

Chapter 5: Local Toxicity: Acute Dermal and Ocular Effects

Introduction

Acute local toxicity refers to the local toxic effects that may result from a single exposure to a chemical or product, by way of the oral, dermal, ocular or inhalation routes. The exposure can be incidental, accidental or deliberate (for example, in the case of cosmetics and certain medicines). The dermal route of exposure can also be a significant route for entry into the systemic circulation, as discussed in the section in Chapter 7 on percutaneous absorption. The main types of toxic effect are dermal and ocular corrosion and irritation, and these are generally assessed in a sequential manner in the context of tiered assessment strategies, such as those adopted by the OECD in 2001(1). In such strategies, dermal effects are generally assessed before ocular effects. Tiered approaches for assessing corrosion and irritation are illustrated in Figures 5.1 (dermal effects) and 5.2 (ocular effects).

Current Status of Alternative Methods for Skin Corrosion

Structure–activity relationships for skin corrosion

Various structure–activity relationships (SARs) for skin corrosion have been reported by Barratt and colleagues (2–5). On the whole, the SARs presented in these studies take the form of principal component (PC) plots, which are based on physicochemical properties, and show a separation between corrosive (C) and non-corrosive (NC) chemicals. Explicit classification models were not presented. Rather than modelling a heterogeneous group of chemicals, separate analyses were performed for acids, bases, electrophiles and neutral organics (defined as uncharged molecules that lack the potential to react covalently, and which do not ionise under biological conditions [Martin Barratt, personal communication]).

The most recent presentation of this approach is given in Barratt *et al.* (4). In addition to PC analyses, discriminant analysis and neural-network analysis were also applied to a group of neutral and electrophilic chemicals (2), and to the acids, bases and phenols (3). Finally, in another study (6), PC plots for acids were based not only on physicochemical properties, but also on *in vitro* cytotoxicity measurements in mouse 3T3 cells. More recently, it was shown that a heterogeneous set of organic

chemicals could be predicted as C or NC on the basis of melting point (mp) and molecular mass (MW), according to the following prediction model (PM; 7):

If mp ≥ 37°C and MW ≤ 123g/mol, predict as C; otherwise predict as NC.

Similar rules have been developed by Gerner and colleagues, who have incorporated a system of decision rules into an expert system used by the German BgVV (8–10). An example is the following PM:

If MW > 1200g/mol, then the substance has no local toxic effects.

In vitro methods for skin corrosion

The current status of alternative methods is summarised in Table 5.1. ECVAM-funded validation studies on *in vitro* tests for skin corrosion have been conducted (11, 12), and the scientific validities of four *in vitro* tests have been endorsed by the ESAC: the rat skin transcutaneous electrical resistance (TER) assay (13), two tests based on the use of commercial reconstituted skin equivalents, EPISKIN™ (13) and EpiDerm™ (14), and CORROSITEX™ (15, 16).

In the EU, a new *Test Method on Skin Corrosion*, incorporating the rat skin TER and human skin model assays, has been included in Annex V of the Dangerous Substances Directive (*Directive 67/548/EEC*; 17), thereby making the use of *in vitro* alternatives for testing the skin corrosion potential of chemicals mandatory in the EU.

A draft Test Guideline (TG) on *in vitro* tests for skin corrosion was submitted to the OECD in late 1998, for consideration by the OECD Member Countries. Following a number of commenting rounds, an expert meeting, held on 1–2 November 2001 in Berlin, agreed that the draft TG on *in vitro* skin corrosion should be divided into two separate TGs: a draft proposal for a new TG 430 (18) on the TER test (not restricted to the rat skin TER test) and a draft proposal for a new TG 431 (19) on the human skin model test. The new TG 430 and TG 431 have now been accepted by the National Coordinators of the OECD Test Guidelines Programme.

Tiered testing strategies for skin corrosion

In 1996, the OECD proposed a tiered (stepwise) approach to hazard identification, which underwent

