Appendix A

Publicly Available Protocols for the IRE Test Method

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Appendix A1

INVITTOX Protocol 85. The Rabbit Enucleated Eye Test Method of Dr. Lesley Earl

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THE RABBIT ENUCLEATED EYE TEST

The isolated eye of a rabbit is exposed to the test compound and assessed for corneal swelling, corneal opacity and fluorescein retention in order to evaluate the eye irritation potential of the compound.

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NOTE
*The protocol presents the standard operation procedure used in the Home Office UK/EEC Validation Study for Alternatives to the Draize Test. It should be noted that this protocol might need to be modified in light of experience gained in the study. Additional information added in the course of producing this **INVITTOX** protocol, e.g. this note, is presented in italics.*

Critical Assessment

The use of isolated rabbit eyes for the assessment of eye irritation potential is a recognized alternative to the Draize eye irritation test, because it closely models the *in vivo* test system, but does not require the exposure to take place in the eyes of living animals. The procedure permits evaluation of undiluted test materials, i.e. as they could enter the *in vivo* eye test, and in this respect provides a major advantage over many other *in vitro* procedures. This test system could be used as part of an *in vitro* test battery for the assessment of eye irritancy, to provide a higher level of testing after initial screening in simple cell culture-based systems. It will not, however, provide information about potential effects of the test substance on the conjunctivae, nor about the rate of recovery from the insult.

An interlaboratory trial (Whittle *et al.*, 1992), using test substances with various degrees of eye irritation potential, showed good agreement between three laboratories, with 22 out of 27 substances being rated within the same one out of the four irritancy ratings used. This consistency occurred in spite of the fact that the three laboratories adopted different *in vitro* grading systems. Two exposure periods were used, 10 seconds and 60 seconds. When results were compared with *in vivo* data, a better correlation was obtained with results from the 10-second exposure. Better predictions of *in vivo* effects were obtained with liquid test substances than with solid test substances.

Basic Procedure

Objectives
The purpose of the test is to assess the irritation potential of substances applied to the isolated rabbit eye. However, the method does not provide information on the effects of materials on the conjunctiva of the eye, or on any recovery of the cornea from damage, which might occur in the eye *in vivo*.

Summary of test method
The eyes of rabbits are enucleated immediately after death of the animal and are mounted in a temperature-controlled chamber which provides optimum conditions for the continuation of *in vivo* physiology. The eyes are left in the maintenance chamber long enough to stabilise. Test materials are then applied in a single dose to the cornea for 10 seconds. The effects of this treatment are assessed at predetermined intervals by four methods:

1) Assessment of corneal opacity.
2) Measurement of corneal thickness to determine corneal swelling.
3) Assessment of the rate at which fluorescein penetrates into the cornea.
4) Histological examination of the cornea to assess any damage to the corneal epithelium.

The degree of damage to the cornea is recorded over a 4 hour period. The stability of the test system during the study is confirmed by the concurrent observation of an untreated eye. At the end of the experiment, the preparation and examination of histological sections of the cornea can be used to confirm the level of corneal damage.

Procedure Details

Preparation of the *in vitro* model

Selection of animals
Eyes from New Zealand White rabbits are used in this study. Suitable eyes show no opacity of the cornea and no imperfections on the corneal surface based on detailed macroscopic and slit-lamp examination. Sufficient animals are required to provide at least three eyes for each test material to be tested, plus one other eye to serve as an untreated control.

Equipment
The equipment used has been described previously (Burton, York and Lawrence, ***Food and Cosmetics Toxicology***, **19**: 471-480, 1981).

Equipment required is as follows:

Fine surgical scissors
Surgical enucleating scissors
Forceps

Perspex clamps for holding eyes: the clamp has an upper arm that can be moved up and down to accommodate the eyeball, and stainless steel pins embedded in the upper arm and base to hold the eye in place. The pins protrude only to about 1 mm, so as to avoid puncturing the globe. The upper arm is cut away, if required, to permit saline to drip onto the upper surface of the cornea.

Superfusion apparatus: this is a perspex maintenance chamber with six cells, each holding one eye. The walls of each cell are made of black perspex for optimal slit-lamp observation. A stainless steel tube leading from each cell is connected to a peristaltic pump and is used to supply isotonic saline at a constant flow to the cell. The perspex clamp with the eye is positioned within the compartment so that saline from the steel tube drips onto the cornea. Saline is pumped out of the cell via two stainless steel drainage tubes in the rear bottom corners. A sliding door at the front of each cell allows for access to the eye. A water jacket surrounds the maintenance chamber and receives water pumped in from a temperature-controlled water bath. The stainless steel tubes used for saline delivery to the cells pass through the water jacket so that the saline can be warmed to the correct temperature.
Slit lamp microscope, e.g. Haag-Streit AG, Liebefeld-Bern, Switzerland
Depth Measuring Attachment for slit lamp, e.g. Attachment No. 1, Haag-Streit

Prior to preparation of the eyes, the water heater/circulator and the remote thermometer are switched on. After a heating period, the heater control is adjusted to give a stable air temperature within a closed cell of the maintenance chamber of 32°C (± 2°C). The peristaltic pump provides a flow rate of saline to each cell of less than 1 ml/min.

Dissection

N.B. Some training is required in order to carry out this dissection. Care is required to avoid loss of intraocular pressure. A trained dissector can expect to lose one out of every three-four eyes dissected, on account of damage. Spare eyes should be prepared to make up for any loss.

The following dissection procedure is carried out on each selected rabbit:

a) The animal is killed by the injection of pentobarbitone solution into the ear vein.

b) Immediately after death, a few drops of physiological saline are applied to the eyes to prevent them drying during dissection.

c) Each eye is dissected by deflecting the nictitating membrane and cutting away the conjunctivae using angled forceps and curved scissors. The eyeball is proptosed by applying pressure above and below the eyeballs. The remaining conjunctival tissue, the orbital muscles and the optic nerve are cut and the eyeball is lifted from the orbit.

d) Adherent tissue is dissected from the globe of the eyeball, and the eyeball is rinsed with physiological saline.

Supply of tissue

If it is impractical or undesirable to use eyes from rabbits killed at the testing facility, then eyes may be obtained from a local industrial laboratory and transported with minimum delay under maintained conditions to the testing facility.

Eyes are enucleated from dead rabbits in the previously described way at the supplying laboratory. Those animals that had previously been used for experimental purposes by the laboratory, were either used in skin irritation tests, to supply tissues other than eyes or as control (untreated) animals. After removal, the eyes are placed in a large insulated flask. The temperature is maintained by sealing 1 litre of water (37°C) in a plastic bag within the flask. To prevent drying of the enucleated eyes, each eye is thoroughly wetted with saline and the humidity maintained by a quantity of freestanding water (37°C) in the bottom of the flask. The eyes are transported to the testing facility (not more than 1 hour) for the continuation of the procedure. The eyes are then placed in the superfusion chambers.

Pretreatment incubation
After dissection, the eyeball is then mounted in a vertical position in a clamp which holds the eye firmly, but without excessive pressure. The clamp is positioned in a cell of the maintenance chamber. The saline drip tube of the cell is positioned so that the drops of saline fall onto the upper margin of the cornea and irrigate the whole surface of the cornea. All the eyes necessary for the test are dissected out and mounted in the chamber in this way.

Immediately after the eye is positioned in the chamber, it is stained with fluorescein solution (1% fluorescein sodium BP, Smith and Nephew Pharmaceuticals, Romford, Essex, UK - or equivalent) for a few seconds, after which it is rinsed with saline to establish if there has been any damage during dissection, i.e. if there is any evidence of penetration of fluorescein into the eye. If the cornea has been damaged during dissection, that eye is rejected as unsuitable for use and a further eye is prepared as a replacement. The corneal thickness of undamaged eyes is then measured (Slit reading -1).

