ECVAM key area topical toxicity: Update on activities

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Abstract
The European Cosmetics Directive and REACH have accelerated the need for alternative tests. Especially the animal testing ban of cosmetic ingredients from 2009 strongly impacts on the timely delivery of relevant methods. ECVAM's key area "topical toxicity" focuses on skin and eye irritation/corrosion, phototoxicity and percutaneous absorption. Regulatory accepted alternative tests are available for skin corrosion, phototoxicity and percutaneous absorption. In the area of skin irritation, ECVAM recently finalised a study that demonstrates the scientific validity of a human reconstituted skin model to fully replace the animal test. In contrast, eye irritation testing will probably require an integrated testing strategy combining different in vitro assays that altogether might replace the animal test. To this end, ECVAM has contributed to the recent validation of organotypic models for detecting ocular corrosives and severe irritants. In parallel, four promising cytotoxicity- and cell-function-based assays are under evaluation by ECVAM. Finally, ECVAM is planning a prospective validation study on two human reconstituted tissue models. Moreover, the key area contributed to the implementation of the REACH legislation by leading the expert group on skin/eye irritation producing technical guidance for industry on toxicity testing under REACH.

Keywords: skin irritation, eye irritation, topical toxicity, validation, ECVAM

1. Introduction
Directive 2003/15/EC, the seventh amendment to the Cosmetics Directive prohibits testing of finished products on animals from 2004 and of ingredients by 2009. This animal testing ban is reinforced by marketing bans of cosmetics tested on animals from 2004 (finished products), 2009 (acute effects) or 2013 (repeated-dose toxicity, toxicokinetics, reproductive toxicity; EU, 2003).

With the REACH legislation, the EU also promotes alternative methods for safety testing (whereas 1 and article 1). In addition, REACH article 25 states that animal testing must be used as a last resort, which encourages the exploitation of useful alternative methods to the absolute maximum. Article 13 states, that information on hazards (especially reg. positives) and risks may be generated even by alternative methods that have not yet been taken up as official regulatory test methods. However, such methods must fulfil the requirements of Annex XI, i.e. must be at least sufficiently well developed according to internationally accepted test development criteria (i.e. ECVAM criteria for entering pre-validation). If such methods are moreover validated, they may be used for identifying positives as well as negatives. At EU level, these two legislations have accelerated the development and optimisation of alternative methods (EU, 2006).

The areas which are coordinated and managed by ECVAM in the field of topical toxicity comprise skin and eye irritation, skin corrosion, phototoxicity and percutaneous absorption.

All these human health effects fall under the 2009 deadline of the 7th amendment and the endpoints on skin and eye irritation/corrosion are part of the standard information required in REACH and could lead to testing, using in vitro methods, of up to 20,000 existing chemicals which are marketed in volumes between 1 and 10 tonnes per year (EU, 2006).

In addition, the key area provides technical support to the policy Directorate-Generals of the Commission, such as DG Enterprise and DG Health and Consumer Protection, in relation to Directive 76/768/EEC on the approximation of the laws of the Member
States relating to cosmetic products, and subsequent amendments, and to the Scientific Committee on Consumer Products (SCCP), respectively.

Similarly, the key area provided technical support in the Reach Implementation Project (RIP) 3.3 on the development of Technical Guidance Documents for industry, by chairing the endpoint working group on skin and eye irritation/corrosion, respiratory irritation, contributing to this draft, and by participating in the Drafting Group to finalise the complete guidance document (Anonymous, 2007).

2. Skin irritation/corrosion

The ECVAM skin irritation validation study (SIVS) started in November 2003 and ended successfully in May 2006 with the last Management Team meeting of the study. Further to peer-review of the study by the ECVAM Scientific Advisory Committee (ESAC), in April 2007, ESAC issued a statement on the scientific validity of the EPISKIN™ assay as being a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint is evaluated by MTT reduction, and for being used as a replacement for the Draize Skin Irritation Test (OECD TG 404 & Method B.4 of Annex V to Directive 67/548/EEC) for the purposes of distinguishing between R38 skin irritant and no-label (non-skin irritant) test substances (ESAC, 2007a). The IL-1α endpoint was regarded as a useful adjunct to the MTT assay, as it has the potential to increase the sensitivity of the test, without reducing its specificity. This endpoint could be used to confirm negatives obtained with the MTT endpoint.

The EpiDerm™ model was considered to reliably identify skin irritants due to its high specificity, but negative results might require further testing [e.g. according to the tiered strategy, as described in the OECD TG 404, (OECD, 2004a)]. The ESAC recommended that improvement of the EpiDerm™ protocol should be made to increase the level of sensitivity.

