

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 ($\pm 2^\circ\text{C}$). 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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