The eyes are maintained in the chamber to equilibrate for 45-60 minutes, after which the corneal thickness is measured again (Slit reading 0). If Slit reading 0 exceeds Slit reading -1 by more than 5%, then that eye is rejected from the experiment.

Treatment
Three eyes are treated with each test material and one eye remains untreated as a control.

Prior to application of the test materials, the eye, held in its clamp, is removed from the chamber and positioned with the cornea uppermost.

Liquid test materials
0.1 ml of the test material is applied to the central part of the cornea. After 10 seconds, the test liquid is removed from the cornea by rinsing the surface with a 20-ml syringe of saline. The eye is then replaced in the chamber. The saline drip is repositioned as before.

Solid test materials

Test materials which are not in a powder or fine granular form should be ground prior to treatment. 25 mg of the test material is sprinkled evenly over the whole surface of the cornea. After 10 seconds, all the test material is removed from the corneal surface by rinsing with 20 ml of saline at room temperature. If particles of test material adhere to the corneal surface, then the cornea is rinsed further. If the particles cannot be removed, even after excess rinsing, this should be noted. The clamped eye is then returned to the maintenance chamber, and the saline drip is repositioned as before.

Assessments
The cornea of each treated eye and the control eye is assessed by the methods detailed below:

Corneal opacity
A slit-lamp biomicroscope is used to examine the cornea for the degree of opacity (the most dense area is taken for reading) using the following scoring system:

No opacity	0
Scattered or diffuse area, details of iris clearly visible	1
Easily discernible translucent area, details of iris slightly obscured	2
Nacreous area, no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through opacity	4

Assessments are carried out immediately after treatment, at 30 minutes, and at 1, 2, 3 and 4 hours after treatment.

Corneal thickness
The thickness of the cornea is measured using a slit-lamp biomicroscope fitted with a depth-measuring attachment, or an ultrasonic pachymeter. Refer to slit-lamp manual for instructions on corneal thickness measurements. The definitive values obtained for each eye are recorded and the degree of corneal swelling caused by treatment is calculated as a percentage of the corneal thickness of the eye immediately before treatment (Slit reading 0). Assessments are carried out at 30 minutes, and at 1, 2, 3, and 4 hours after treatment.

Slit-lamp examination of the cornea
Using the slit-lamp set with a narrow slit, the treated corneas are examined for evidence of damage based on reflection of light from different parts of the slit image. The effects are assessed on the following scale:

Slit image identical to control eye	0
Light reflection from one or more regions of the slit image	1

Increased reflection of light suggests some form of corneal damage has occurred.

Assessments are carried out 30 minutes after treatment, and at 1, 2, 3 and 4 hours after treatment.

Penetration of fluorescein into the cornea
One drop of fluorescein solution is applied onto the cornea of each eye for 10 seconds and then rinsed off with saline. The cornea is then examined using a slit-lamp biomicroscope and the staining and diffusion characteristics are assessed according to the following criteria:

No staining	0
Bright green staining of anterior edge of cornea but no penetration	1
Bright green anterior edge to cornea, gradual diffusion of stain through cornea	2

Assessments are carried out at 30 minutes and 4 hours after treatment.

NB In circumstances where grade 3 or grade 4 corneal opacities are present, evaluation of fluorescein penetration is unnecessary.

Supplementary observations
The parameters detailed above provide the minimum requirements for evaluation of effects on the isolated rabbit eye. Solutions of solid substances may also be tested. Further parameters, such as the histological examination of the epithelium, may be recorded. Photography of the eye may also be useful for comparison of responses.

Recording data
The attached score sheet is used to record the individual raw data for each chemical. Further descriptive information can be recorded on a separate sheet, which should be marked with the study number, the compound being evaluated, the date of the experiment and the signature of the operator.

Amendments

Experimental variation
Where the investigator determines that an individual eye has elicited a different response from the other two, similarly treated, eyes, the experiment must be repeated. The data from all six eyes will then be used to calculate the mean values to be used in the overall assessment of damage.

Swelling of control eyes
Control (untreated) eyes are used as an indication of the stability of the test system equipment. They should remain stable without significant change in corneal thickness during the 4 hour experimental observation period. If the corneal thickness of a control eye changes by more than 7% during the 4 hour observation period, then the experiment must be rejected and repeated.

Results

Interpretation
The opacity scores that are obtained with this method are defined in the same way as the Draize corneal opacity scores. The other scores, with the exception of corneal swelling, are numerical representations of qualitative descriptions. No one score is sufficient in itself to assess the effect of a test substance. Damage is assessed by means of different parameters, depending on the nature of the effects observed. For example, when severe opacity is the primary effect in the test, opacity and its time of onset are the important factors to be evaluated. On the other hand, when no opacity is observed, other factors, such as corneal swelling are used in the assessment.

References

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The *in vitro* assessment of severe eye irritants.
Fd. Cosmet. Toxicol. **19**: 471-480.

York M., Lawrence R.S., and Gibson G.B. (1982)
An *in vitro* test for the assessment of eye irritancy in consumer products - preliminary findings.
Int. J. Cosmet. Sci. **4**: 223-234.

Whittle E., Basketter D., York M., Kelly L., Hall T., McCall J., Botham P., Esdaile D., and Gardner J. (1992)
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Appendix A2

Table of IRE Protocols from the Reviewed Literature

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Table of IRE Protocols from the Reviewed Literature

