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Improvement of the Bovine Corneal Opacity and Permeability (BCOP) assay as an *in vitro* alternative to the Draize rabbit eye irritation test

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ABSTRACT

Measurement of ocular irritancy is a necessary step in the safety evaluation of both industrial and consumer products. Assessment of the acute eye irritation potential is therefore part of the international regulatory requirements for testing of chemicals.

The Bovine Corneal Opacity and Permeability (BCOP) assay is generally accepted as a valid *in vitro* alternative method to the Draize eye irritation test to detect corrosive and severe eye irritants (category 1), but has not proven sensitive enough to discriminate accurately moderate (category 2A/2B) to mild and non-irritating compounds. In the currently accepted BCOP assay, opacity is determined by the amount of light transmission through the cornea, and permeability is determined by the amount of sodium fluorescein dye that passes through all corneal cell layers. Both measurements are used to assign an *In Vitro* Irritancy Score (IVIS) for prediction of the *in vivo* ocular irritation potential of a test substance. Nowadays, opacity is measured by an OP-KIT opacitometer providing a center-weighted reading of light transmission by measuring changes in voltage when the transmission of white light passes through the cornea alters. As a consequence, this may underestimate opacity that develops as spots or heterogeneous opaque areas on the periphery of an isolated cornea.

A prototype of a laser light-based opacitometer (PLLBO) allowing better measurement of opacities was developed by Van Goethem et al. (2010). This new device showed improved sensitivity to detect subtle changes in corneal transparency. Furthermore, the new opacitometer allowed the analysis of the complete corneal surface and was able to detect more efficiently opaque spots located along the sides of the excised corneas.

A further improved prototype of the PLLBO was constructed in combination with a camera and a speckle noise reducer. Treatment conditions of the corneas in the cornea holders were optimized in order to mimic more the real *in vivo* situation. A set of test compounds with irritancy potencies especially in the mild and moderate range was tested. The improved LLBO showed some promising features which potentially could improve the usefulness of the BCOP test. Adaptation of cornea holders showed to be of limited value and only restricted to concentrations up to 15% which mimics more test conditions in industry.

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1. Introduction

The human eye is a complex organ comprised of various different tissues and cell types. The cornea, constituting the outer barrier of the eye, and the conjunctivae are the tissues which are most often affected after exposure of the eye to ocular irritants (Hackett and McDonald, 1991). Eye irritation is the result of changes in the eye following the application of a test chemical to the anterior surface of the eye and which are fully reversible within 21 days of application. Measurement of ocular irritancy is a necessary step in the safety evaluation of both industrial and consumer products.

Irritation testing using laboratory animals has largely remained unchanged for many years. The Draize eye irritation test (Draize et al., 1944) became a governmentally endorsed methodology and is described in OECD testing guideline (TG) 405 (OECD, 2002). Advances in ocular toxicology are challenging the validity, precision, and relevance of the Draize eye irritation test (Wilhelmus, 2001). Significant levels of variability were observed since the test is based on a subjective scoring procedure (Weil and Scala, 1971). Besides the fact that the test causes considerable discomfort and pain to animals, it is also recognized that the response in the

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rabbit is not always predictive of that found in humans (Curren and Harbell, 2002; Freeberg et al., 1986; Griffith et al., 1980). Taken together, both ethical and scientific reasons stimulated the development and validation of several alternative *in vitro* methods to assess eye irritation (Balls et al., 1999; Worth and Balls, 2002).

The status of alternative methods for eye irritation was described by Eskes et al. (2005) (Eskes et al., 2005) and although some of the assays showed considerable promise as screens for ocular irritancy, the outcome of many validation studies concluded that no single test was capable of replacing the range of injuries measured in the Draize rabbit eye test. The main reason for this is the difficulty of comparing *in vitro* test results with historical animal data which are often of insufficient quality for validation purposes. Further, one should realize that the *in vitro* tests only partially models the complex *in vivo* eye irritation response. However, a number of tests, including the BCOP (Bovine Corneal Opacity and Permeability), HET-CAM (Hen's Egg Test-ChorioAllantoic Membrane), IRE (Isolated Rabbit Eye), and ICE (Isolated Chicken Eye) tests, are accepted by European Union (EU) national regulatory authorities, on a case-by-case basis, for the identification of corrosive and severe eye irritants (EU, 2004).

The BCOP assay is an *in vitro* alternative which is routinely used in several industrial and contract testing laboratories in the context of workplace safety and product safety applications and is described in OECD TG 437 (OECD, 2009). However, the BCOP assay seems to be not sensitive enough to discriminate among moderate and mildly irritating materials when applying the standard protocol (Cooper et al., 2001; Eskes et al., 2005; Jones et al., 2001).