Table 5.1: An overview of *in vitro* methods for skin corrosion and irritation

Method	Test system	Endpoint	Applicability	Formal status
Skin corrosion				
Rat skin transcutaneous electrical resistance (TER) assay	Excised rat skin	Electrical resistance	General; additional dye-binding step for surfactants and solvents	Validated and accepted by regulatory authorities for skin corrosion testing in the EU and OECD member countries
EPISKIN™ human skin model (commercial system)	Reconstructed human epidermal equivalent	Cell viability (MTT reduction assay)	General; a few materials may interfere with MTT reduction	Validated and accepted by regulatory authorities for skin corrosion testing in the EU and OECD member countries
EpiDerm™ human skin model (commercial system)	Reconstructed human epidermal equivalent	Cell viability (MTT reduction assay)	General; a few materials may interfere with MTT reduction	Validated and accepted by regulatory authorities for skin corrosion testing in the EU and OECD member countries
CORROSITEX™ (commercial system)	Reconstituted collagen matrix	Colour or physical change in indicator “chemical detection system”	Mainly acids, bases and derivatives	Validated and endorsed (US and EU) for skin corrosion testing of acids, bases and their derivatives
Skin irritation				
EPISKIN™ human skin model (commercial system)	Reconstructed human epidermal equivalent	Cell viability (MTT reduction assay)	General; a few materials may interfere with MTT reduction	Protocol modification and prevalidation (validation study under discussion)
EpiDerm™ human skin model (commercial system)	Reconstructed human epidermal equivalent	Cell viability (MTT reduction assay)	General; a few materials may interfere with MTT reduction	Protocol modification and prevalidation (validation study under discussion)
Pig ear test	Pig ear	Trans-epidermal water loss (TEWL)	General	Further development necessary
Mouse skin integrity function test (SIFT)	Excised mouse skin	TEWL and electrical resistance (ER)	General; a few materials may interfere with either TEWL or ER determination	Protocol modification and prevalidation (validation study under discussion)

revisions in 1998 and in 2001 (1). In the EU, a tiered testing strategy for skin corrosion/irritation is being proposed for incorporation into Annex V of *Directive 67/548/EEC*. This could be achieved during 2002 by means of the 29th Adaptation to Technical Progress of the directive (Juan Riego-Sintes, personal communication).

An evaluation of a two-step strategy, based on the sequential use of pH measurements and *in vitro* data, indicated that the use of pH data in addition to TER or EPISKIN data, improves the ability to predict corrosion potential (20). An evaluation of a three-step strategy, based on the sequential use of QSARs, pH measurements and *in vitro* data, indicated that tiered

approaches provide an effective means of classifying chemicals, while at the same time reducing and refining the use of animals (21). A study carried out by ECVAM confirmed the usefulness of pH as a predictor of skin corrosion potential, and provided a new PM for identifying chemicals that are corrosive by a pH-dependent mechanism (22).

Skin Corrosion: Summary, Conclusions and Recommendations

Alternative methods for skin corrosion have been validated and accepted for regulatory use in the EU,

so animal testing should not be performed for this endpoint. The hazard identification (classification and labelling) of skin corrosives should be based on the use of a pH test, where appropriate, and an *in vitro* test (rat skin TER assay, human skin model assay or, for qualifying test chemicals, CORROSI-TEX). For risk-assessment purposes (dose–response investigations, coupled with assessments of skin-irritation potential at doses negative in skin corrosion tests), the rat skin TER or a human skin model assay is recommended.

Short-term prospects

1. The achievement of acceptance by the OECD Council of the new TGs on *in vitro* tests for skin corrosion, although this is not a prerequisite for the use of these tests in the context of chemicals testing in the EU.
2. The validation of QSARs and/or expert system rulebases for skin corrosion.

Current Status of Alternative Methods for Skin Irritation

Structure–activity relationships for skin irritation

Relatively few QSAR studies for skin irritation have been reported in the literature. Barratt (2) reported a QSAR for predicting the primary irritation index (PII) of organic chemicals, but this had little predictive value ($r^2 = 0.42$). In the same study, discriminant analysis was shown to discriminate between irritant and non-irritant chemicals, as defined by EU classification criteria, with an accuracy of 67%.

Hayashi *et al.* (23) reported two QSARs for predicting the molar-weighted PII of phenols. One model, based on absolute hardness, was proposed for chemicals with negative lowest unoccupied molecular orbital (LUMO) energies, whereas the other model, based on the logarithm of the octanol–water partition coefficient ($\log P$), was proposed for chemicals with positive LUMO energies. These models had correlation coefficients of 0.72 and 0.82, respectively (i.e. r^2 values of 0.52 and 0.67).

Smith *et al.* (24) analysed a data set for 42 esters for which human skin irritation data were available, and for which 19 physicochemical properties had been calculated. Best-subsets regression was used to select variables for subsequent inclusion in discriminant models. The best variables were water solubility (lower for irritants than non-irritants), a dispersion parameter (higher for irritants), a hydrogen-bonding parameter (higher for irritants),

the sum of partial positive charges (lower for irritants), and density (lower for irritants). A discriminant model based on all five parameters had a sensitivity of 85% and a specificity of 92%.