Reference	Unilever Safety & Environmental Assurance Centre (SEAC)	SafePharm Laboratories, Contract Research Organization in the United Kingdom (SOT 2003/2004 posters and Appendix to Unilever protocol)	INVITTOX Protocol #85 (EC/HO Validation Study)	Chamberlain et al. (1997) -- IRAG Evaluation (1 data set)	Cooper et al. (2001)
TEST METHOD COMPONENT					
Eye selection and preparation performed at testing laboratory	Not noted	Not noted	Not noted	Note: Procedure based on Burton et al. (1981). Submitted data based on Lewis et al. (1994)	Not noted
Rabbit strain	New Zealand White	New Zealand White of either sex	New Zealand White	Not noted	Not noted
Eyes inspected on live animal and method of inspection	Suitable eyes show no opacity of the cornea and no imperfections on the corneal surface based on macroscopic and slit-lamp examination	Biomicroscopic examination of cornea using slit-lamp; assessment of corneal uptake of sodium fluorescein; measurement of corneal thickness using ultrasonic pachymeter	Cornea examined for opacity and surface imperfections with slit lamp	Not noted	Not noted
Method of killing animal	Pentobarbitone solution injected into ear vein	Pentobarbitone solution injected into ear vein	Pentobarbitone solution injected into ear vein	Not specified; "humanely sacrificed"	Not noted
Eye dissection	Some training is required in order to carry out this dissection. Care is required to avoid loss of intraocular pressure. Immediately after animal death, saline is applied to eye to prevent drying during dissection. Nictitating membrane and conjunctiva are cut away, and the eyeball is proptosed by applying pressure above and below the eyeball. Orbital muscles and the optic nerve are cut and the eyeball is lifted from the orbit. Excess tissue is dissected from the eyeball. Eyeball is rinsed with physiological saline.	Similar to INVITTOX protocol	Saline applied to eye to prevent drying during dissection. Training recommended. Nictitating membrane and conjunctiva are cut away, and the eyeball is proptosed by applying pressure above and below the eyeball. Orbital muscles and the optic nerve are cut and the eyeball is lifted from the orbit. Excess tissue is dissected from the eyeball.	Not noted	Performed on the premises of the rabbit supplier
Eyes purchased from supplier					
Supplier	Eyes are enucleated in the supplier's facility from rabbits used for other testing purposes (i.e., skin irritation tests, untreated control animals, or tissue supply for studies not involving the eye)	Not noted	Rabbits used for other testing purposes in the supplier's laboratory (i.e., skin irritation tests, untreated control animals, or tissue supply for studies not involving the eye)	Not noted	Eyes were enucleated from animals that had been used for other purposes at a nearby laboratory, then transported to the testing facility with minimum delay
Maintenance of eyes during shipment	After removal, eyes are placed in a large insulated flask. The temperature is maintained by sealing 1 L of water (37°C) in a plastic bag within the flask. Each eye is thoroughly wetted with saline and humidity maintained by free-standing water (37°C) in the bottom of the flask. Eyes are transported to the testing facility within 2 hours.	Not noted	After removal, eyes are placed in an insulated flask, that is maintained at 37°C. Saline is applied to eyes, and added to the bottom of the flask to maintain humidity. Eyes are transported to testing facility within 1 hour.	Not noted	Not noted
Pretreatment equilibration in superfusion apparatus	Eye is mounted in a vertical position in metal clamp that holds the eye firmly, but without excessive pressures. The clamp has metal rings on which the eye sits; it is positioned in a cell of the maintenance chamber. The saline drip tube of the cell is positioned so that drops of saline fall onto the upper margin of the cornea and irrigate the whole surface of the cornea. Peristaltic pump provides a flow rate of saline to each cell of 0.1 - 0.2 mL/min.	Enucleated eyes mounted in perspex clamps and placed in superfusion chamber for equilibration. Peristaltic pump supplies 0.9% saline solution at approximately 32°C	Eye is mounted in a vertical position in a clamp with stainless steel pins embedded in the upper arm and base to hold the eye in place. The pins protrude to about 1 mm, so as to avoid puncturing the globe. Each holder is placed in a cell of a maintenance chamber; saline is dripped onto the cornea at a rate of less than 1 mL/minute.	Eyes are maintained in a superfusion system which maintains them bathed with saline at a constant temperature	On arrival at the testing facility, eyes were placed clamps and mounted in a maintenance chamber; the anterior corneal surface was bathed with a saline drip
Duration	45 - 60 minutes	30 or more minutes	45 - 60 minutes	45 - 60 minutes	Short period to stabilize; otherwise not specified
Temperature	31°C (± 1°C)	32°C (± 1.5°C)	32°C (± 2°C)	About 32°C	31°C

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TEST METHOD COMPONENT					
Method of detecting damaged enucleated eyes prior to use in test	Immediately after the eye is positioned in the chamber, it is stained with 1% fluorescein sodium BP for a few seconds, after which it is rinsed with saline; if any fluorescein penetrates into the eye, the eye is rejected for use and a suitable replacement prepared	Eyes are re-examined after 30 minutes to ensure damage was not caused during dissection. Eyes are rejected if corneal thickness has increased greater than 10% relative to the *in vivo* measurement or if the cornea has stained with fluorescein sodium drops.	1% fluorescein sodium BP applied for a few seconds and rinsed with saline; if any fluorescein penetrates into the eye, the eye is rejected	Enucleated eyes are examined with a slit lamp before use in a test and any with abnormalities are rejected	Eyes were observed during the stabilization period, and any damaged eyes were discarded
First corneal thickness measurement (when performed)	Corneal thickness measured after fluorescein test with slit/pachymeter reading set at -1	*In vivo* then after equilibration.	Corneal thickness measured after fluorescein test with slit reading set at -1	Corneal thickness measured after slit lamp examination with the depth measuring attachment for the slit lamp.	Pretreatment corneal thickness measurement performed, but no details provided
Additional corneal thickness measurements prior to treatment	After equilibration, corneal thickness is measured again (slit/pachymeter reading set at 0). If slit reading 0 exceeds slit reading -1 by more than 4%, the eye is rejected from the experiment.	After equilibration, just before treatment.	After equilibration, corneal thickness is measured again (slit reading set at 0). If slit reading 0 exceeds slit reading -1 by more than 5%, the eye is rejected.	Repeated measurements (to the nearest 0.01 units) are made at the corneal apex while the eye is in the superfusion apparatus. After equilibration, corneal thickness is measured again, and any eyes that have swollen more than 4% relative to the first reading are rejected.	Not noted
Treatment of eyes					
No. of eyes used/test substance	3	3	3	2	3
No. of untreated controls	1	2	1	Not noted	1
Liquid substances	Viscous liquids should be layered onto the cornea to ensure even coverage.	-	-	-	Shampoo formulations
Amount applied	1) 20 µL of test material is applied to the upper margin of the cornea every 10 seconds up to 60 seconds (120 µL total amount applied). Usually, application of liquids to the eye is *in situ* with the eye clamped in the maintenance chamber. The saline drip tube is deflected from the eye during treatment. ***OR*** 2) 20 µL of test material is applied for 10 seconds.	0.1 mL applied evenly to the cornea	The eye in its clamp is removed from the superfusion chamber for treatment; eye is treated with cornea facing upward. 0.1 mL applied to central part of cornea (prior to application of test material, the eye, held in its clamp, is removed from the chamber and positioned with the cornea uppermost	0.1 mL applied to cornea	20 µL of test material applied to the cornea every 10 seconds up to 60 seconds (120 µL total amount applied)
Concentration tested	100%	100%	100%	100%	Formulations were tested at 100% and as 10% (w/v) solutions in distilled water
Exposure duration	10 seconds or 60 seconds	10 seconds	10 seconds	10 seconds	60 seconds
Rinsing procedure	Test material is removed from the cornea with at least 20 mL of physiological saline from a syringe. The saline drip is repositioned to irrigate the eye as before.	Test material is washed off cornea using 20 mL of saline solution warmed to approximately 32°C	Cornea rinsed with 20 mL of saline	Cornea rinsed with 20 mL or more of warmed saline	Not noted