The assay is based on the methods described by Muir (1984, 1985, 1987) and Tchao (1988) and was developed by Gautheron et al. (1992) for the prediction of the irritation potential of process intermediates and compounds in development. In general, the BCOP assay can test a wide range of physical forms and solubility characteristics. For example, Vanparys et al. (1993) showed that when fifty pharmaceutical and commercially available substances were evaluated representing both liquids (miscible and immiscible) and solids (soluble and insoluble), a concordance of 96% was obtained when irritants were discriminated from non-irritants.

Up to now opacity readings are performed by using the OP-KIT opacitometer, which is a white (polychromatic) light, dual-beam opacitometer. This type of opacitometer provides a center-weighted reading of light transmission through the cornea. It was found that with the OP-KIT opacitometer different values on opacity were obtained (specially with alcohols and solids) depending where the spot was located, leading to misclassification of compounds for their eye irritating potential (Van Goethem et al., 2010). Another observation was that the OP-KIT opacitometer is not sensitive enough to differentiate between moderate (category 2A/2B), mild, and non-irritating compounds (narrow scale for ranking compounds). Furthermore, the OP-KIT device is difficult to calibrate and is not stable over time.

Recognizing the limitations of the conventional OP-KIT opacitometer, a prototype laser light-based opacitometer (PLLBO) was developed that uses an adjustable laser beam in combination with a calibrated photocell instead of visible light. This new opacitometer was designed to provide a more even distribution of light across the corneal surface which resulted in an improved method of opacity assessment when optical linearity and local effects (opaque spot induction) were analyzed (Van Goethem et al., 2010). Additional studies are required to determine if such instruments provide an improvement over the conventional opacitometer. This proof of concept should be considered as a first step towards a technical optimization in which further possible advantages of laser light technology should be investigated. For that reason, an improved PLLBO was constructed by using the laser light-based opacitometer (LLBO) in combination with a camera and a speckle noise reducer and by modifying the cornea holders so that test compounds and formulations can be diluted whether or not in function of time as happens *in vivo* by excessive tearing in the eye of man at exposure which attempt to wash out irritants that may have come into contact with the eye.

A set of compounds with irritancy potencies especially in the mild and moderate range were tested and compared with the results of the original PLLBO when possible. The improved LLBO showed promising features which potentially could improve the usefulness and applicability domain of the BCOP test.

2. Materials and methods

2.1. Test compounds

The eye irritating potential of nine reference chemicals (Table 1) selected from the recommended substances list for demonstrating technical proficiency with BCOP of OECD TG 437 (OECD, 2009) was assessed. The selected reference chemicals, for which high quality *in vivo* rabbit eye test data (ECETOC, 1998; Gautheron et al., 1994) and high quality *in vitro* BCOP data (Balls et al., 1999; Gautheron et al., 1994) exist, covered the range of irritant categories from non to severe (category 1) and represent different chemical classes.

The eye irritating potential of another 20 compounds (Table 2) selected from literature was assessed to evaluate further the performance of the new LLBO (Balls et al., 1999; Gautheron et al., 1994). The selection of test compounds focused on the non- to moderate (category 2A/2B) irritant categories and represent different chemical classes.

To determine the suitability of the adapted cornea holders, concentration series (15–100%) of ethyl acetoacetate (Table 2) and N,N-dimethylformamide (DMF, CAS: 68-12-2) were used. The latter is also used as positive control for liquid test items in the BCOP assay.

Also cosmetic formulations and simple formulations with different irritancy potencies were tested. Since it was not possible to obtain formulations from industry, it was decided to test three commercial kids shampoos labeled as 'does not sting the eyes'. The shampoos were tested as the actual formulation as they are on the market. Simple formulations were made of irritating compounds diluted in artificial tear fluid (Hyabak). A concentration series was tested for one severely irritating (pyridine), one moderately irritating (γ-butyrolactone), and one mildly irritating (1,2,4-trimethylbenzene) compound. All three compounds were previously tested as pure products (100%) by others.

2.2. BCOP assay

The BCOP assay was performed according to OECD TG 437 (OECD, 2009). A brief overview can be found in the paper of Van Goethem et al. (2010).

2.3. Construction of an improved laser light-based opacitometer (LLBO)

2.3.1. Conventional opacitometer (OP-KIT)

Up to now, opacity readings are performed by using the OP-KIT opacitometer (MC2, Clermont Ferrand Cedex, France), which is based on the use of a white (polychromatic) light, dual-beam opacitometer. This type of opacitometer provides a center-weighted reading of light transmission through the cornea. More detailed technical information on this device can be found in the paper of Van Goethem et al. (2010).