                  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 24. INT - Quinoline                 | S             | NC                    | Mild (4)                                   | 0                                    | 0    | 0                                       | 0    | 2.4  | 3.7   | 5.0   | 7.1   | Residual test material present                            | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 25. API - Sulphone                  | S             | NC                    | Minimal (3)                                | 0                                    | 0    | 0                                       | 0    | 2.9  | 2.7   | 3.2   | 4.4   | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 26. INT - Indole                    | S             | NC                    | Mild (4)                                   | 0                                    | 0    | 1                                       | 3    | 5.9  | 9.8   | 10.9  | 14.8  | Residual test material present                            | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 27. RM - Acetophenone               | S             | NC                    | Mild (4)                                   | 0                                    | 0    | 0                                       | 0    | 6.1  | 3.4   | 2.0   | 2.5   | Residual test material present                            | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 28. RM - Lactam                     | S             | NC                    | Mild (4)                                   | 0                                    | 0    | 0                                       | 0    | 1.3  | 0.2   | 0.4   | 2.0   | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 29. API - Sulfonamide               | S             | NC                    | Minimal (3)                                | 0                                    | 0    | 0                                       | 0    | 1.5  | 1.1   | 1.4   | 2.0   | Normal  | Non-severe irritant | Over predicted |  |                                 |                 |                           |
| 30. RM - Aliphatic carboxylic ester | L             | NC                    | Mild (4)                                   | 0                                    | 0    | 0                                       | 0    | 16.8                                       | 22.8  | 30.7  | 39.4  | Slight pitting  | Severe irritant     | Over predicted |  |                                 |                 |                           |
| 31. RM - Sodium Hydroxide 10%       | L             | R41                   | Extremely severe (8)                       | 4                                    | 4    | 4                                       | 4    | 110.5                                      | NA    | NA    | NA    | Rippling, loosening of epithelium                         | Severe irritant     | Correct        |  |                                 |                 |                           |
| 32. RM - Trichloroacetic acid 30%   | L             | R41                   | Extremely severe (8)                       | 4                                    | 4    | 4                                       | 4    | 12.2                                       | 21.1  | 38.2  | 54.1  | Roughening of epithelium                                  | Severe irritant     | Correct        |  |                                 |                 |                           |
| 33. RM - Acetone                    | L             | R36                   | NA   | 2                                    | 3    | 3                                       | 4    | 19.3                                       | 24.5  | 39.0  | 49.6  | Loosening/roughening of epithelium                        | Severe irritant     | Over predicted |  |                                 |                 |                           |
| 34. RM - Alkyl alcohol              | L             | R36                   | NA   | 3                                    | 3    | 4                                       | 4    | 41.0                                       | 56.1  | 68.5  | 77.2  | Pitting, sloughing  | Severe irritant     | Over predicted |  |                                 |                 |                           |
| 35. RM - 1-Butanol                  | L             | R36                   | Severe (8)                                 | 3                                    | 4    | 3                                       | 4    | 54.6                                       | 68.3  | 81.4  | 91.8  | Pitting, loosening of epithelium                          | Severe irritant     | Over predicted |  |                                 |                 |                           |
| 36. RM - Ammonium nitrate           | S             | R36                   | Moderate (5)                               | 0                                    | 0    | 0                                       | 0    | 10.5                                       | 12.2  | NA    | 15.0  | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 37. RM - Cetylpyridium bromide      | S             | R36                   | Moderate (5)                               | 2                                    | 3    | 3                                       | 3    | 49.0                                       | 48.8  | 61.0  | 31.1+ | Loosening of epithelium                                   | Severe irritant     | Over predicted |  |                                 |                 |                           |
| 38. RM - Methyl ethyl ketone        | L             | R36                   | Moderate (5)                               | 3                                    | 4    | 4                                       | 3    | 34.8                                       | 62.6  | 85.6  | 104.7 | Loosening of epithelium                                   | Severe irritant     | Over predicted |  |                                 |                 |                           |
| 39. RM - Butyl acetate              | L             | NC                    | Mild (4)                                   | 1                                    | 3    | 2                                       | 1    | 19.5                                       | 17.2  | 21.5  | 30.1  | Normal  | Severe irritant     | Over predicted |  |                                 |                 |                           |
| 40. RM - Toluene                    | L             | NC                    | Mild (4)                                   | 0                                    | 0    | 0                                       | 0    | 7.4  | 9.8   | 13.4  | 14.6  | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 41. RM - Glycerol                   | L             | NC                    | Minimal (3)                                | 0                                    | 0    | 0                                       | 0    | 13.1                                       | 15.5  | 16.5  | 21.4  | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 42. RM - Propylene glycol           | L             | NC                    | Minimal (3)                                | 0                                    | 0    | 0                                       | 0    | 10.1                                       | 10.2  | 10.8  | 16.3  | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 43. RM - 4,4'-Methylene bis         | S             | NC                    | Practically non-irritating (2)             | 0                                    | 0    | 0                                       | 0    | 9.4  | 12.3  | 12.8  | 15.1  | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |
| 44. RM - Polyethylene glycol 400    | L             | NC                    | Non-irritant (1)                           | 0                                    | 0    | 0                                       | 0    | 12.7                                       | 13.4  | 16.9  | 17.8  | Normal  | Non-severe irritant | Correct        |  |                                 |                 |                           |

<sup>1</sup> *In vivo* data for compounds nos. 31 - 44 from ECETOC Technical Report No. 48, Eye Irritation: Reference Chemicals Data Bank (August 1992). ECETOC, Brussels.

Figure 2 - Examples of Untreated and Treated Enucleated Eyes