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TEST METHOD COMPONENT					
Solid substances	The eye to be treated is removed from the maintenance chamber fixed in its clamp and positioned horizontally in a petri dish.	-	Solutions of solids may be tested in addition to finely ground or powder forms	-	None tested
Form of solid	Not noted	Not noted	Test materials are applied as a powder or fine granular form	Not noted	Not noted
Amount applied	50 mg	0.1 mL or a maximum of 100 mg sprinkled evenly over the cornea	25 mg	25 mg applied to cornea	Not noted
Concentration tested	Not noted	Not noted	Not noted	Not noted	Not noted
Exposure duration	"Specified exposure period"	10 seconds	10 seconds	10 seconds	Not noted
Method of application	Sprinkled evenly over entire surface of cornea	Sprinkled evenly over entire surface of cornea	Sprinkled evenly over entire surface of cornea	Not noted	Not noted
Rinsing prodedure	All particles are removed from the corneal surface by rinsing with at least 20 mL of physiological saline from a syringe. The clamped eye is returned to the maintenance chamber and saline drip repositioned to irrigate the eye.	Test material is washed off cornea using 20 mL of saline solution warmed to approximately 32°C	Cornea rinsed with 20 mL of saline at room temperature; the cornea is rinsed further if particles stick to surface; if particles cannot be removed completely, this is noted	Cornea rinsed with 20 mL or more of warmed saline	Not noted
Endpoints assessed					
Corneal opacity					
Timepoints after treatment	0.5, 1, 2, 3 and 4 hours after treatment	1, 2, and 4 hours after treatment	0.5, 1, 2, 3 and 4 hours after treatment	Not noted	At regular intervals (not specified) up to 4 hours
Scoring system used	Most dense area taken for reading; macroscopic and microscopic examinations conducted. 0 = No opacity or Normal; 1 = Scattered or diffuse area, details of iris clearly visible or Very slight; 2 = Easily discernible translucent area, details of iris slightly obscured or Slight; 3 = Nacreous (gray/white) area, no details of iris visible, size of pupil barely discernible or Moderate; 4 = Opaque cornea, iris not discernible through opacity or Severe	McDonald-Shadduck system used, which measures the severity of corneal cloudiness and the area of the cornea involved. CORNEAL CLOUDINESS: 0 = Normal cornea; 1 = Some loss of transparency; 2 = Moderate loss of transparency; 3 = Involvement of the entire thickness of the stroma (endothelial surface still visible); 4 = Involvement of the entire thickness of the stroma (endothelial surface not visible). AREA: 0 = normal cornea with no area of cloudiness; 1 = 1 - 25% of stromal cloudiness; 2 = 26 - 50% area of stromal cloudiness; 3 = 51 - 75% area of stromal cloudiness; 4 = 76 - 100% area of stromal cloudiness	Draize system for scoring corneal opacity; 0 = no opacity, 1 = scattered or diffuse, 2 = discernible transluscent area, 3 = nacreous area, and 4 = opaque cornea	Not noted	Draize system for scoring corneal opacity; 0 = no opacity, 1 = scattered or diffuse, 2 = discernible transluscent area, 3 = nacreous area, and 4 = opaque cornea
Instrumentation	Slit lamp biomicroscope is used to examine cornea for degree of opacity	Slit lamp biomicroscope is used to examine cornea for degree of opacity	Slit lamp biomicroscope is used to examine cornea for degree of opacity	Not noted	Not noted
Corneal thickness					
Timepoints after treatment	0.5, 1, 2, 3 and 4 hours after treatment	1, 2, and 4 hours after treatment	0.5, 1, 2, 3 and 4 hours after treatment	Not specifed in report; intervals up to 5 hours after application of test substance	At regular intervals (not specified) up to 4 hours
Instrumentation	Slit lamp biomicroscope fitted with a depth-measuring device, or an ultrasonic pachymeter	Ultrasonic pachymeter (DGH Technology Incorporated, Solana Beach, California)	Slit lamp biomicroscope fitted with a depth-measuring device, or an ultrasonic pachymeter	Slit lamp biomicroscope fitted with a depth-measuring device	Ultrasonic pachometer (Teknar Ophthsonic pachometer, Mentor O&O Inc., MA, USA)

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TEST METHOD COMPONENT					
Method of evaluating degree of swelling as a result of treatment	Value obtained for each eye is recorded; degree of corneal swelling caused by treatment is calculated as a percentage of the corneal thickness of the eye just prior to treatment (slit reading 0)	Not described	Value obtained for each eye is recorded; degree of corneal swelling caused by treatment is calculated as a percentage of the corneal thickness of the eye just prior to treatment (slit reading 0)	Corneal thickness is measured and expressed as percentage of corneal swelling relative to pretreatment corneal thickness value (a continuous variable)	Corneal thickness is measured and expressed as percentage corneal swelling throughout the 4 hour observation time using the pretreatment thickness value
Fluorescein penetration/staining					
Timepoints after treatment	60 minutes	4 hours after treatment (assessment of corneal uptake of sodium fluorescein)	0.5 and 4 hours after treatment (Not conducted when grade 3 or 4 corneal opacities are present)	Not noted	Performed, but few details provided
Method of application	1 drop of fluorescein solution is applied to the cornea for 10 seconds, then is rinsed off with saline	Not described	1 drop of fluorescein solution is applied to the cornea for 10 seconds, then is rinsed off with saline	Not noted	Not noted; the extent to which fluorescein penetrated the cornea was assessed visually by using a Zeiss slit lamp
Scoring system used	N = negligible (occasional punctate staining with no diffusion of stain into the stroma); M = marginal (punctuate staining across cornea with some evidence of slight diffusion into cornea); D = distinct (pale continuous staining of the epithelium with slow diffusion into the stroma); L = bright area of stain to extreme outer edge of cornea, with no penetration into cornea; S = intense staining of the epithelium and anterior stroma with very rapid diffusion into the remainder of the stroma; E = intense staining of very badly damaged cornea, which appears yellow/orange as opposed to bright green of previous grades; O = other effect	0 = Absence of fluorescein staining. 1 = Slight fluorescein staining confined to a small focus. 2 = Moderate fluorescein staining confined to a small focus. 3 = Marked fluorescein staining that may involve a larger portion of the cornea. 4 = Extreme fluorescein staining. (More detail provided in Appendix to Unilever protocol)	Staining and diffusion characteristics are assessed as follows: 0 = no staining, 1 = bright green staining of anterior cornea edge but no penetration, 2 = bright green anterior edge to cornea and gradual diffusion of stain through cornea	Not noted	Fluorescein penetration is expressed using a graded scoring system (not specified)
Macroscopic examination of cornea	Not noted	Not noted	Not noted	Any changes in the normal appearance of the cornea are carefully noted	Not noted
Timepoints after treatment	Not noted	Not noted	0.5, 1, 2, 3 and 4 hours after treatment	Not noted	Not noted
Instrumentation	Not noted	Not noted	Slit lamp	Not noted	Not noted
Histology performed?	After the final assessments and measurements have been taken (240 minutes), each eye is removed from its chamber cell, and the cornea is dissected, fixed, processed, and embedded in paraffin wax for sectioning. Sections are cut and stained. Corneal evaluation is divided into 2 distinct areas: epithelial and stromal response.	Not noted	Histological examination of corneal epithelium is noted as a supplementary observation that may be performed	Not noted	After 4 hour observation period, the corneas were excised and fixed for histological assessment of epithelial and stromal responses; the number of epithelial cell layers that had eroded and evidence of other histopathological changes were recorded

