

# **Alternative (Non-animal) Methods for Chemicals Testing: Current Status and Future Prospects**

**A Report prepared by ECVAM and the  
ECVAM Working Group on Chemicals**

*Edited by  
Andrew Worth and Michael Balls*

*European Centre for the Validation of Alternative Methods,  
Institute for Health & Consumer Protection,  
European Commission Joint Research Centre, 21020 Ispra (VA), Italy*



# Preface

## List of Contributors

### Members of the ECVAM Staff

Michael Balls  
Alessia Bogni  
Susanne Bremer  
Silvia Casati  
Sandra Coecke  
Chantra Eskes  
Pilar Prieto  
Enrico Sabbioni  
Andrew Worth  
Valérie Zuang

### Members of the ECVAM Working Group on Chemicals

Martin Barratt	Marlin Consultancy, Bedford, UK
Bas Blaauboer	Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
Philip Botham	Syngenta, Macclesfield, UK
Robert Combes	Fund for the Replacement of Animals in Medical Experiments (FRAME), Nottingham, UK
Johannes Doehmer	GenPharmTox BioTech AG, Munich, Germany
Julia Fentem	Safety and Environmental Assurance Centre, Unilever, Sharnbrook, UK
Manfred Liebsch	ZEBET, Berlin, Germany
Horst Spielmann	ZEBET, Berlin, Germany

### Other External Partners

David Basketter	Safety and Environmental Assurance Centre, Unilever, Sharnbrook, UK
Richard Clothier	School of Biomedical Sciences, University of Nottingham, Nottingham, UK
Mark Cronin	School of Pharmacy & Chemistry, Liverpool John Moores University, Liverpool, UK
Per Garberg	Biovitrum, Stockholm, Sweden
Nicola Gilmour	Safety and Environmental Assurance Centre, Unilever, Sharnbrook, UK
Rosalind Hanway	Health & Safety Executive, Bootle, UK
Ian Kimber	Syngenta, Macclesfield, UK
Camilla Pease	Safety and Environmental Assurance Centre, Unilever, Sharnbrook, UK
Walter Pfaller	Institute of Physiology, Innsbruck University, Innsbruck, Austria
Barry Phillips	Royal Society for the Prevention of Cruelty to Animals (RSPCA), Horsham, UK
Vera Rogiers	Vrije Universiteit, Brussels, Belgium
Helmut Tritthart	University of Graz, Graz, Austria
Erik Walum	Biovitrum, Stockholm, Sweden

## Overview

The principal aim of this report is to summarise the current status of alternative tests in contributing to the assessments of the potential toxicological (human health) effects of substances, as currently required by European Union (EU) legislation on chemicals. These assessments are also likely to be required when the proposed EU Chemicals Policy is implemented. The effects covered are: acute lethal toxicity; dermal and ocular irritation and corrosion; skin and respiratory sensitisation; target organ and target system toxicity; genotoxicity and carcinogenicity; reproductive toxicity; and the biokinetic endpoints of absorption, distribution and metabolism. The emphasis of the report is on methods that can be used to replace or partially replace existing animal-based tests. For each toxicological effect, the report identifies alternative methods that can be used immediately, either because they have been scientifically validated as definitive tests, or because they are considered to be sufficiently well-established for use in the prioritisation of further testing. For most of the toxicological effects considered, the incorporation of alternative methods into tiered testing strategies offers the most immediate promise for replacing, reducing and refining the use of animals, without compromising the protection of human health. In some cases, such as dermal and ocular irritation and corrosion, tiered testing strategies can be implemented immediately.

The second main aim of the report is to make recommendations for the further development and validation of alternative tests and testing strategies, with emphasis on the expectations that could realistically be met in the short-term (by the end of 2003), medium-term (end of 2006), and long-term (end of 2010), if sufficient and appropriate human and financial resources were made available. This time-frame takes into account the duration of the forthcoming Sixth and Seventh EU Framework Programmes.

The report was compiled by the European Centre for the Validation of Alternative Methods (ECVAM), on the basis of contributions from a large number of experts in the fields of toxicity testing and alternative methods, including members of the ECVAM staff, the ECVAM Working Group on Chemicals and associated working groups, and other experts in the field.