In vitro methods for skin irritation

At present, there are no validated *in vitro* tests for skin irritation. Acting on a recommendation by the ECVAM Skin Irritation Task Force (25), ECVAM has supported prevalidation studies on five *in vitro* tests for acute skin irritation: EpiDerm, EPISKIN, PREDISKIN, the pig-ear test, and the mouse-skin integrity function test (SIFT; 26, 27). The outcome of these studies was that none of the tests was ready for progression to formal validation, but that appropriate modifications to certain test protocols might enable them to meet the criteria for early inclusion in a future validation study.

Various follow-up activities to the prevalidation study are currently in progress, with the objective of having test protocols suitable for inclusion in a formal validation study before the end of 2002. An extended ECVAM Skin Irritation Task Force meeting was held in May 2001, to agree and prioritise the activities required before the setting up of a validation study (28). On the basis of additional work conducted after the prevalidation studies, it is hoped that a common EpiDerm/EPISKIN protocol and a SIFT protocol could meet the performance criteria defined for progression to a validation study.

Several testing strategies have been proposed/evaluated (1, 25, 29), some of which involve human volunteer studies (human 4-hour patch test; 30). Although it may be appropriate to conduct such studies on a case-by-case basis for risk-assessment purposes, human testing has no role in the early screening and hazard identification of chemicals.

In vitro methods for phototoxicity

Phototoxicity is a broad term covering a number of endpoints, including acute photoirritation, photogenotoxicity and photocarcinogenicity. Although not currently part of the testing requirements for chemicals, some phototoxicity endpoints are required for the assessment of cosmetic and pharmaceutical products.

Acute photoirritation refers to the toxic response that may be induced in the skin upon exposure to light, following earlier exposure to certain chemicals either by the topical or the systemic route (31, 32). The 3T3 neutral red uptake (NRU) assay for acute photoirritation has been successfully validated (33, 34) and endorsed by the ESAC (35, 36). A

test guideline based upon the validated protocol has been accepted as an Annex V method in relation to *Directive 67/548/EEC*, and an equivalent test guideline has recently been accepted into the OECD Test Guidelines Programme for acceptance as an OECD Test Guideline (37).

Several approaches have been developed for photomutagenicity testing. For example, Dean *et al.* (38) proposed the combined use of a bacterial mutation test in *Escherichia coli* and a mammalian cytogenetics assay in Chinese hamster ovary (CHO) cells. Photomutagenicity tests based on bacterial, yeast and mammalian cells have also been proposed by Chetelat *et al.* (39, 40).

Skin Irritation: Summary, Conclusions and Recommendations

In vitro methods for skin irritation testing could be used immediately for priority setting. The human skin model assays (for example, EpiDerm and EPISKIN) and the mouse SIFT appear to be the most promising methods (28).

For risk-assessment purposes, there is a need to identify and evaluate the usefulness of new, mechanistically based endpoints that are more predictive of skin irritation than are simple cytotoxicity determinations. The existing *in vitro* models also need to be improved, so that they are more representative of the skin *in vivo*.

Short-term prospects

1. The validation of modified test protocols for the human skin models and SIFT, to determine whether any of these existing methods can adequately distinguish acute skin irritants from non-irritants. The key activities required before organising a validation study, which should be coordinated by ECVAM, are: a) identification of test chemicals (irritants, non-irritants) for use in (pre)validation studies from the New Chemicals Database (European Chemicals Bureau, Joint Research Centre, Ispra); and b) assessment under blind conditions of a common protocol for the EPISKIN and EpiDerm tests, and of a modified protocol for the SIFT, to check whether these are ready for formal validation. It is envisaged that this will be a small study in a single laboratory for each test, with approximately 20 chemicals not previously used in this work.
2. The further development of (Q)SARs and/or expert system rulebases for skin irritation.
3. The acceptance during 2002 by the OECD Council of a revised TG 404 (acute dermal irri-

tation/corrosion), which includes as a supplement the OECD tiered testing strategy for skin irritation and corrosion.

Medium-term prospects

The validation of QSARs and/or expert system rulebases for skin irritation.