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Reference	Unilever Safety & Environmental Assurance Centre (SEAC)	SafePharm Laboratories, Contract Research Organization in the United Kingdom (SOT 2003/2004 posters and Appendix to Unilever protocol)	INVITTOX Protocol #85 (EC/HO Validation Study)	Chamberlain et al. (1997) -- IRAG Evaluation (1 data set)	Cooper et al. (2001)
TEST METHOD COMPONENT					
Other observations	Slit-lamp examination of the cornea at 0.5, 1, 2, 3, 4 hours after treatment. Using the slit-lamp set with a narrow slit, the treated corneas are examined for evidence of damage based on reflection of light from different parts of the slit image. The effects are scored as follows: N = normal; BG = more reflection than control eye, most intense at anterior margin decreasing gradually towards the posterior margin; BD = distinct bright line on anterior margin and little reflection from remainder of cornea; BT = intense reflection throughout cornea reflecting presence of significant primary opacity. Increased reflection of light suggests some form of corneal damage has occurred.	Corneal epithelium observations	Slit-lamp examination of the cornea at 0.5, 1, 2, 3, 4 hours after treatment. Using the slit-lamp set with a narrow slit, the treated corneas are examined for evidence of damage based on reflection of light from different parts of the slit image. The effects are scored as follows: 0 = slit image identical to control eye; 1 = light reflection from one or more regions of the slit image. Increased reflection of light suggests some form of corneal damage has occurred. Photography of the eye may be useful for comparing responses	-	-
Criteria for an acceptable test	There are no criteria set for the control eyes post treatment; the eyes are checked pretreatment and this has been found to be sufficient to weed out any damaged eyes. If, however, there is an unusual degree of change in the control whether by swelling, macro, or even micro observation, the test would be repeated, with consideration made on a case-by-case basis.	Not described	Control eyes should remain stable without > 7% change in corneal thickness during the 4 hour observation period	Not noted	Not noted
Irritancy classification	Normal = no effects; Very slight = No significant effects on any category (<11% swelling and/or 1-2 cell layers lost); Slight = Any unusual effect, slight opacity (>11% swelling and/or 3-4 cell layers lost); Moderate = Slight/moderate opacity and/or >25% swelling and/or 5-6 cell layers lost; Severe = Moderate/severe opacity and/or >35% swelling and/or 7-8 cell layers lost.	Any parameter that meets or exceeds the following cut-off values indicates a severe eye irritant. *Cut-off Values to Detect Severe Eye Irritants*: Maximum corneal opacity (corneal cloudiness x area) ≥ 4; Maximum fluorescein uptake (intensity x area) ≥ 4; Mean corneal swelling (60, 120, 240 minutes) ≥ 25%; Corneal epithelium observations = any with pitting, mottling or sloughing	Damage is assessed by means of different parameters, depending on the effects observed.	Any chemical causing >15% corneal swelling at any time after treatment is considered to have the potential to cause severe ocular irritation *in vivo*	The classification is generally based on the weight of evidence from the opacity score, the % corneal swelling, and the number of epithelial cell layers eroded, with any one endpoint triggering the higher classification. Very slight irritant (opacity = 0, or corneal swelling < 11%, or 0-2 epithelial cell layers lost); Slight (opacity = 1-2, or corneal swelling = 12-25%, or 3-4 epithelial cell layers lost); Moderate (opacity = 2-3, or corneal swelling = 26-35% or 5-6 epithelial cell layers lost); Severe (opacity = 3-4, or corneal swelling = >35% or 7-8 epithelial cell layers lost)
Conducted in compliance with GLPs	Not noted	Not noted	Not noted	Not noted	Not noted
Other Notes	-	-	-	-	-

Table of IRE Protocols from the Reviewed Literature

Reference	Gettings et al. (1996)
TEST METHOD COMPONENT	
Eye selection and preparation performed at testing laboratory	Not noted
Rabbit strain	New Zealand White
Eyes inspected on live animal and method of inspection	Not noted
Method of killing animal	Not noted
Eye dissection	Performed on the premises of the rabbit supplier
Eyes purchased from supplier	
Supplier	A supplier was used, but specific supplier not noted
Maintenance of eyes during shipment	Eyes were transported to the laboratory under humid conditions at 31°C
Pretreatment equilibration in superfusion apparatus	On receipt at testing facility, each eye was mounted in a vertical position in a perspec clamp. The clamp was positioned in a cell of a maintenance chamber at 31°C and the corneal surface bathed with a saline drip.
Duration	Approximately 30 minutes
Temperature	31°C

Table of IRE Protocols from the Reviewed Literature

Reference	Gettings et al. (1996)
TEST METHOD COMPONENT	
Method of detecting damaged enucleated eyes prior to use in test	Eyes were stained with 2% fluorescein, examined using a slit lamp, and those retaining fluorescein were discarded
First corneal thickness measurement (when performed)	Corneal thickness measured after slit lamp examination with the depth measuring attachment for the slit lamp (slit reading -1)
Additional corneal thickness measurements prior to treatment	After equilibration, corneal thickness is measured again (slit reading set at 0). If slit reading 0 exceeds slit reading -1 by more than 4%, the eye was rejected.
Treatment of eyes	
No. of eyes used/test substance	3
No. of untreated controls	1
Liquid substances	Surfactant-based formulations
Amount applied	20 µL of test material was applied to the upper margin of the cornea every 10 seconds up to 60 seconds (120 µL total amount applied)
Concentration tested	100%
Exposure duration	60 seconds
Rinsing procedure	Test material was removed by rinsing with 20 mL saline

Table of IRE Protocols from the Reviewed Literature

Reference	Gettings et al. (1996)
TEST METHOD COMPONENT	
Solid substances	-
Form of solid	Not noted
Amount applied	Not noted
Concentration tested	Not noted
Exposure duration	Not noted
Method of application	Not noted
Rinsing prodedure	Not noted
Endpoints assessed	
Corneal opacity	
Timepoints after treatment	Immediately after treatment and at 0.5, 1, 2, 3, and 4 hours after treatment
Scoring system used	Macroscopic examination; Scoring system not described
Instrumentation	Not noted
Corneal thickness	
Timepoints after treatment	At 0.5, 1, 2, 3, and 4 hours after treatment
Instrumentation	Corneal thickness measured with the depth measuring attachment for the slit lamp

Table of IRE Protocols from the Reviewed Literature

Reference	Gettings et al. (1996)
TEST METHOD COMPONENT	
Method of evaluating degree of swelling as a result of treatment	Post-treatment corneal thickness values were compared with the pretreatment value and expressed as the percentage increase in thickness
Fluorescein penetration/staining	
Timepoints after treatment	1 hour after treatment
Method of application	Fluorescein solution is applied and initial staining of cornea and diffusion into corneal stroma assessed by slit lamp
Scoring system used	Not noted
Macroscopic examination of cornea	Slit lamp examination using both open and narrowed slit settings to assess any damage to the corneal epithelium
Timepoints after treatment	Immediately after treatment and 0.5, 1, 2, 3, 4 hours after treatment
Instrumentation	Slit lamp
Histology performed?	Performed but not described

Table of IRE Protocols from the Reviewed Literature

Reference	Gettings et al. (1996)
TEST METHOD COMPONENT	
Other observations	-
Criteria for an acceptable test	Not noted
Irritancy classification	Report states that "test materials were classified into four groups ranging from no significant effects to maximal response." However, no other information was provided.
Conducted in compliance with GLPs	Not noted
Other Notes	-

Table of IRE Protocols from the Reviewed Literature

Reference	Jones et al. (2001)	Koeter and Prinsen (1985)	Lewis et al. (1994a)	Price and Andrews (1985)	Whittle et al. (1992) - method A
TEST METHOD COMPONENT					
Eye selection and preparation performed at testing laboratory	Not noted	Rabbits that had been used in primary skin irritation or eye irritation studies were used as eye donors	Not noted	Not noted	Interlaboratory study of 3 laboratories, but not all labs used same methods
Rabbit strain	Not noted	New Zealand White	New Zealand White albino	Not noted	New Zealand White
Eyes inspected on live animal and method of inspection	Not noted	Only animals that were in good health and free of any eye defects were used	Eyes were examined *in vivo* for suitability before testing	Rabbits with microscopically normal eyes were selected and corneal thickness was measured using a Zeiss photoslit-lamp microscope, specially modified to take photographs through the pachometer	Corneal thickness of eyes was measured *in vivo*
Method of killing animal	Not noted	Not noted	Animals were humanely killed; no other information provided	An iv overdose of sodium pentobarbitone	Lethal dose of pentobarbitone sodium was administered via the marginal ear vein
Eye dissection	Performed on the premises of the rabbit supplier	Not noted	Immediately after death, a few drops of saline (0.85%) were applied to the eyes to prevent them from drying during dissection. The eyes were dissected carefully, the eyeball was proptosed, the adjacent conjuntival tissue, orbital muscles and the optic nerve were cut, and the eyeball was lifted from the socket.	Dissected as described in Burton et al. (1981)	Immediately after death, each eye was dissected carefully but rapidly, avoiding contact with or drying of the corneal surface
Eyes purchased from supplier					
Supplier	Eyes were enucleated from animals that had been used for other purposes at a nearby laboratory, then transported to the testing facility with minimum delay	Not noted	Not noted	Not noted	Not noted
Maintenance of eyes during shipment	Not noted	Not noted	Not noted	Not noted	Not noted
Pretreatment equilibration in superfusion apparatus	On arrival at the testing facility, eyes were placed in clamps and mounted in a maintenance chamber; the anterior corneal surface was bathed with a saline drip	Not noted	Each eyeball was mounted in a vertical position in a perspex clamp held within a chamber that was fitted with a pump that delivered saline (about 32°C) at regular intervals to the surface of the cornea	The apparatus used to maintain eyes was similar to that described in Burton et al. (1981). Enucleated eyes were lightly supported by clamps within temperature-regulated chambers and warm saline was dripped continuously over their surfaces.	The eye was mounted in a perspex clamp within a temperature-controlled superfusion chamber, such that the cornea was in a vertical position facing the observer. Each compartment of the chamber was equipped such that isotonic saline solution dripped onto the cornea and flowed down over the cornea surface
Duration	Short period to stabilize; otherwise not specified	Not noted	45 - 60 minutes	Approximately 30 minutes	30 - 45 minutes
Temperature	31°C	Not noted	About 32°C	Not noted	32 ± 1.5°C

