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Discussion

# The Draize Eye Test and in vitro alternatives; a left-handed marriage?

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## 1. Variability of the Draize Test

No other animal test like the Draize Eye Irritation Test has been as controversial to replacement by in vitro methods, while initially it was believed to be one of the 'simplest' animal tests to be replaced. Since the early 1980s numerous alternatives have been developed, with some being submitted to validation, but without finding a single test or set of tests for replacing the animal test. Why is this? For many of the alternatives, it soon became clear that the chosen test system had not enough relevance with respect to eye irritation as was hoped for. For instance, a test system measuring decreased sperm mobility/motility provides some information on cytotoxicity in general, but not specifically on toxicity to corneal or conjunctival tissue. The fact that the toxicity measured by the test system has to be translated to specific (rabbit) ocular toxicity is the basis for most of the problems encountered. Furthermore, the variability of the Draize Eye Test, especially in the middle range of irritancy adds to this problem. The factors contributing to this variability are meanwhile well-known and recognized by the scientific world. The variability is mainly caused by the subjective scoring by different observers and interlaboratory variability.

## 2. Exposure conditions in the Draize Test

What is not highlighted in the discussions so far, however, is surprisingly enough the conduct and course of the test itself, although several investigators have discussed the unrealistic exposure conditions of the Draize Eye Test, i.e., instillation in the conjunctival cul-de-sac of the rabbits eye, compared to potential human exposure (Freeberg et al., 1986; Roggeband et al., 2000).

For most routine acute and repeat toxicity tests, standard exposure times and/or delivery of dosage (orally, intravenously, etc.) are well-defined. In the dermal irritation test, for example, the entire dosage is held by a patch onto the skin for an exact period of time. In the eye irritation test, however, neither of these well-defined conditions exists. For liquids, pastes and solids, it is impossible to estimate how much and for how long the test substance stays in contact with the eye. For aqueous, non-viscous formulations the standard instillation of 0.1 ml in the conjunctival cul-de-sac of the rabbit and the holding of the eye-lids for 1 s, results in a rapid removal of the material within seconds/minutes through blinking with the nictitating membrane (third eye-lid) and grooming by the rabbit.

This contrasts with the situation for sticky pastes for example, which cannot be removed that easily. The most dramatic variation in contact time and dosage occurs with solids. Even if applied as a 0.1 ml equivalent (the content of the cul-de-sac), the actual amount of a powder/solid that stays in contact with the eye is unpredictable. More importantly, the contact time may vary from a couple of minutes to 24 h, because rinsing the eye is

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not allowed before the 24-h reading (only recently changed to 1 h for solids in the 2002 update of OECD guideline no. 405).

## 3. Testing of solids and variability

From ethical and scientific points of view, it is unbelievable that this situation still exists. Having carried out the test since 1981, it became increasingly difficult for me to adhere to this non-rinsing practice. Unintentionally, I discovered that the problem could be solved by manipulation of the eye-lids of the rabbit at the 1 h observation time point in such a way that any remnants of the test material present could be removed without rinsing. This process was always recorded in our reports, but never resulted in any comment on this deviation from the guideline. It is striking how few reports on eye irritation even mention the presence of remnants of powders/solids in the eye at the 1-h and/or 24-h observation time points, whereas it should be a common finding. The enclosure of solid materials up to 24 h in the conjunctival cul-de-sac, sometimes in combination with mechanical damage, can have a devastating effect on the eye. In the case of poorly water-soluble solids with distinct cytotoxic properties, the entrapped solid can rapidly cause a considerable and increasing degree of swelling of the conjunctivae, making it even more difficult for the animal to remove the material. If, at the 1-h observation, the lower eye-lid is not pulled away far enough by the observer, it can stay unnoticed that a bulk of test material lays deeply hidden in the conjunctival cul-de-sac.