Recommendations for research and development

More resources should be provided for research aimed at the identification of new markers for skin irritation, and ongoing activities should be coordinated, with a view to having identified promising toxicological endpoints and developed new toxicity tests for validation by 2006. This research should be undertaken in parallel with the validation of existing test protocols for hazard identification. One approach to this research is through the application of genomics and proteomics. For example, COLIPA has started a three-year programme on specific aspects of proteomics and genomics.

Current Status of Alternative Methods for Eye Irritation and Corrosion

In vitro methods for eye corrosion

The tests for skin corrosion described above are assumed to identify chemicals that would also be corrosive to the eye. This should be clearly spelled out in Annex V of *Directive 67/548/EEC*, in the description of the methods for skin corrosivity testing (17).

In vitro methods for eye irritation

Six major validation or evaluation studies took place between 1991 and 1997.

1. The European Commission/Home Office (EC/HO) study (41).
2. The European Cosmetic, Toiletry & Perfumery Association (COLIPA) study (42).
3. The Bundesgesundheitsamt/German Department of Research and Technology (BGA/BMBF) study (43–45).
4. The Cosmetics, Toiletries and Fragrance Association (CTFA) study (46–49).
5. The Interagency Regulatory Alternatives Group (IRAG) study (50).

6. The Japanese Ministry of Health and Welfare/Japanese Cosmetic Industry Association (MHW/JCIA) study (51), in which 12 alternative methods (not counting variations on the various methods) have been evaluated.

The outcome of each of these studies has been summarised by Balls *et al.* (52). No test was found capable of replacing the Draize rabbit eye test, but some of the assays showed considerable promise as screens for ocular irritancy. For example, a validation study carried out in Germany led to the conclusion that the combined application of the HET-CAM and NRU tests could be used to identify severe eye irritants (chemicals with EU risk phrase R41). Subsequent analyses suggested that the endpoints of the 3T3 NRU and HET-CAM tests can also be used to distinguish between non-irritants and irritants (i.e. chemicals with EU risk phrases R36 and R41), although the PMs, based on the data generated in the German validation study, have not yet been subjected to a formal validation (53).

The use of *in vitro* methods as screening tests is widespread in industry, since there is much confidence that a number of alternative tests do work in-house. However, it has proved impossible to establish this satisfactorily by conducting validation studies in which *in vitro* test results are compared with historical animal data. The main reason for this is the subjective scoring of tissue lesions in the eye in the Draize test, which provides variable estimates of eye irritancy. Other possible contributing reasons for the outcomes of recently completed validation studies are: a) the *in vitro* tests only partially modelled the complex *in vivo* eye irritation response; b) the protocols and PMs might have been insufficiently developed; and c) the choice of statistical approaches for analysing the data might not have been appropriate (52).

Table 5.2 summarises those alternative methods that are currently the most developed and the most widely used. The Irritection system (formerly EYTEX™) is not recommended, due to the lack of a standardised protocol and poor *in vitro*–*in vivo* correlations.

It is generally considered that a battery of alternative tests is required for the assessment of eye irritation, since there are multiple mechanisms of eye irritation. For example, corneal opacity can result from increased corneal hydration due to inhibition of the sodium–potassium ATPase pump in the corneal endothelium (54). Such batteries should be based on *in vitro* tests that model different mechanisms and therefore give complementary results.

Structure–activity relationships for eye irritation

In a study by Cronin *et al.* (55), the application of linear regression analysis to a data set of 23 physico-

chemical properties for 53 organic liquids led to the development of statistically significant QSARs (based on log P) for predicting the molar eye score (MES) of alcohols and acetates. The MES is the modified maximum average score (MMAS) corrected for the number of molecules applied to the rabbit eye.

Subsequently, Abraham *et al.* (56) used the solvatochromic parameters (molar refraction, polarisability, and hydrogen-bond acidity and basicity), which are derived from chromatography experiments, to model a data set comprising 38 of the 53 organic liquids previously analysed by Cronin *et al.* (55). A QSAR for predicting the MES, based on these parameters and a vapour solubility parameter, had an r^2 value of 0.89.

A different approach to the prediction of the molar eye score was adopted by Kulkarni & Hopfinger (57). In addition to using parameters based on solute properties, they also used a molecular dynamics method to generate intermolecular membrane–solute interaction properties. QSARs based on these properties were then derived by using a genetic algorithm. A QSAR based on three parameters (two energy terms and a topological index) had an r^2 value of 0.92. The 16 chemicals used to derive the model were aliphatic and aromatic hydrocarbons, and aliphatic ketones, alcohols and acetates.