Table of IRE Protocols from the Reviewed Literature

Reference	Jones et al. (2001)	Koeter and Prinsen (1985)	Lewis et al. (1994a)	Price and Andrews (1985)	Whittle et al. (1992) - method A
TEST METHOD COMPONENT					
Method of detecting damaged enucleated eyes prior to use in test	Eyes were observed during the stabilization period, and any damaged eyes were discarded	All eyes were examined with a slit-lamp microscope just before treatment	A pretreatment measurement of corneal thickness was taken using a slit lamp and pachymeter (Carl Zeiss, 30 SL)	Eyes were examined and only those within an *in vitro* corneal thickness measurement within 2 machine units of the *in vivo* reading were used.	After equilibratioin, two drops of 1% (w/v) fluorescein solution were applied to the eye and washed off with saline after a few seconds. Corneal thickness was measured. Eyes were rejected if they either retained fluorescein stain or had a corneal thicknesss 4% or greater than *in vivo* reading.
First corneal thickness measurement (when performed)	Pretreatment corneal thickness measurement performed, but no details provided	Pretreatment corneal thickness measurement performed, but no details provided	Just before equilibration period	*In vivo*. First performed on enucleated eye just after equilibration period.	*In vivo*. First performed on enucleated eye just after equilibration period.
Additional corneal thickness measurements prior to treatment	Not noted	Not noted	Just after equilibration period. The percentage corneal swelling was calculated and any eyes that had swollen more than 4% relative to the first reading were rejected.	Not noted	Not noted
Treatment of eyes					
No. of eyes used/test substance	3	4	2	6 or more	3 eyes
No. of untreated controls	1	2	Not noted	Used, but a specific number not noted	1 eye
Liquid substances	Shampoo and conditioner formulations	-	-	-	-
Amount applied	20 µL of test material applied to the cornea every 10 seconds up to 60 seconds (120 µL total amount applied)	100 µL	100 µL applied directly to the cornea	100 µL of test substance was dripped onto the surface of the eye	100 µL applied to the eye using a 1 mL syringe
Concentration tested	All formulations were tested at 100% and the shampoos were also tested as 10% (w/v) solutions in distilled water	Not noted	100%	100%	100%
Exposure duration	60 seconds	5 - 10 seconds	10 seconds	Approximately 10 seconds	10 seconds
Rinsing procedure	Not noted	The corneal surface was rinsed thoroughly with approximately 20 mL of isotonic saline	Test chemical was removed by rinsing the surface of the cornea with at least 20 mL warmed saline	Excess test substance was washed off using warm saline (usually 5 drops from an eye dropper, but sometimes a greater volume and/or force was used, if necessary)	Test substance was washed off using saline at about 32°C

Table of IRE Protocols from the Reviewed Literature

Reference	Jones et al. (2001)	Koeter and Prinsen (1985)	Lewis et al. (1994a)	Price and Andrews (1985)	Whittle et al. (1992) - method A
TEST METHOD COMPONENT					
Solid substances	None tested	-	-	None tested	-
Form of solid	Not noted	Not noted	Not noted	Not noted	Not noted
Amount applied	Not noted	100 mg	25 mg applied directly to the cornea	Not noted	25 mg applied directly to the cornea
Concentration tested	Not noted	Not noted	100%	Not noted	100%
Exposure duration	Not noted	5 - 10 seconds	10 seconds	Not noted	10 seconds
Method of application	Not noted	Solids were dusted onto the eyes	Not noted	Not noted	For solids, the eye was removed from the superfusion chamber, and placed so that the cornea faced upwards
Rinsing prodedure	Not noted	The corneal surface was rinsed thoroughly with approximately 20 mL of isotonic saline	Test chemical was removed by rinsing the surface of the cornea with at least 20 mL warmed saline	Not noted	While the eye was still outside the superfusion apparatus, the solid test substance was washed off with saline; then the eye was returned to its chamber
Endpoints assessed					
Corneal opacity					
Timepoints after treatment	At regular intervals (not specified) up to 4 hours	30, 75, 120, 180, 240 minutes	Before dosing and at 0.5, 1, 2, 3, 4, 5 hours after dosing	Not evaluated	Immediately after treatment and at 30, 60, 120, 180, 240 and 300 minutes
Scoring system used	Draize system for scoring corneal opacity; 0 = no opacity, 1 = scattered or diffuse, 2 = discernible transluscent area, 3 = nacreous area, and 4 = opaque cornea	0 = no effect or negligible effect, 1 = slight degree of corneal opacity, 2 = moderate degree of corneal opacity, 3 = marked degree of corneal opacity (the final score = the sum of scores for each of the 4 eyes and was interpreted as follows: 1-5 = slight effects, 6-9 = moderate effect, 10-12 = severe effect)	The cornea of each eye was assessed by macroscopic examination for evidence of opacification of the cornea; no additional information was provided	Not noted	Area most dense used for scoring. No opacity = 0; scattered or diffuse areas, details of iris visible = 1; easily discernible translucent area, iris slightly obscured = 2; severe corneal opacity, iris not visible, pupil barely discernible = 3; complete corneal opacity, iris invisible = 4.
Instrumentation	Not noted	Not noted	Not noted	Not noted	Not noted
Corneal thickness					
Timepoints after treatment	At regular intervals (not specified) up to 4 hours	30, 75, 120, 180, 240 minutes	Before dosing and at 0.5, 1, 2, 3, 4, 5 hours after dosing	1, 2, 3, 4, 5 hours	Immediately after treatment and at 30, 60, 120, 180, 240 and 300 minutes
Instrumentation	Ultrasonic pachometer (Teknar Ophthsonic pachometer, Mentor O&O Inc., MA, USA)	Depth-measuring device mounted on a slit-lamp microscope	Not noted	Zeiss photoslit-lamp microscope, equipped with a pachometer, specially modified to take photographs through the pachometer	Not noted