Often, this forced continuous exposure for the next 24 h results in a complete closure of the eye-lids by the abundant production of colloidal discharge which often forms a sealing crust. Upon opening these sealed eye-lids, purulent discharge, and other inflammatory debris are released. The degree of swelling of the conjunctivae can be sufficiently severe such that removal of any remains of the test substance is hardly possible anymore. In the majority of these cases, the eye is permanently damaged or can only be saved by applying special care, such as regular daily cleaning and rinsing of the eye and eye-lids, often including cutting off the eye-lashes to prevent further sealing. In general, keeping the eye-lids open is essential for the recovery process, otherwise the enclosed inflammatory exudate will further damage the cornea. If no further extensive remedial treatment is given to the animal, the described exposure conditions can easily cause an initial opacity score of 1 or 2 to develop into a score of 3 or 4. Also, the eye can become vulnerable to microbiological infection (the so-called secondary inflammatory process), causing initial mild to moderate effects during the first days after exposure developing into more severe and prolonged effects during the 21 day observation period.

Without doubt such events will have occurred in other laboratories in the past, and probably will continue to occur, even with application of the present 1-h rinsing protocol for solids now in place. The events described here are of course not typical for all solids. Many of the solids are inert and form an unharmful bulk which can easily be removed by the animal or the observer, or they are well water-soluble and have already disappeared at the 1-h observation time point. However, the overall problem makes the Draize Eye Test highly variable, even *before* the actual scoring of effects takes place. Therefore, even if the scoring could be made more objective and less variable, the scores recorded will still represent a large variation. To my knowledge, this important source of variability has never been discussed, while its implication for any validation of alternative in vitro methods is very important.

## 4. Draize Test results and validation

Does this mean that we cannot use the data from the Draize Eye Test for validation purposes at all? It seems logical to assume that non-irritating or severely irritating hydrophilic liquids and non-irritating solids produce reliable reactions in the Draize Eye Test. Extremely variable results, however, will be obtained with sticky pastes and solids in the moderate to severe range of irritancy and with hydrophobic solutions. Such data will be unsuitable for the use as benchmarking data in the validation of in vitro methods. Apart from that, the kind of entrapment of solids in the rabbit eye has little relevance, because it is highly unlikely to occur in humans, accidentally or intentionally. For that reason, the low-volume eye test (LVET) has been developed by the Procter and Gamble company (Griffith et al., 1980). In this test, one-tenth of the original volume of 0.1 ml is administered directly onto the cornea of the rabbit and this is believed to mimic human exposure more realistically.

From a safety standpoint, it is understandable that the Draize Eye Test is still required by regulatory agencies, mostly because of the perceived higher sensitivity of the rabbit eye compared to the human eye. However, this perception has more to do with these exaggerated exposure conditions rather than with specific ocular tissue sensitivity. In that light, it is praiseworthy that, several years ago, European regulatory agencies took the initiative to accept in vitro screening of severe eye irritants by using isolated eyes or corneas, or the Hen's-egg chorioallantoic membrane (HET-CAM assay).

Recently, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) of the United States of America initiated a programme to officially adopt these alternatives

into the US-guidelines for the screening of severe eye irritants. In this programme, the methods and the data available are evaluated by a panel composed of national and international experts. Although there was a general awareness amongst the panel members concerning the variability of the Draize Eye Test, the general attitude was still to attempt to fit in the in vitro methods with the Draize Eye Test, rather than to address the validity of the latter test. All emphasis is again on the statistical evaluation of the in vivo and in vitro data. It is true that the nature and quality of the in vivo and in vitro data was examined in more detail, but mostly with the intention of modifying/optimizing the in vitro assays, rather than questioning the relevance of the in vivo data. To some extent the flaws of the rabbit test were acknowledged, but this did not lead to any real changes to the conduct of the test itself, whereas in vitro methods are still judged against this "Gold Standard" and repeatedly forced to have their methodologies adapted to the rather unrealistic conditions of the Draize Eye Test.