Chapter 1 summarises the background to the future Chemicals Policy, by referring to the main recommendations of the European Commission White Paper, to the conclusions of the European Environment Council, and to the opinions of the European Parliament and its Economic and Social Committee. This chapter also explains the role that ECVAM has played in the formulation of the policy, and the role that ECVAM is expected to play in its future implementation.

This report refers to a number of completed and proposed prevalidation and validation studies. Therefore, Chapter 2 provides background information on the principles of validation, and on the way in which these principles are applied by ECVAM, which has the duty of coordinating the validation of alternative tests at the EU level. This chapter also explains the important concept of the prediction model, which enables the data generated by alternative tests to be interpreted in terms of potential toxic hazard either in whole animals or in humans.

Chapter 3 describes the risk-assessment process, in which the use of alternative tests is becoming increasingly significant. The concepts of hazard prediction and risk assessment are explained, and the possible uses of alternative tests in the various components of the risk-assessment process are described. In addition, reference is made to the procedures of reverse risk assessment and read-across, which can play a useful role in reducing the need for animal testing.

The general aim of Chapters 4–10 is to address the current status of alternative tests in different areas of toxicity testing. Each chapter includes an assessment of the short-term, medium-term and long-term possibilities for the development and validation of alternative tests and testing strategies.

Chapter 4 deals with acute lethal toxicity testing, which is conducted as part of acute toxicity testing, and as a basis for making decisions on the need for further testing. There is a good medium-term prospect for the (partial) replacement of the classical LD50 test and other equivalent animal tests, since a validation study on the use of basal cytotoxicity tests (for certain kinds of chemicals) is to be initiated in 2002, under the auspices of ECVAM and the US NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), which will take into account long experience in the use of *in vitro* cytotoxicity tests and the positive outcomes of a number of evaluation studies. In this chapter, the outline of a tiered testing approach is also recommended.

Chapter 5 summarises the status of alternative tests for acute dermal and ocular irritation and corrosion. Significant progress has already been made with respect to skin corrosion, since guidelines based on two *in vitro* tests have been accepted at the EU and Organisation for Economic Cooperation and Development (OECD) levels as replacements for the Draize skin-corrosion test. Several alternative tests for skin irritation have been evaluated in an ECVAM prevalidation study, which is a high priority for follow-up in 2002. For eye irritation, it has not proven possible to formally establish the scientific validity of one or more replacement tests, applicable across the full range of eye-irritation potency, despite a significant validation effort in this area. Thus, eye irritation remains a priority area for further research, test development and validation.

Chapter 6 covers dermal and respiratory sensitisation, for which non-animal methods have yet to be validated. Considerable progress has been made in increasing the understanding of the biological mechanisms underlying the sensitisation response, and a number of promising computer-based and *in vitro* systems are in the course of development. Thus, sensitisation is also a priority area for further research, test development and validation. In the meantime, an animal-based (refinement and reduction) alternative, the murine local lymph node assay (LLNA), is regarded as scientifically valid for skin sensitisation testing.

Chapter 7 addresses the various biokinetic endpoints that relate to the absorption, distribution and metabolism of chemicals. Biokinetic parameters are important determinants of systemic toxicity, and their incorporation into biologically based kinetic models will ultimately provide efficient tools for risk-assessment purposes. This chapter refers to a number of promising computer-based and *in vitro* models of membrane permeability that are considered ready for validation or regulatory acceptance and application. For the assessment of metabolism, both computer-based and *in vitro* approaches are discussed, and a tiered approach is recommended, based on the sequential analysis of: a) enzyme pathways, including enzyme activation and inhibition; b) induction effects; and, where appropriate, c) enzyme polymorphisms.

The large and diverse area of target organ and target system toxicity is examined in Chapter 8, which refers to nephrotoxicity and neurotoxicity by way of illustration. For effects such as these, it is clear that stepwise testing strategies, based on the use of complementary *in vitro* endpoints, will need to be designed and evaluated, and this will require a substantial and long-term effort. For neurotoxicity testing, a tiered approach based on the sequential assessment of basal cytotoxicity and neurospecific endpoints is recommended, and is approaching readiness for prevalidation. Chapter 8 also describes developments in the field of *in vitro* repeat-dose toxicity testing, which is recommended as a priority area for future research.