In addition to the derivation of regression models, attempts have also been made to develop classification models for eye irritation. For example, Cronin *et al.* (55) applied linear discriminant analysis to the data set for 53 organic liquids, but found no linear combination of physicochemical properties capable of discriminating between irritant and non-irritant chemicals (as defined by EU classification criteria). However, in a PC plot based on all 23 variables, the irritant chemicals appeared to form an embedded cluster within the non-irritant chemicals. Similar findings were subsequently reported by Barratt (58).

The phenomenon of embedded clustering of irritant chemicals was investigated further by Cronin (59), this time by using the technique of cluster significance analysis (CSA) to determine whether the embedded clustering was statistically significant. Out of a total of 23 physicochemical descriptors, it was reported that the five most significant were log P, log P², the heat of formation, the dipole moment, and a topological index. Subsequently, the method of “embedded cluster modelling” was developed (60), which generates elliptical prediction models for the embedded clusters, and was applied to an eye-irritation data set (61).

The physicochemical determinants of eye irritation potential were also investigated by Rosenkranz *et al.* (62). In comparison with non-irritant chemicals, these workers reported that irritant chemicals have significantly lower molecular masses, higher aqueous solubilities, lower log P values, and greater

Table 5.2: An overview of *in vitro* methods for eye irritation

Method	Test system	Endpoint	Reference
Bovine corneal opacity and permeability (BCOP) test	Excised cornea from the bovine eye	Opacity and permeability of the cornea	Gautheron <i>et al.</i> (69)
Hen's egg test-chorioallantoic membrane (HET-CAM)	Hen's egg	Damage to the chicken chorioallantoic membrane	Lüpke (66)
Chorioallantoic membrane-trypan blue staining (CAM-TBS)	Hen's egg	Damage to the chicken chorioallantoic membrane	Hagino <i>et al.</i> (71)
Isolated rabbit eye (IRE) test	Isolated rabbit eye	Corneal swelling, corneal opacity and fluorescein retention	Whittle <i>et al.</i> (72)
Isolated chicken eye (ICE) test	Isolated chicken eye	Corneal swelling, corneal opacity and fluorescein retention	Burton <i>et al.</i> (73)
Fluorescein leakage (FL) test	Madin-Darby canine kidney (MDCK) cells	Damage caused to the tight junctions in MDCK monolayers	Tchao (74)
Neutral red uptake (NRU) test	3T3-L1 cells	Cell viability	Borenfreund & Puerner (75)
Neutral red release (NRR) test	Rabbit corneal fibroblasts or mouse 3T3-L1 cells or normal human epidermal keratinocytes	Damage to the cell membrane	Reader <i>et al.</i> (76)
Red blood cell (RBC) haemolysis test	RBCs from calf blood samples	Damage to cytoplasmic membrane (haemolysis) in combination with damage of liberated cellular proteins (denaturation)	Pape & Hoppe (77)
Agarose diffusion method	L-929 mouse fibroblast cells	Cell death	Wallin <i>et al.</i> (78)
EpiOcular™	Reconstituted human corneal epithelium	Cell viability, release of inflammatory mediators, membrane permeability	Stern <i>et al.</i> (79)

molecular-orbital energy gaps (absolute hardness values). On the basis of the last-named observation, it was concluded that chemical reactivity does not appear to be a requirement for eye irritation. The authors also used the Multi-CASE expert system to identify biophores (substructures which occur with a significantly greater frequency in irritants than in non-irritants) and biophobes (substructures which occur significantly more frequently in non-irritants). The major structural determinants (biophores) included primary, secondary and tertiary amine groups (i.e. basic groups), as well as carboxylate, organosulphate and sulphonate groups (i.e. acidic groups).

In contrast to the studies summarised above, which aimed to develop (Q)SARs for non-surfactant chemi-

cals, an investigation by Patlewicz *et al.* (63) focused on surfactants. In this study, neural network analysis indicated that the maximum average score (MAS) of cationic surfactants is positively correlated with surfactant concentration and critical micelle concentration, and negatively correlated with log P.

The ECVAM Workshop on Eye Irritation and Follow-up Activities

In 1999, ECVAM organised a workshop on *Eye Irritation Testing: The Way Forward* (52). Four parallel activities were suggested as a means of making progress toward the short-term reduction and refinement of animal use, and the long-term

replacement of the Draize test: a) an evaluation of the reference standards (benchmarking) approach; b) a review of tiered testing strategies; c) further analyses of the data obtained in previous validation and evaluation studies; and d) further research on the mechanisms of eye irritation. The following sections describe the progress made in these activities.