Table of IRE Protocols from the Reviewed Literature

Reference	Jones et al. (2001)	Koeter and Prinsen (1985)	Lewis et al. (1994a)	Price and Andrews (1985)	Whittle et al. (1992) - method A
TEST METHOD COMPONENT					
Method of evaluating degree of swelling as a result of treatment	Corneal thickness is measured and expressed as percentage corneal swelling throughout the 4 hour observation time using the pretreatment thickness value	Corneal thickness is measured and expressed as percentage corneal swelling throughout the 4 hour observation time using the pretreatment thickness value; the interpretation of the observed swelling was based on the mean maximum swelling for all 4 eyes and also on the time of occurrence	The mean percentage corneal swelling relative to the pretreated (control) value was calculated for each treated pair of eyes	Not noted	Not noted
Fluorescein penetration/staining					
Timepoints after treatment	Performed, but few details provided	Before treatment and 30 minutes after treatment	4 hours	If used, fluorescein was applied 4 hours after dosing	240 minutes posttreatment
Method of application	Not noted; the extent to which fluorescein penetrated the cornea was assessed visually by using a Zeiss slit lamp	2% fluorescein sodium solution was applied to the surface of the cornea for a few seconds followed by rinsing with isotonic saline	Not noted	Not noted	Not noted
Scoring system used	Fluorescein penetration is expressed using a graded scoring system (not specified)	0 = none or a few cells permeable, 1 = small number of cells permeable, 2 = individual cells and areas of the cornea permeable, 3 = entire cornea permeable (the final score = the sum of scores for each of the 4 eyes and was interpreted as follows: 1-5 = slight effects, 6-9 = moderate effect, 10-12 = severe effect)	Not noted	The rate and degree of penetration of the stroma were assessed	No fluorescein retention = 0; small number of cells retaining fluorescein = 1; individual cells and areas of the cornea retaining fluorescein = 2; large areas of the cornea retaining fluorescein =3
Macroscopic examination of cornea	Not noted	Pitting of corneal epithelial cells, loosening of epithelium, roughening of the corneal surface, and sticking of the test substance to the cornea; the final score for these effects was subjective and represented the mean value of all 4 eyes	Not noted	Any qualitative changes in the appearance of the cornea were noted and/or photographed	During exposure, eyes were examined for any macroscopic signs of damage
Timepoints after treatment	Not noted	Not noted	Not noted	Not noted	Not noted
Instrumentation	Not noted	Not noted	Not noted	Not noted	Not noted
Histology performed?	After 4 hour observation period, the corneas were excised and fixed for histological assessment of epithelial and stromal responses; the number of epithelial cell layers that had eroded and evidence of other histopathological changes were recorded	Not noted	Not noted	Not noted	After 300 minutes posttreatment, lab A and lab B removed the corneas from the eyes, fixed the corneas in Bouins fixative, mounted them in wax blocks, and sectioned using standard histological techniques. The number of cell layers eroded from the corneal epithelium was noted.

Table of IRE Protocols from the Reviewed Literature

Reference	Jones et al. (2001)	Koeter and Prinsen (1985)	Lewis et al. (1994a)	Price and Andrews (1985)	Whittle et al. (1992) - method A
TEST METHOD COMPONENT					
Other observations	-	-	-	-	-
Criteria for an acceptable test	Not noted	Not noted	Not noted	Not noted	Not noted
Irritancy classification	The classification is generally based on the weight of evidence from the opacity score, the % corneal swelling, and the number of epithelial cell layers eroded, with any one endpoint triggering the higher classification. Very slight irritant (opacity = 0, or corneal swelling < 11%, or 0-2 epithelial cell layers lost); Slight (opacity = 1-2, or corneal swelling = 12-25%, or 3-4 epithelial cell layers lost); Moderate (opacity = 2-3, or corneal swelling = 26-35% or 5-6 epithelial cell layers lost); Severe (opacity = 3-4, or corneal swelling = >35% or 7-8 epithelial cell layers lost)	The final *in vitro* irritancy grade was assessed by averaging the final scores of permeability, corneal opacity, corneal swelling, and the macroscopic effects	Any chemical causing more than 15% corneal swelling at any time after treatment was considered to have the potential to cause severe ocular irritancy *in vivo*	Grade I = <20% increase in corneal thickness in 5 hours, Grade II = ≥20% increase in corneal thickness in 5 hours, Grade III = ≥20% increase in corneal thickness in 2 hours, Grade IV = ≥20% increase in corneal thickness in 1 hour. The grade is increased by 1 if eyes stain with fluorescein. The grade for a test substance is the overall mean for 6 eyes.	LAB A: No significant effects (<11% swelling, 0-2 epithelial cell layers eroded) = 1; effects but no opacity (>11% corneal swelling and/or 3-4 epithelial cell layers eroded) = 2; slight-moderate opacity and/or >25% corneal swelling and/or 5-6 epithelial cell layers eroded = 3; immediate opacity or moderate-severe opacity that develops over time and/or >35% swelling and/or 7-8 epithelial cell layers = 4. LAB B: Grading was based on a subjective judgement of the measured parameters, each of which influenced the grading to a greater or lesser extent, such that the significance of the % corneal swelling > epithelial cell erosion ≥ corneal opacity > fluorescein retention. LAB C: <20% corneal swelling within 5 hours = 1; ≥20% corneal swelling by 5 hours = 2; ≥20% corneal swelling within 2 hours = 3; ≥20% coreal swelling within 1 hour or if corneal opnacity was visible to the naked eye = 4
Conducted in compliance with GLPs	Not noted	Not noted	Not noted	Not noted	Not noted
Other Notes	-	-	-	-	Each laboratory adopted an approach to the assessment of results based on previous experience with the technique in their laboratory.

Table of IRE Protocols from the Reviewed Literature

Reference	Whittle et al. (1992) - method B	York et al. (1994)	CEC (2001)
TEST METHOD COMPONENT			
Eye selection and preparation performed at testing laboratory	Interlaboratory study of 3 laboratories, but not all labs used same methods	Not noted	Interlaboratory study of 3 laboratories, but not all labs used same methods
Rabbit strain	New Zealand White	Not noted	New Zealand White
Eyes inspected on live animal and method of inspection	Corneal thickness of eyes was measured *in vivo*	Not noted	Corneal thickness measured in vivo in all laboratories
Method of killing animal	Lethal dose of pentobarbitone sodium was administered via the marginal ear vein	Not noted	Lethal dose of Euthesate or sodium pentobarbitol via the marginal ear vein
Eye dissection	Immediately after death, each eye was dissected carefully but rapidly, avoiding contact with or drying of the corneal surface	Not noted	Immediately after death, each eye was dissected in approximately two minutes with extreme care to avoid touching the corneal surface. Left sufficient length of optic nerve to prevent rupture and loss of intra-ocular pressure
Eyes purchased from supplier			
Supplier	Not noted	Eyes were purchased from another establishment where rabbits had been used for other purposes that would not adversely affect the eyes.	For I.H.S. Proefstations voor Veeteelt (Merelbeke, Belgium)
Maintenance of eyes during shipment	Not noted	Eyes were dissected immediately after animal's death, and transported quickly to testing facility under warm, moist conditions.	Not noted
Pretreatment equilibration in superfusion apparatus	The eye was mounted in a perspex clamp within a temperature-controlled superfusion chamber, such that the cornea was in a vertical position facing the observer. Each compartment of the chamber was equipped such that isotonic saline solution dripped onto the cornea and flowed down over the cornea surface	After each eye had been mounted in the perfusion chambers, the procedures were consistent with Burton et al. (1981)	45-60 Minutes at 32 C
Duration	30 - 45 minutes	Not noted	45-60 minutes
Temperature	32 ± 1.5°C	Not noted	32 ± 1.5°C