Several background documents to the study are available on the ECVAM website (http://ecvam.jrc.ec.europa.eu under "Download study documents") and the manuscripts on the validation study, as well as on the chemicals selection, were published (Spielmann et al., 2007; Eskes et al., 2007).

An EU test method guideline on human skin model assays for skin irritation testing was drafted and submitted to the EU National Coordinators of Testing Methods. Submission of the test guideline by the Commission at OECD level is also foreseen whether in parallel, or after regulatory acceptance of the method in Europe.

Follow-up studies to the SIVS are currently being planned such as e.g., the evaluation of the between-laboratory reproducibility of the second endpoint IL-1 alpha release and the identification/optimisation/development of alternative methods which could classify chemicals according to the Globally Harmonised System for classification and labelling of dermal irritancy, i.e. as strong (category 2), mild (category 3) and non-irritants (no category).

2.1 Similar tests

With regard to the validation of similar tests to the validated ones for the purposes of skin irritation and/or skin corrosion testing, several other reconstituted human epidermis models are at different stages of evaluation/validation:

The SkinEthic human epidermal model was endorsed by ESAC as being able to distinguish between corrosive and non-corrosive chemicals within the context of the OECD Test guideline 431 in 2006 (ESAC, 2006). The same model but applied to skin irritation testing will be evaluated in a joint validation project by ECVAM's topical toxicity key area and the reference laboratory CORRELATE during 2008.

The EST-1000 skin irritation assay (Cellsystems) is currently undergoing an external catch-up validation study and the results will be submitted to ECVAM. The same model, but applied to skin corrosion testing is currently being peer-reviewed by ESAC.

2.2 Research

To further the development of mechanistic parameters, ECVAM assessed two promising new predictive technologies in the framework of its in-house research: toxicogenomics as well as toxico-metabonomics. The outcome of the toxicogenomics approach was published in 2007 (Borlon et al., 2007). The study investigated the expression of 240 genes following subcytotoxic treatments of 4 skin irritants and 4 non-irritants and used the validated human reconstituted epidermis model EPISKIN™. Results indicated that about 50 genes showed differential expression for at least one test chemical when compared to the seven others. Among these, there were genes taking part in stress response, cell signalling, inflammation, protein metabolism, etc. Interestingly, it could be observed that the genes found to be significantly upregulated are not direct inducers of apoptosis, stressing that the genes identified are important in the relevant processes triggered by chemically induced trauma, i.e. inflammation or mild tissue remodelling, including cell division. It was also possible to discriminate specific compounds effects from more general ones. Sixteen genes were down-regulated in the tissues exposed to irritant compounds and up-regulated, or unchanged in tissues exposed to non-irritant compounds when compared to control tissues. These genes could represent useful biomarkers of transcriptomic response triggered by irritant chemicals and could serve to discriminate irritant from non-irritant chemicals.

Although the first results are promising, only 8 chemicals were tested. Expanding the number of chemicals assessed will show whether such
3. Eye irritation

ECVAM has focused its work programme in the area of eye irritation on:
- the recommendations on the necessary efforts to progress alternative methods validation and animal test replacement for eye irritation (Eskes et al., 2005) outlined in the report on "Alternative (Non-animal) Methods for Cosmetics Testing: Current Status and Future Prospects" (Eskes & Zuang, 2005)
- the recommendation from the ECVAM Task Force on Eye Irritation which met several times since its establishment in June 2004.

Substantial progress has been made in the different areas of activities as described below. In addition ECVAM is working in close collaboration with ICCVAM, COLIPA and industry in general in order to streamline and harmonise global validation efforts and avoid duplication of work.

3.1 Evaluation of in vitro assays

Two organotypic assays, the Bovine Corneal Opacity and Permeability (BCOP) assay, and the Isolated Chicken Eye (ICE) test were endorsed by ESAC in April 2007 for the identification of severe irritants (ESAC, 2007b). Such statement was based on the results and conclusions from the ICCVAM-led retrospective study carried out in collaboration with ECVAM from 2003-2006. Following the ESAC statement, EU Test Guidelines on the two assays are currently under preparation. For the two other organotypic assays evaluated, the Hen's Egg Test on the Chorio-allantoic Membrane (HETCAM) assay and the Isolated Rabbit Eye (IRE) test, ESAC requested that further work was performed before a statement on their validity to identify severe eye irritants could be made. It is important to note nevertheless that all four have been accepted already in 2004 by EU competent authorities for the identification of severe eye irritants (EC, 2004). With regard to the evaluation of these four organotypic assays for identifying mild or non irritants, a retrospective validation of the collected data is foreseen.