The reference standards approach

Following the outcomes of numerous validation and evaluation studies, it is now widely considered that the adoption of a reference standards approach could considerably improve the predictive abilities of *in vitro* tests for which no relevant and reliable *in vivo* benchmark data exist. To investigate the applicability of such an approach in the validation of *in vitro* tests, ECVAM established a Reference Standards Working Group, which decided that an initial evaluation of the benchmarking approach should be made by concentrating on eye irritancy as the toxicological endpoint.

Subsequently, an ECVAM Reference Standards Study was initiated in 1998, with the objective of assessing the feasibility of using reference standards to demonstrate the scientific validity of *in vitro* tests for eye irritation. Five *in vitro* methods were included in the study: a) the isolated chicken eye (ICE) test; b) the bovine corneal opacity and permeability (BCOP) test; c) the combined use of the HET-CAM and NRU test; d) EpiOcular™ (a reconstituted human corneal epithelium); and e) the red blood-cell (RBC) haemolysis test. Four groups of chemicals were tested: a) neutral organics (BCOP); b) alcohols and esters (HET-CAM/NRU, EpiOcular); c) surfactants (ICE, HET-CAM/NRU, RBC); and d) siloxanes (BCOP, ICE). Sets of five unknowns were tested against sets of five reference standards.

The outcome of the study revealed that the BCOP results were good for neutral organics, and that the RBC and NRU results were good for surfactants. Results for alcohols and esters were not as good, but data obtained with the HET-CAM/NRU test were better than data obtained with the EpiOcular test. Siloxanes were the most difficult group included in this study, because their physical properties made them difficult to handle consistently in some of the assays. The BCOP performed slightly more predictably than the ICE. This might be explained by the fact that, for this set, the *in vivo* score was presented not as the MMAS, but as a differently weighted PII. This might have reduced the *in vivo-in vitro* correlation with the ICE, which generates a score intended to be an MMAS equivalent (64).

The study led to the following conclusion.

1. The reference standards approach is feasible, but further investigations into its applicability are required.

2. The classifications of chemicals into different chemical classes in the study were too crude and too broad, and needed redefinition, since the assumption that reference standards would permit the testing of broad classes of chemicals with different mechanisms of effect proved to be false.
3. The scientific validities of existing and new *in vitro* methods for eye irritation could be validated by using the reference standards approach.

The management team of the reference standards study made the following recommendations.

1. With a view to re-defining chemical classes by identifying more sub-groups or classes of chemicals, the *in vivo* data should be analysed in terms of discrimination between different mechanisms of chemical action, by focusing on the effects of chemicals on various components of the eye, and on different time-scales of effect, regardless of apparent chemical similarity.
2. The limitations of each assay should be defined in terms of chemical class. The existing *in vitro* data should be re-examined in the light of the chemicals that succeeded or failed in each method, to identify how to better define groups of chemicals suitable for testing in each *in vitro* assay.
3. The domains of application of specific methods should be narrowed, and a decision tree for method selection should be developed.
4. A database of reference standard chemicals should be established.

Tiered testing strategies

In the hazard identification of chemicals, which is different from the safety assessment of ingredients and mixtures of ingredients used in a wide range of industrial, pharmaceutical and consumer products, the purpose of testing is to classify eye irritation potential according to classification schemes defined by regulatory authorities.

In 1996, the OECD proposed a stepwise approach to hazard identification, which underwent revisions in 1998 and in 2001 (1). According to the OECD strategy, new chemicals can be classified as irritating to the eye on the basis of results from non-animal tests, including structure-activity relationships, physicochemical tests (such as the pH test), and *in vitro* tests. Testing in animals is only required as a last step to confirm negative results generated by the non-animal tests applied in earlier steps. The step-

wise process is therefore intended to reduce and refine the use of animals, but the *in vivo* test is not replaced. In the EU, a tiered testing strategy for eye corrosion/irritation is being proposed for incorporation into Annex V of *Directive 67/548/EEC*. As with tiered testing for skin corrosion, this could be achieved during 2002, by means of the 29th Adaptation to Technical Progress of the directive (Juan Riego-Sintes, personal communication).

Computer-based simulations of the OECD approach, involving real data and prediction models, have shown that testing strategies based on the stepwise use of SARs, the pH test, an *in vitro* test, and the Draize test, can provide a satisfactory means of identifying eye irritants, while at the same time reducing and refining the use of rabbits (7, 65).