Genotoxicity and carcinogenicity are covered in Chapter 9, which presents the outline of a tiered testing strategy based on computer-based models and *in vitro* tests for detecting point mutations, clastogenicity, aneuploidy and non-genotoxic carcinogenicity. It is argued that genotoxic chemicals should automatically be regarded as carcinogenic, to avoid the need to conduct time-consuming and

expensive rodent studies. Efforts should be focused on the development and validation of *in vitro* methods for the detection of non-genotoxic carcinogens, and on the validation of computer-based models for predicting genotoxicity and carcinogenicity.

Chapter 10 discusses alternative methods for reproductive toxicity testing. This is another area in which testing strategies will need to be based on a number of critical and complementary *in vitro* endpoints, to provide a means of assessing the effects on different components of the reproductive cycle. Encouraging progress has been made in the area of developmental toxicity, since three alternative methods for predicting embryotoxic potential have been endorsed by the ECVAM Scientific Advisory Committee as scientifically valid and ready for consideration for regulatory acceptance and application. Future efforts should focus on the further development and validation of alternative tests for assessing other critical aspects of the male and female reproductive systems, and on the design and evaluation of testing strategies that incorporate these tests.

Chapter 11 discusses the current status of animal and non-animal methods for the detection of so-called “endocrine disruptors”, i.e. chemicals that alter the function of the endocrine system and cause adverse health effects. International activities initiated by the US Environmental Protection Agency (EPA) and the OECD are described, with particular reference to three animal tests: the uterotrophic test, the Hershberger test and an enhanced repeat-dose protocol (OECD Test Guideline 407). In addition, various non-animal approaches are described, including computer-based and *in vitro* methods. This is an area in which more research and development are required, so that suitably developed and validated non-animal tests can eventually be incorporated into tiered testing strategies that will reduce, refine and replace the use of animals.

Finally, Chapter 12 provides a summary of the current situation in relation to each toxicological endpoint, and brings together the main conclusions and recommendations presented elsewhere in the report. A distinction is made between prospects for validation on the one hand, and recommendations for research and test development on the other. In addition, a number of recommendations are directed at specific organisations or expert groups, and some general recommendations are made for the use of alternative methods in the future EU Registration, Evaluation and Authorisation (REACH) system.