A retrospective validation of four cytotoxicity- and cell function-based assays, i.e., the Neutral Red Release, the Red Blood Cell test, the Fluorescein Leakage assay and the Cytosensor Microphysiometer was initiated in October 2005 and is expected to be finalised by early 2008. The study is coordinated by an International Validation Management Group, and is based on the retrospective collection of existing data compiled according to the ECVAM Modular Approach to Validation and Weight-of-Evidence principles (Hartung et al., 2004; Balls et al., 2006).

Based on the final results, i.e. if there is sufficient evidence of sufficiently high quality to support the validity of the methods, the assays may proceed towards ESAC for peer review.

Two Human Reconstituted Tissue models, the SkinEthic Human Corneal Epithelium and the EpiOcular™ OCL-200 model were positively reviewed by the ECVAM Eye Irritation Task Force which recommended protocol improvements and the organisation of a workshop to plan for a formal validation study. The two assays are currently undergoing protocol optimisation and assessment in a multi-industrial trial coordinated by COLIPA. When available, the findings from the multi-industrial trial will be considered for the planning of an ECVAM prospective validation study. In the meantime, ECVAM is setting up the necessary actions groups (validation management team, chemicals selection group) required to perform the formal and independent validation trial.

Additional assays which were submitted to ECVAM are also currently under evaluation. These encompass the Low Volume Eye Test (LVET) as a refinement assay, the Ocular Irritection assay and the Slug Mucosal Irritation (SMI) assay amongst others. A first submission of the LVET was received in 2003. Following the ECVAM request for further information and the recommendations from the
ECVAM Task Force on Eye Irritation, a final dossier based on retrospective evidence compiled according to the Modular Approach was submitted to ECVAM by the test proposer in February 2007. The assay is currently under peer-review by ESAC. With regard to the Ocular Irritation assay, a dossier based on retrospective and prospective information is currently being prepared according to the Modular Approach to Validation by the test method proposer. Finally, for SMI assay the results of a prevalidation study co-organised by university and industry, have just been submitted to ECVAM for review and consideration.

3.2 Testing Strategies
Promising test strategies for eye irritation have been suggested during an ECVAM Expert Meeting comprising more than 30 participants from industries, CROs, regulators, academia and welfare organisations (Scott et al., in preparation). The performances and applicability domains which will be determined in the evaluation of individual alternative methods, as mentioned above, will be combined based on the proposed strategies and evaluated in order to determine the most suitable strategies that utilise the strengths of specific in vitro assays to classify test substances according to their irritation potential.

3.3 Mechanistically-based assays
Although the assays currently under evaluation appear promising for specific purposes and applicability domains, they may not model completely all aspects and mechanisms of ocular toxicity in vivo, e.g. tissue remodelling, modelling the full inflammatory response. To achieve full replacement of the animal test, it is recommended beside the use of test strategies, to advance in parallel the development of mechanistically-based models in order to address the currently existing mechanistic gaps (Eskes et al., 2005).

In May 2005, a joint ICCVAM-ECVAM symposium was organised on "Mechanisms of Chemically-Induced Ocular Injury and Recovery" where 76 scientists from governmental institutions, academia and industry participated. The participants discussed and identified those mechanistic aspects of ocular irritation where further investigation and development are required (Hamernik et al., 2006). Currently, the COLIPA eye irritation research program addresses some of these mechanistic features (Jones et al., 2006). In addition, individual efforts exist within the scientific community to develop more complex test systems and biomarkers for eye irritation. ECVAM is closely following these efforts to ensure that the most mechanistically relevant assays can proceed towards validation according to internationally agreed principles and eventually contribute, in combination with the most advanced assays to the full replacement of the animal test.

4. Acute phototoxicity
Further to an international EU/ECVAM/COLIPA validation exercise it was proven that the phototoxic potential of chemicals in humans can correctly be predicted by the 3T3-NRU in vitro phototoxicity test (3T3-NRU-PT; Spielmann et al., 1994; Spielmann et al., 1998). This in vitro test gained regulatory acceptance in all EU Member States in 2000 (EC, 2000) and in the OECD Member States in 2004 (OECD, 2004b). The test is now widely used in the chemical and cosmetic industries.

Subsequently, the usefulness of reconstructed human skin model assays as a supplementary assay to the 3T3-NRU PT test was demonstrated in several studies (Jones et al., 2003; Lelièvre et al., 2007; Liebsch et al., 1995; Liebsch et al., 1997; Liebsch et al., 2005; Portes et al., 2002).

