

THE BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY - METHOD OF GAUTHERON

The effects of a test compound on the opacity and permeability of a freshly collected bovine cornea can be used as a measure of eye irritancy potential.

Contact

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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

This technique has the advantage over the Draize test that both of the end-points used are objective and quantitative, in contrast to the subjective scoring used in the *in vivo* test. In contrast to cell-culture-based systems, the three-dimensional structure of the tissue is preserved, thus giving a closer approximation of the *in vivo* situation. The sacrifice of animals is not required, as slaughterhouse material is used. It does not require any special conditions or facilities for cell culture and is therefore inexpensive and relatively simple to implement.

The protocol includes a method to be used for the testing of solids, which may present some difficulties. Interference with opacity measurements may be caused by highly coloured test compounds which can stain the cornea.

General

This assay was developed in the Merck Sharp & Dohme Research Laboratories to assess the ocular irritancy potential of process intermediates. The test utilizes bovine corneas from eyes freshly collected in a local abattoir, and measures two end-points, namely opacity and permeability. The objective values obtained from both parameters are combined, and the *in vitro* irritancy scores are compared to a previously established scale of ocular irritancy. For in-house products, irritancy is classified into three broad categories: mild, moderate and severe.

Equipment

1. opacitometer, e.g. Electro-Design, RIOM, France
2. corneal holders (15) in polypropylene
3. dissection equipment (scissors, forceps, scalpels)
4. plastic containers for collection of eyes
5. electric screwdriver
6. vacuum pump
7. water-bath
8. spectrophotometer
9. mortar and pestle
10. common tissue culture and laboratory equipment

Materials

1. Hank's balanced salt solution with Ca^{++} , Mg^{++} , (HBSS, Sigma H-1387), supplemented with 0.350 g/l sodium bicarbonate according to the supplier's recommendation.
2. Fetal bovine serum (FBS)
3. Eagle's Minimum Essential Medium (MEM, Sigma M-3024). This is routinely prepared from powder, supplemented with 2.2 g/l sodium bicarbonate and 0.292 g/l (2 mM) glutamine, according to the supplier's recommendations, and stored refrigerated (one-week stock). In experiments, the medium also contains 1% FBS, prepared daily, and is used at 32°C. In this protocol, MEM medium always refers to complete medium which should be preheated to 32°C before use.
4. Dulbecco's phosphate-buffered saline (DPBS Sigma-D5780).
5. Na-fluorescein (Sigma F-6377). The dye is used as a 0.4 or 0.5% solution in DPBS (4 or 5 mg/ml).
6. Saline, always refers to 0.9% NaCl in distilled water.

Bovine eyes

Eyes, excised by an abattoir employee, are collected in a plastic jar containing one litre of HBSS for approximately 25 eyes. Buffer storage and transportation of eyes to the laboratory are performed at room temperature. The eyes are generally used within two hours after killing the animals.

Procedure Details

1. Preparation of corneas

During dissection, great care should be taken to avoid damage to corneal surfaces (epithelial and endothelial). All eyes are carefully examined, and those presenting defects, such as neovascularization, pigmentation, opacity or scratches are discarded. Eye balls are first dissected free of surrounding tissues (lids, conjunctiva, ocular muscles and glands) and placed in a jar containing fresh HBSS. Selected corneas are dissected with a 2-3 mm rim of sclera for easier handling, and stored in a petri dish containing HBSS until use. Corneas are then mounted in holders, the endothelial side being placed onto the O-ring of the posterior part of the holder. The anterior part of the holder is placed on the cornea and held in place with three screws. Compartments are then filled (the posterior part first) with MEM medium and corneas are incubated for one hour in a water-bath at 32°C.

2. Basal opacity

Immediately after incubation, anterior and posterior compartments are refilled with fresh medium, and opacity is determined (the method to measure opacity is described below). It should be very close to zero, thus permitting the elimination of any damaged or folded corneas: the limits for selecting good corneas are below or equal to 3 and above or equal to -3.

3. Treatment

Medium is removed from the anterior compartment, using a needle (with the point cut to remove the liquid completely) attached to a vacuum pump, and replaced by the test compound or an appropriate vehicle. Two treatment protocols are used, depending on the physical state (liquid or solid) of the product evaluated:

Protocol 1 : 10 min. treatment - **for liquids and surfactants**

Protocol 2 : 240 min, treatment - **for all solids**

In both protocols, substances are prewarmed at 32°C for a few minutes before being applied to the cornea. This is particularly important for liquids since the treatment time is only 10 minutes. For solids, this step is sometimes difficult; they are prepared in a mortar and very insoluble substances might be very sticky, preventing their transfer into a tube. In this case, place the mortar into warm (32°C) water for a few minutes.

3.1. Protocol 1

Liquid substances are applied neat (0.750 ml). If dilutions are requested, the solvent can be saline, for water-miscible products. PEG-600 or Triacetin can be used for immiscible liquids; triacetin may be preferred when possible (i.e. if miscible with the test liquid), because PEG-600 may enhance the penetration of some substances.

Surfactants are usually applied at 10% in saline, or at the dilution provided, and 0.750 ml is applied onto each cornea. Other concentrations (in saline) can also be tested as required.

Because some compounds, for example certain organic solvents, may be aggressive to plastic, it is recommended that glass syringes be used for all chemicals. In order to apply compounds uniformly onto the corneas, slightly rotate the holder, maintaining the cornea in a horizontal position (holes should be closed with the caps provided). Corneas are incubated in a horizontal position for 10 minutes at 32°C in a water bath. The holders should be completely immersed in water to ensure a uniform temperature.