To give an example, ICCVAM evaluated the data of the EC/HO validation study (Balls et al., 1995) in which four candidates (Isolated Chicken Eye test, Isolated Rabbit Eye test, HET-CAM assay, and Bovine Corneal Opacity and Permeability test) selected by ICCVAM participated. Twenty-two out of the 59 substances examined in this study were severe irritants and, for the purpose of selecting in vitro methods for screening severe irritants, the data of these compounds (tested by four labs per method) were useful. For the Isolated Chicken Eye test (ICE), the ICCVAM panel concluded that it could identify severe irritants but with a high false-negative rate, especially for solids. Of the 22 compounds, the ICE identified 13 as severely irritating, 4 as irritating and 5 as non, or borderline, irritating. The latter five compounds were all solids. Remarkably, most of the in vitro methods participating in the EC/HO study also did not identify these compounds as severe irritants. Instead of questioning the in vivo exposure conditions of solids, ICCVAM considered this to be a deficiency of the in vitro method. Therefore, ICCVAM recommended that the test method needed to be optimized with respect to the exposure conditions for solids. Considering the fact that we have to deal with in vivo exposures to solids ranging from a couple of minutes up to 24 h, then standardization of the Draize Eye Test would be an appropriate recommendation.

## 5. How to proceed?

All suggestions for optimization/modification—how ever well intended they may seem to be—are driven by the thought that they are needed because there is not sufficient correlation with the in vivo "Gold Standard" test. The fact that these are totally uncontrolled and non-standardized conditions in the in vivo test, which cannot be modeled accurately by any of the in vitro tests, seems to be ignored or of no concern to regulatory bodies or to validation bodies like ICCVAM and ECVAM (European Centre for the Validation of Alternative Methods). Until the problems with the Draize Test discussed in this paper are solved and taken account of, all efforts to validate in vitro tests as complete replacements for the in vivo test will be doomed to fail.

Since the first international (pilot) validation (Commission of the European Communities, 1991) of alternatives for eye irritation was started in 1988, numerous validations using optimized/modified/standardized in vitro protocols have been carried out without any substantial success. We seem to be caught in a vicious circle and, by now, after almost 18 years of validation, I think it is time to conclude that further attempts will be futile, if we keep on using "old" in vivo data or new data generated by the current protocol for comparison. In fact, since the very first validation, most of the in vitro tests have been used in practice for decision making by many companies and have been accepted in Europe for screening of severe irritants. Having carried out the Draize Eye Test since 1981 and applied an in vitro/ex vivo screen prior to any in vivo testing since 1992, my recommendation would be a multi-way approach, as follows:

(a) Immediate implementation in the guidelines (legislation) of the most current in vitro methods in the testing strategy for screening of severe irritants, following current EC practice (CA, 2002). Many contract or company laboratories already have extensive experience with the existing in vitro alternatives. Moreover, severe irritancy is not based on the in vitro screen alone but often confirmed by other tests, such as skin irritation/corrosion (in vivo and in vitro) and acute dermal toxicity. Also indications of the possible (severe) irritating properties of a compound are often known by the manufacturer. Furthermore, most new substances will be tested in a battery of acute toxicity tests, covering skin and eye irritation, oral, dermal and inhalatory toxicity and skin sensitization, which require a tiered decision-making process by the investigator with respect to dose and test concentration selection. For that purpose, it is always useful to know as early as possible if one is dealing with an irritating (reactive) compound or not.
(b) Internationally, the Draize Eye Test should be re-evaluated taking a more realistic procedure like the LVET into consideration. The exposure conditions should be standardized for liquids and solids, i.e., a fixed exposure time, amount and mode of instillation (directly onto the cornea instead of in

the conjunctival cul-de-sac). In the EC guidelines there is the provision that substances causing eye irritation may also be examined for the effect of rinsing after a fixed exposure time, but in practice this possibility seems not to be followed. For exceptional circumstances, like ocular therapeutics or pesticides, the non-rinsing protocol could be maintained because in daily practice rinsing the eyes after (accidental) exposure to pesticides by the user may not always be possible.

(c) Together with the immediate implementation of in vitro methods and standardization of the Draize Eye Test, the possibilities for a more mechanistically-based development and optimization of in vitro methods should be an ongoing process. The parallel testing mentioned under point (a) would also offer the unique possibility to further validate the in vitro methods for the non-severe irritating category of compounds, and to test any new (mechanistically-based) modification both in vitro and in vivo.

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