These studies showed that a true phototoxic potential of a chemical which is correctly predicted in the 3T3-NRU-PT may be modified and modulated when applied topically to the skin at low concentrations (e.g. in formulations) and additional information on phototoxicity linked to barrier function, tissue distribution and bioavailability of the chemical in the skin may be needed.

The reconstructed human skin model assays represent a useful tool for confirmation of non-phototoxic and phototoxic samples identified by the 3T3 NRU PT. However, further investigations are currently on-going by testing a wider range of chemicals.

5. Skin absorption/penetration
In vitro tests for the determination whether a chemical penetrates through skin received regulatory acceptance at OECD level in 2004 (OECD, 2004c) and were introduced into Annex V through the 3OTATP at EU level in 2007. In vitro methods for skin absorption measure the diffusion of chemicals across excised human or pig skin in flow through or static diffusion cells. An OECD guidance document for the conduct of skin absorption studies describes the circumstances in which the use of the in vitro method would be appropriate (OECD, 2004d).

In 2007, preliminary results on an in vitro test based on reconstituted human epidermis (RHE) models were submitted to ECVAM for evaluation.

The advantages of this test method are the adequate availability of RHE models, the shorter duration of the experiments and possibly also the investigation of skin metabolism in skin tissue of human origin (Schäfer-Korting, 2006).

6. Policy support
6.1 Directive 76/768/EEC on the regulation of cosmetic products
Scientific and technical support is continuously provided to ECVAM's customer Directorate Generals
International Cooperation on Cosmetics Regulation

the Cosmetics Directive, and in the recently created
the validation and legal acceptance of alternative methods
work is further described in Hoffmann
concepts in the design and evaluation of ITS. The
ECB have been developing ITS for skin irritation,

further develop endpoint-specific ITS, ECV AM and
potential costs (e.g. consequence of incorrect
in more detail, and to assess their impacts, including
all REACH endpoints. Since these strategies are
complete guidance document.

Toxicity chaired the endpoint working group on skin
health and environmental endpoints.

Guidance Documents for industry on eleven human
Project (RIP 3.3-2) on the development of Technical

In the REACH guidance documentation, Intelligent
recommendations to its policy DGs, especially with
regard to cosmetics testing.

6.2 REACH

ECVAM steered the REACH Implementation Project (RIP 3.3-2) on the development of Technical Guidance Documents for industry on eleven human health and environmental endpoints.

In particular, the ECVAM key area on Topical Toxicity chaired the endpoint working group on skin and eye irritation/corrosion and respiratory irritation and participated in the Drafting Group to finalise the complete guidance document.

In the REACH guidance documentation, Intelligent Testing Strategies (ITS) have been developed for all REACH endpoints. Since these strategies are described at a general level, considerable research is still needed to develop endpoint-specific strategies in more detail, and to assess their impacts, including their benefits (e.g. reduced animal testing) and potential costs (e.g. consequence of incorrect predictions). As an illustration of research needed to further develop endpoint-specific ITS, ECVAM and ECB have been developing ITS for skin irritation, as a means of exploring the applicability of novel concepts in the design and evaluation of ITS. The work is further described in Hoffmann et al. (2008).

7. Conclusions

Important progress was made in the development, validation and legal acceptance of alternative methods in the field of topical toxicity. Indeed, many successes took place in this field during the last seven years and most of the current validated and accepted alternative methods belong to the field of topical toxicity. These are methods for skin corroboration, phototoxicity and skin penetration and more recently, the successful ECVAM validation study, where a full replacement method to the regulatory animal test was validated for the purposes of skin irritation testing.

In the field of eye irritation, an ambitious programme was set up in 2004 in order to be able to meet the deadline of 2009 imposed by the 7th amendment to the Cosmetics Directive. Twelve methods are currently under validation and test strategies combining the different tests according to their strengths and applicability domains were identified. The scientific validity of in vitro methods for the identification of severe eye irritant chemicals were endorsed by ESAC in April 2007.

The next 2-3 years will be dedicated to the validation of in vitro methods for the other ranges of eye irritation, i.e. mild and non-irritant. Once the evaluations of the individual assays will be completed, the results will be combined and statistically evaluated in order to determine the most suitable strategies to classify test substances according to their irritation potential and ultimately replace the Draize rabbit eye test.

With regard to policy support, the key area will continue to provide technical and scientific recommendations to its policy DGs, especially with regard to cosmetics testing.

References


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