During the development and validation of a non-animal method intended for use as a screen in a stepwise testing strategy, it should be sufficient that the test can place chemicals into two or more categories of eye irritation potential, without generating too many false-positive results. There is less concern about the generation of false-negatives, because these would be identified by the animal test(s) carried out in the last step of the process. The revised OECD TG 405 (acute eye irritation/corrosion) is not in itself mandatory in OECD Member Countries, but it may become mandatory in an individual Member Country, if the regulatory authority of that country so decides.

The breakout group on *Acute Toxicity Testing Local: Skin and Eye* at the First International Symposium on Regulatory Testing and Animal Welfare, held in Ottawa, Canada, in June 2001, concluded that, although the use of screening tests is very common in industry, this is not necessarily identical to the use of non-animal tests in the OECD stepwise strategy or in the Globally Harmonised System for classification. *In vitro* alternatives are extensively used by industry for product selection, but in-house experience is not being shared.

The development and implementation, in a regulatory testing framework, of appropriate testing strategies for eye irritation, which limit the use of the Draize rabbit test to the final step, are critically dependent on the availability of one or more scientifically validated *in vitro* tests for inclusion in the testing strategy. Therefore, in the short-term, the *in vitro* tests currently being used in-house must be demonstrated to be valid for the purposes to which they are being used, or new *in vitro* tests will need to be developed and validated.

Research on the mechanisms of eye irritation

The COLIPA Eye Irritation Task Force has developed a research programme in collaboration with external academic partners, and funded by the cos-

metics industry (66). The aims of this programme are to: a) identify new *in vitro* endpoints that are more predictive of the *in vivo* response of the human eye than is the Draize test; and b) define the circumstances when it is appropriate to use different types of test systems, including simple epithelial cultures and three-dimensional cultures. Experiments will be conducted on human corneal cell cultures, rabbit corneas (from abattoirs), and human corneas which are unsuitable for transplantation, taking into account the experience obtained from earlier in-house research and from previous validation studies.

The International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) is also organising activities on alternative tests for eye irritation, such as the development of a mechanistically based eye-irritant classification scheme (67). Further information is available from the ILSI Web site: <http://hesi.ilsil.org>.

The acceptance of alternative methods by national regulatory agencies

ECVAM has conducted a survey to establish the uses for which alternative methods for eye irritation are formally accepted by regulatory authorities in the EU Member States. The outcome of the study revealed the following.

1. In Belgium, the use of the BCOP test is accepted by the Pharmaceutical Commission for the identification of drug formulations that are eye irritants. For pesticides, the *in vivo* methods in Annex V of *Directive 67/548/EEC* are generally used, although positive results obtained in *in vitro* tests are also considered. For new chemicals, only the methods defined in Annex V are accepted, whereas, for existing chemicals, literature data are accepted, if the results are positive and considered to be adequate. To date, very few *in vitro* data have been received for the notification of new chemicals, and *in vitro* data have never been received for the registration of pesticides or biocides.
2. In Germany, the BgVV accepts *in vitro* data obtained from the HET-CAM test for the classification and labelling of severely eye-irritating chemicals. For severe eye irritants, classifications may also be based on the results obtained in the BCOP, the isolated rabbit eye (IRE) and the ICE tests. In addition, chemicals that are corrosive to the skin or that are very acidic or basic (low or high pH in solution) can be classified as severely irritating to the eye, without additional *in vitro* or *in vivo* testing.
3. In The Netherlands, the regulatory authorities accept the ICE test for the screening of severe