# Contents

---

<b>Chapter 1: Background</b> .....	1
<i>The White Paper on a Strategy for a Future Chemicals Policy</i> .....	1
Follow-up to the White Paper .....	2
<i>Directive 86/609/EEC</i> and ECVAM .....	2
Alternative Methods and Their Application .....	2
The Role of ECVAM in the Formulation of the EU Chemicals Policy .....	5
The Role of ECVAM in the Implementation of the EU Chemicals Policy .....	7
Appendix 1.1: List of ECVAM Workshops .....	10
<b>Chapter 2: The Principles and Procedures of Validation</b> .....	13
Introduction .....	13
Alternative Methods, Prediction Models and Validation .....	13
The Evolution of Alternative Methods and the ECVAM Validation Process .....	13
ECVAM's Criteria for Test Development and Validation .....	15
Practical and Logistical Aspects of Prevalidation and Validation Studies .....	16
Examples of Prevalidation and Validation Studies .....	16
The Time Required for Validation and Regulatory Acceptance .....	16
<b>Chapter 3: The Scientific Basis of Chemical Risk Assessment</b> .....	21
The Conventional Risk-assessment Paradigm .....	21
Shortcomings of Conventional Risk Assessment Approaches .....	21
Advances in the Scientific and Technological Basis for Risk Assessment .....	21
Approaches for Reducing the Amount of Testing .....	22
The Use of Alternative Methods in Hazard and Risk Assessment .....	23
<b>Chapter 4: Acute Lethal Toxicity</b> .....	27
Introduction .....	27
<i>In Vitro</i> Methods for Acute Lethal Toxicity .....	27
<i>In Vitro</i> Cytotoxicity Databases .....	27
The MEIC and EDIT projects .....	29
The International Workshop on <i>In Vitro</i> Methods for Acute Systemic Toxicity .....	30
Structure–Activity Relationships for Acute Lethal Toxicity .....	30
A Tiered Testing Strategy for Acute Lethal Toxicity .....	31
Acute Lethal Toxicity: Summary, Conclusions and Recommendations .....	32
<b>Chapter 5: Local Toxicity: Acute Dermal and Ocular Effects</b> .....	35
Introduction .....	35
Current Status of Alternative Methods for Skin Corrosion .....	35
Skin Corrosion: Summary, Conclusions and Recommendations .....	36
Current Status of Alternative Methods for Skin Irritation .....	37
Skin Irritation: Summary, Conclusions and Recommendations .....	38
Current Status of Alternative Methods for Eye Irritation and Corrosion .....	38
The ECVAM Workshop on Eye Irritation and Follow-up Activities .....	40
Eye Irritation: Summary, Conclusions and Recommendations .....	43
<b>Chapter 6: Local Toxicity: Sensitisation</b> .....	49
Introduction .....	49
Current Status of Alternative Methods for Skin Sensitisation .....	49
A Tiered Testing Strategy for Skin Sensitisation .....	50
Skin Sensitisation: Summary, Conclusions and Recommendations .....	52
Current Status of Alternative Methods for Respiratory Sensitisation .....	52
Respiratory Sensitisation: Summary, Conclusions and Recommendations .....	52

## Contents continued

---

<b>Chapter 7: Biokinetics</b> .....	55
Introduction .....	55
Barrier Function .....	55
Barrier Function: Summary, Conclusions and Recommendations .....	60
Xenobiotic Metabolism .....	61
Biokinetic Modelling .....	65
Biokinetic Modelling: Summary, Conclusions and Recommendations .....	66
<b>Chapter 8: Target Organ and Target System Toxicity</b> .....	71
Introduction .....	71
Current Status of Repeat-dose Toxicity Testing .....	71
The ECVAM Workshop on <i>In Vitro</i> Methods for Long-term Toxicity Testing .....	71
Repeat-dose Toxicity: Summary, Conclusions and Recommendations .....	74
Current Status of Nephrotoxicity Testing .....	74
Nephrotoxicity: Summary, Conclusions and Recommendations .....	75
Current Status of Neurotoxicity Testing .....	75
Testing Strategies for <i>In Vitro</i> Neurotoxicity .....	76
Neurotoxicity: Summary, Conclusions and Recommendations .....	78
<b>Chapter 9: Genotoxicity and Carcinogenicity</b> .....	83
Introduction .....	83
Current Approaches to Genotoxicity Testing .....	83
Current Approaches to Carcinogenicity Testing .....	85
Tiered Testing Strategies for Genotoxicity and Carcinogenicity .....	89
Issues Requiring Further Consideration .....	89
Genotoxicity and Carcinogenicity: Summary, Conclusions and Recommendations .....	90
<b>Chapter 10: Reproductive Toxicity</b> .....	95
Introduction .....	95
Scientific Background .....	95
Current Status of Alternative Methods for Reproductive Toxicity .....	97
Projects Funded by the European Commission .....	99
Reproductive Toxicity: Summary, Conclusions and Recommendations .....	99
<b>Chapter 11: Endocrine Disruption in Humans</b> .....	103
Introduction .....	103
Scientific Background .....	103
Tests for Endocrine Disruptors .....	104
Existing Testing Strategies for Endocrine Disruptors .....	108
Discussion .....	110
Endocrine Disruptors: Summary, Conclusions and Recommendations .....	111
<b>Chapter 12: Summary</b> .....	115
Introduction .....	115
The Current Situation .....	115
Prospects for Prevalidation and Validation .....	120
Recommendations for Research and Development Activities .....	121
Recommendations to Specific Organisations .....	124
General Recommendations for the Use of Alternatives in the EU REACH System .....	125