Table of IRE Protocols from the Reviewed Literature

Reference	Whittle et al. (1992) - method B	York et al. (1994)	CEC (2001)
TEST METHOD COMPONENT			
Method of detecting damaged enucleated eyes prior to use in test	After equilibratioin, two drops of 1% (w/v) fluorescein solution were applied to the eye and washed off with saline after a few seconds. Corneal thickness was measured. Eyes were rejected if they either retained fluorescein stain or had a corneal thicknesss 4% or greater than *in vivo* reading.	Not noted	Fluorescein sodium 2% (w/v) applied to corneal surface for a few seconds and then rinsed off with 5-10 mL of isotonic saline at 32 ° C
First corneal thickness measurement (when performed)	*In vivo*. First performed on enucleated eye just after equilibration period.	Not noted	Not noted
Additional corneal thickness measurements prior to treatment	Not noted	Not noted	After fluorescein staining for damage assessment, then post-equilibration, then at 30, 75, 120, 180a nd 240 minutes after test substance application (Shell used 60 instead of 30 and 75 minutes)
Treatment of eyes			
No. of eyes used/test substance	3 eyes	1 Eye for 10 sec. treatment + 1 eye for 60 sec. Treatment	3 Eyes for each test substance
No. of untreated controls	1 eye	1 Eye	1 Eye
Liquid substances	-	Not tested	-
Amount applied	20 µL of test material applied to the cornea every 10 seconds up to 60 seconds (120 µL total amount applied over 6 applications)	Not noted	100 µL was applied to the cornea for 10 seconds; then rinsed with 20 mL of isotonic saline
Concentration tested	100%	Not noted	100% unless otherwise specified
Exposure duration	60 seconds	Not noted	10 seconds
Rinsing procedure	Not noted	Not noted	20 mL isotonic saline

Table of IRE Protocols from the Reviewed Literature

Reference	Whittle et al. (1992) - method B	York et al. (1994)	CEC (2001)
TEST METHOD COMPONENT			
Solid substances	-	Eyes were removed from the temperature-controlled chambers and arranged so that the cornea faced upwards.	-
Form of solid	Not noted	Not noted	Not noted
Amount applied	25 mg applied directly to the cornea	50 mg	100 mg
Concentration tested	100%		100% unless otherwise specified
Exposure duration	60 seconds	10 seconds and 60 seconds	10 seconds
Method of application	For solids, the eye was removed from the superfusion chamber, and placed so that the cornea faced upwards	Sprinkled over the cornea.	Sprinkled to cover the entire cornea
Rinsing prodedure	While the eye was still outside the superfusion apparatus, the solid test substance was washed off with saline; then the eye was returned to its chamber	The test material was rinsed from each eye using an excess (usually 20 mL) of warm isotonic saline then returned to its chamber, and the saline perfusion restarted	The test material was rinsed from each eye using 20 mL of warm isotonic saline then returned to its chamber, and the saline perfusion restarted
Endpoints assessed			
Corneal opacity			
Timepoints after treatment	Immediately after treatment and at 30, 60, 120, 180, 240 and 300 minutes	Immediately after treatment and at 4 hours	Immediately after treatment and at 60 , 120, 180, and 240 minutes; except Shell used 60, 120, 240 and 300 minutes and I.H.E used 60, 120, 180 and 240 minutes
Scoring system used	Area most dense used for scoring. No opacity = 0; scattered or diffuse areas, details of iris visible = 1; easily discernible translucent area, iris slightly obscured = 2; severe corneal opacity, iris not visible, pupil barely discernible = 3; complete corneal opacity, iris invisible = 4.	Opacification scored immediately after treatment and maximum corneal opacity. Based on Draize et al. (1944) for corneal assessment of corneal opacity *in vivo*	Area most dense used for scoring. No opacity = 0; scattered or diffuse areas, details of iris visible = 1; easily discernible translucent area, iris slightly obscured = 2; severe corneal opacity, iris not visible, pupil barely discernible = 3; complete corneal opacity, iris invisible = 4.
Instrumentation	Not noted	Not noted	Not noted
Corneal thickness		Maximum corneal swelling	Maximum corneal swelling
Timepoints after treatment	Immediately after treatment and at 30, 60, 120, 180, 240 and 300 minutes	4 hours after treatment	30 ,75, 120, 180 and 240 minutes after treatment of eyes; except Shell used 60, 120, 180, 240 minutes
Instrumentation	Not noted	Slit lamp with a pachometer attachment	Slit lamp by TNO-CIVO and Shell; ultrasonic pachometer at I.H.S.

Table of IRE Protocols from the Reviewed Literature

Reference	Whittle et al. (1992) - method B	York et al. (1994)	CEC (2001)
TEST METHOD COMPONENT			
Method of evaluating degree of swelling as a result of treatment	Not noted	Not noted	Percent increase in thickness at each time point relative to Tzero was calculated
Fluorescein penetration/staining			
Timepoints after treatment	240 minutes posttreatment	Performed, but few details provided	30, 240 minutes
Method of application	Not noted	Not noted	Drops of 2% (w/v) fluorescein sodium applied to cornea for a few seconds, then rinsed off with 5-10 mL of isotonic saline at 32ºC
Scoring system used	No fluorescein retention = 0; small number of cells retaining fluorescein = 1; individual cells and areas of the cornea retaining fluorescein = 2; large areas of the cornea retaining fluorescein =3	Assessment was qualitative	No fluorescein retention = 0; small number of cells retaining fluorescein = 1; individual cells and areas of the cornea retaining fluorescein = 2; large areas of the cornea retaining fluorescein =3
Macroscopic examination of cornea	During exposure, eyes were examined for any macroscopic signs of damage	Not noted	During exposure, eyes were examined for any macroscopic signs of damage
Timepoints after treatment	Not noted	Not noted	Not noted
Instrumentation	Not noted	Not noted	Not noted
Histology performed?	After 300 minutes posttreatment, lab A and lab B removed the corneas from the eyes, fixed the corneas in Bouins fixative, mounted them in wax blocks, and sectioned using standard histological techniques. The number of cell layers eroded from the corneal epithelium was noted.	Histological evaluation of loss of corneal epithelial cells was performed.	No

Table of IRE Protocols from the Reviewed Literature

Reference	Whittle et al. (1992) - method B	York et al. (1994)	CEC (2001)
TEST METHOD COMPONENT			
Other observations	-	-	-
Criteria for an acceptable test	Not noted	Not noted	Not noted
Irritancy classification	LAB A: No significant effects (<11% swelling, 0-2 epithelial cell layers eroded) = 1; effects but no opacity (>11% corneal swelling and/or 3-4 epithelial cell layers eroded) = 2; slight-moderate opacity and/or >25% corneal swelling and/or 5-6 epithelial cell layers eroded = 3; immediate opacity or moderate-severe opacity that develops over time and/or >35% swelling and/or 7-8 epithelial cell layers = 4. LAB B: Grading was based on a subjective judgement of the measured parameters, each of which influenced the grading to a greater or lesser extent, such that the significance of the % corneal swelling > epithelial cell erosion ≥ corneal opacity > fluorescein retention. LAB C: <20% corneal swelling within 5 hours = 1; ≥20% corneal swelling by 5 hours = 2; ≥20% corneal swelling within 2 hours = 3; ≥20% coreal swelling within 1 hour or if corneal opnacity was visible to the naked eye = 4	Emphasis was placed on the development of corneal opacity that was visible immediately after the test material was rinsed from the treated eye.	Although independent standard methods were used in each laboratory to perform the calculations, an overall *in vitro* irritancy grade was assigned as follows: A = Not irritant; B = Slightly irritating; C = Moderately irritating; D = Severely irritating.
Conducted in compliance with GLPs	Not noted	Not noted	Not noted
Other Notes	Each laboratory adopted an approach to the assessment of results based on previous experience with the technique in their laboratory.	-	Each laboratory adopted an approach to the assessment of results based on previous experience with the technique in their laboratory.