- eye irritants. The same applies to the BCOP and other tests based on isolated eyes, although these studies are rarely submitted to the Dutch Competent Authority. Although data from the HET-CAM test have not yet been submitted, this test will be accepted for the classification of severe eye irritancy, provided that the chemical under investigation belongs to classes of chemicals for which the model has been shown to be relevant. Since the neutral red release (NRR) assay and the agarose-diffusion method have no clear prediction models for severe eye irritation, which could result in the overprediction or underestimation of R41 labelling, the Dutch Competent Authority has a preference for the other methods (isolated eyes, isolated corneas and HET-CAM). No *in vitro* method is accepted as a stand-alone test for the classification of irritating (R36) and non-irritating substances.
4. In the UK, the Health & Safety Executive (HSE) accepts the use of the IRE, BCOP and HET-CAM assays for the detection of severe eye irritants. These *in vitro* tests are acceptable for the testing of new and existing substances, as well as biocides. The position of the HSE is that, where a positive result is obtained, the substance is considered a severe eye irritant (R41), and no further testing can be justified, on animal welfare grounds. Where a negative result is obtained, an *in vivo* test may be required. The HSE prefers that tests are conducted in a GLP environment, and that the full test report and study protocol are provided in the regulatory submission.
 5. The Irish Competent Authority has accepted *in vitro* data from the BCOP assay. *In vitro* data generated by other tests have not yet been submitted to the Authority.
 6. In Finland, there is no official position on the general acceptance of methods other than those mentioned in Annex V of *Directive 67/548/EEC*. However, the reliability and relevance of non-validated *in vitro* methods are assessed on a case-by-case basis. In particular, if an *in vitro* test indicated that a chemical is irritating to the eye, the chemical could be classified as an eye irritant on the basis of this test result. However, it is unlikely that a chemical would be classified as non-irritating on the basis of an *in vitro* method alone: a negative finding would have to be confirmed by using a method included in Annex V. The Finnish Competent Authority also stresses the importance of conducting all non-clinical health and environmental safety studies for regulatory purposes in accordance with GLP principles.
 7. In France, the BCOP, HET-CAM, IRE and ICE tests are accepted for positive classification (R41 with EU classification system or irritant category 1 with the Globally Harmonised System). If a method were able to differentiate a moderate degree of irritation, an R36 classification could be envisaged. In this case, the French authorities would require extensive documentation of the method in a validation process. The NRR assay and the agarose-diffusion method are accepted for the evaluation of cosmetic products.
 8. The Greek Competent Authority only accepts official testing methods that are included in Annex V.
 9. The Spanish Competent Authority stated that no *in vitro* data have up to now been submitted to them. Its position is not to accept data from alternative methods, unless considerable experience with the methods exists, or they have been validated and officially accepted. In very special cases, however, they would be prepared to consider the *in vitro* data.
 10. Denmark has not yet officially accepted any specific alternative method for the prediction of eye irritation, but positive results from alternative methods have already been used, together with results from *in vivo* experiments.
 11. In Sweden, the National Chemicals Inspectorate is prepared to accept *in vitro* data in combination with existing *in vivo* data, pH measurements and SAR data in an integrated testing strategy, such as the one proposed by the OECD (the test strategy attached to OECD TG 405) on a case-by-case basis. However, the National Chemicals Inspectorate has not yet been faced with this situation. For cosmetics, the Medical Products Agency applies the Scientific Committee on Cosmetic Products and Non-food Products (SCCNFP) *Notes of Guidance for Testing of Cosmetic Ingredients for their Safety Evaluation* (68).
- It is important to note that all the National Competent Authorities are waiting for the relevant bodies, such as ECVAM and other Commission services, and/or the OECD, to validate, accept and publish test guidelines on alternative methods, to facilitate acceptance of the *in vitro* methods.

Eye Irritation: Summary, Conclusions and Recommendations

Progress in the validation of alternative tests for eye irritation has been hampered by a lack of *in vivo*

data of sufficient quality for use in validation studies. However, data from a number of tests, including the BCOP, HET-CAM, IRE and ICE tests, are already accepted by national regulatory authorities, on a case-by-case basis, for the identification of severe eye irritants. Furthermore, the tiered testing of eye irritation is accepted by OECD Member Countries as a means of reducing and refining the use of the Draize eye test in rabbits.

Short-term prospects

1. The acceptance during 2002 by the OECD Council of a revised TG 405 (acute eye irritation/corrosion), which includes as a supplement the OECD tiered testing strategy for eye irritation and corrosion.
2. Further investigations on the applicability of the reference standard approach (see above).
3. The validation of QSARs and/or expert system rulebases for eye irritation.

Recommendations

In the short term, EU national regulatory authorities should consider harmonising their positions on the acceptance of the BCOP, HET-CAM, IRE, ICE, and other non-animal tests for eye irritation. It is therefore recommended that ECVAM should commission weight-of-evidence reviews on the use of the BCOP, HET-CAM, IRE and ICE tests, for eventual consideration by the ESAC.

In the medium-term and long-term, the application of genomics and/or proteomics technologies could lead to the identification of promising new endpoints for eye irritation, provided that attempts were made to relate the genomics/proteomics data to *in situ* eye-irritation endpoints. Since more than one tissue of the eye is usually injured, the integrated use of different types of test should be explored. In particular, it would be useful to include a test that measures the time-course and degree of recovery of the eye. Time-to-recovery is a piece of information that affects classification and labelling.

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