The test substance is then removed, and the epithelium is washed at least three times, until the medium is clear, with approximately 4 ml of MEM. The anterior compartment is refilled with medium, and opacity is measured. Corneas are again incubated at 32°C for a period of 2 hours. Both compartments are refilled with fresh medium and opacity is again determined. The values obtained at this time-point (120 min) are the only ones used in calculations.

3.2. Protocol 2

Solutions or suspensions of solid products are prepared at 20% (in practice, 1 g plus 5 ml saline), using a mortar and pestle for homogenous preparations (start grinding in the mortar with a small volume of liquid). A volume of 0.750

ml is applied onto the epithelium with an appropriate syringe and needle. For sticky suspensions, it may be necessary to use a needle with a large diameter, or even to unscrew the anterior glass for pasty substances. Corneas are placed in a horizontal position for 4 hours at 32°C. The holders should be completely immersed in a water bath. The test compound is then removed and the epithelium is washed at least three times, until the cornea is free of particles; gentle swirling movements of the holders are sometimes necessary. It is also possible to remove the anterior glass if product is still present in the chamber. Both compartments are refilled with fresh medium and the opacity measurement is performed immediately without any further incubation.

N.B. This is the general procedure testing substances. In the EC/UK Study, however, all liquids and surfactants were tested neat and all solids at 20% in saline.

3.3. Number of corneas used

The number of corneas used per experiment is generally 15, but more or less may be used depending on the availability of eyes, holders and the number of test compounds. Each experiment includes a control group treated with saline (or with triacetin or PEG-600 if one of these has been used as solvent), a positive control group treated with a reference substance (see section "Positive controls") and several (generally 3) groups of corneas treated with the test substance. Each group is composed of three corneas.

4. Opacity measurement

The opacitometer determines changes in light transmission passing through the corneas, and displays a numerical opacity value (arbitrary units).

4.1. Calibration

This operation is performed with no cornea in the Opacitometer (Electro-Design, RIOM, France), but using the calibration devices. The electrical zero (balance between photocells) is adjusted with the "balance" knob, and the apparatus is set to "75" with a standardized opaque sheet of polyester.

4.2. Measurement

The lateral glasses of the holders should be dried. Changes in corneal opacity are determined by comparison with "basal opacity" measured before treatment (t=0 opacity).

Each corneal holder is placed in the experimental (positive) compartment of the apparatus with *no holder* in the control (negative) compartment. Thus, the value obtained (for control or treated corneas) represents the absolute opacity value for a given cornea, but not the difference between a treated and a control cornea, as was determined in previous studies.

5. Permeability

This second step of the assay is performed immediately after the measurement of opacity. The medium is removed from the anterior compartment, and replaced by 1 ml of fluorescein solution (0.4% for liquids and surfactants, 0.5% for solids). Corneas are incubated in a horizontal position for 90 minutes, immersed in a water-bath at 32°C. Medium from the posterior chamber is then removed, and its optical density (O.D.) determined with a spectrophotometer at 490 nm.

6. Data calculation

6.1. Opacity

Opacity values measured at a given time-point (120 minutes in protocol 1, 240 minutes in protocol 2) are *first* corrected (individually for each cornea in the experiment) for basal opacity ($t=0$), i.e. " $t=120 - t=0$ " and " $t=240 - t=0$ ". Then each individual cornea in the experimental groups, including the positive controls, is corrected for the mean value of saline-treated corneas (negative control) at this time-point. The values obtained are therefore the "corrected values" of opacity.

6.2. Permeability

The mean O.D. of saline-treated corneas is calculated, and then the individual value for each experimental cornea is corrected for this mean to give the "corrected value" of permeability.

6.3 In-vitro score

This score is calculated with corrected values of opacity and permeability *for each individual cornea*, using the formula:

$$\text{score} = \text{opacity} + (15 \times \text{O.D.})$$

For a given substance, the final in-vitro score will be the mean \pm SD of three corneas.

7. Quality control

7.1. Basal opacity

Only corneas with a basal opacity of ≤ 3 and ≥ -3 are selected for the experiment.

7.2. Fluorescein solution

Stock fluorescein solutions prepared at 4 mg/ml or 5 mg/ml in DPBS should be diluted to 10 $\mu\text{g/ml}$ in complete MEM (the medium used throughout the experiment to fill the posterior compartment), and O.D. determined before application to corneas. An acceptable range is between 1.610 and 1.910, otherwise the dilution should be performed again, and, if necessary, a new solution prepared. The actual value for each experiment has to be reported on the data sheets.

7.3. Positive controls

A group of three corneas treated with a known reference substance has to be included in each experiment. The substance selected will depend on the broad category of products to be evaluated:

- a) BENZALKONIUM CHLORIDE (=BAK) (Sigma, # B-1383) 5% solution in saline, for surfactants
- b) N,N,-DIMETHYLFORMAMIDE (=DMF) (Aldrich, # 15,481-4), 100%, for liquids.
- c) IMIDAZOLE (=IMDZ) (Aldrich, # I-20-2), 20% in saline, for solids.

The limits (mean of three corneas) for a valid experiment are as follows:

Compound	Opacity	Permeability	Score
Benzalkonium chloride	> 60	> 3.000	> 110
N,N-Dimethylformamide	> 70	> 1.500	> 100
Imidazole	> 35	> 2.000	> 70

8. Data interpretation (optional)

Based on experience at MS&D with reference and in-house compounds, and on data from collaborative studies, the following classification system was established:

In-vitro score:

from	0	to	25	=	mild irritant
from	25.1	to	55	=	moderate irritant
from	55.1	to	80	=	severe irritant
>=	80			=	very severe irritant

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