Validation & International Regulatory Acceptance of *In Vitro* Methods for Toxicity Testing

Chantra Eskes, PhD Eng.
ESTIV president
Member of OECD expert groups
ACTIP executive secretary
SeCAM, Switzerland

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Why validation?
How many rabbits does Revlon blind for beauty’s sake?

Henri Spira

1959: The 3Rs Concept

Refinement alternatives alleviate or minimise potential pain, suffering and distress

Reduction alternatives obtain a comparable level of information from the use of fewer animals, or more information from the same number of animals

Replacement alternatives permit a given purpose to be achieved without using animals


Article 4.1: Member States shall ensure that, wherever possible, a **scientifically satisfactory method or testing strategy**, not entailing the use of a live animal, shall be used instead of a procedure (~ use of animal for experimental or other scientific purposes)

Article 13.1: Member States shall ensure that a procedure is not carried out if another method or testing strategy for obtaining the results sought, not entailing the use of a live animal, is **recognised under the legislation of the Union**.

Article 47: The Commission and the Member States shall **contribute to the development and validation of alternative approaches** which could provide the same or higher levels of information as those obtained in procedures using animals, but which do not involve the use of animals or use fewer animals or which entail less painful procedures, and they shall take such other steps as they consider appropriate to encourage research in this field.

Article 48: **European Union Reference Laboratory**
Regulatory drivers (EU)

- **Cosmetics: EU Directive 2003/15/EC (Regulation 1223/2009)**
  - Ban on animal testing for finished products (2004)
  - Ban on animal testing for cosmetic ingredients (2009)
  - Marketing ban on cosmetics tested on animals (2013)

- **New & existing chemicals ($\geq 1$ t/y): REACH Reg. 1907/2006**
  - Skin & eye irritation: *in vitro* testing only for substances 1–10 t/y
  - General rules for adaptation: *in vitro* tests (annex XI)
    - Validated assays: non- and hazardous effects
    - Suitable assays: only hazardous effects
    - Other assays: mechanistic insights
Example of Integrated Testing Strategy: Skin and Eye Irritation

1. Existing data on physico-chemical properties

2. Existing human data

3. Existing animal data from skin/eye irritation/corrosion studies

4. Existing data from acute dermal toxicity (skin: also sensitization studies)

5. Existing (Q)SAR data and read-across

6. Existing *in vitro* data

7. Weight of evidence analysis

8 & 9. New *in vitro/ex vivo* test skin and eye corrosion and irritation

9 or 10. New *in vivo* test for skin/eye irritation (annex VIII)

If *in vitro* validated full replacement(s) available
United Nations Globally Harmonized System

- From 2013 (5th revision): incorporates weigh-of-evidence evaluation
  1. existing human or animal skin corrosion/irritation data,
  2. other existing skin data in animals
  3. existing ex vivo / in vitro data (REACH: position 6 after SAR)
  4. pH-based assessment (and acid/alkaline reserve of the substance) (REACH: position 1)
  5. validated SAR methods
  6. weight of evidence

Although information can be gained from the single parameters within a tier, the totality of existing information shall be considered to make an overall weight of evidence determination, especially when there is conflict in information available on some parameters.
Mixtures: EU CLP Regulation (1272/2008)

- Introduction of GHS classification in the EU
  2010: substances, 2015: mixtures
- Use of tiered weight-of-evidence strategy encouraged
- Validated in vitro methods required to confirm classification of extreme pH formulations with alkaline or acid reserve indicating non-corrosion
CLP additivity approach: lower Generic Concentration Limits Impact on mixture classification

- Many products not previously labelled may become so under EU CLP
- Use of in vitro methods may allow to better reflect the hazard of formulations

E. g. mixture containing R41/Eye Cat. 1 ingredient

- ≥ 5% to < 10%, "Irritant" (Irritating to eyes)
- ≥ 10%, "Irritant" (Risk of serious damage to eyes)
- ≥ 3%, DANGER (Eye Cat. 1) "Causes serious eye irritation"
- ≥ 3%, DANGER (Eye Cat. 1) "Causes serious eye damage"

- ≥ 1% to < 3%, WARNING, (Eye Cat. 2) "Causes serious eye irritation"
- < 1% no label
- < 5% no label

- ≥ 5% to < 10%, "Irritant" (Irritating to eyes)
- ≥ 10%, "Irritant" (Risk of serious damage to eyes)

EU DPD

% ingr.

EU CLP

> 2015
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**Biocides: Regulation 528/2012**

- Testing on vertebrates only as a last resort
- Toxicological information required: **testing strategy, existing information, reduction and refinement assays**
- General rules for adaptation: *in vitro* tests (annex VI)
  - Validated assays: hazardous effects
  - Suitable assays: mechanistic insights
  Non-hazardous effects: confirmation may be requested on a case-by-case basis
Validation principles
2005: OECD Guidance Documents 34 on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment

34 member countries & many countries with relationship
The Validation Process

Research & Development

Pre-validation

Validation

Peer review

Regulatory Acceptance

Applications

Academia & Industry

Validation bodies, associations & test developers

Validation bodies

Regulators

Regulators & Industry

Collaboration, communication & joint progress
Definition of Validation

“…to establish the **reliability** and **relevance** of the method for a particular purpose”
General Principles of Validation

1. An alternative method can only be judged valid if two conditions are met:
   a) the method is reliable
   b) the method is relevant

2. The prediction model should be defined in advance by the test developer

3. Performance criteria should be set in advance by the management team (for a prospective validation study rather than retrospective review)

4. Performance is assessed by using coded chemicals (Phase III of prevalidation; all of validation)

5. There should be independence in:
   a) the management of the study
   b) the selection, coding and distribution of test chemicals
   c) the data collection and statistical analysis

6. Laboratory procedures should comply with GLP and GCCP criteria
Current status
Regulatory Acceptance of Alternative Methods

**OECD**

**Skin Corrosion**
- 2006: OECD TG 435
- 2013 & 2014: Revised TG 431 & 430

**Skin Sensitisation**
- 2002: OECD TG 429 (EU B.42)
- 2010: OECD TG 442A,B (EU B.50, B.51 - 2012)
- 2010: Revised TG 429
- 2015: In silico & in vitro TG 442C,D

**Skin Irritation**
- 2009: EU B.46 (OECD TG 437 - 2010)
- 2013 & 14: Revised TG 439

**Skin absorption**
- 2004: OECD TG 428 (EU B.45)

**Phototoxicity**

**Genotoxicity**
- 80's: OECD TG 471, 473, 476, 479, 480, 481, 482 (non validated) (EU B.13/14, B.10, B.17, B.19, B.15, B.16, B.18)
- 2010: OECD TG 487 (EU B.49 – 2012)
- 2014: Revised TG 487 & 473

**Acute toxicity**
- 2001: OECD TG 420 & 423 (EU B.1bis & B.1tris)
- 2008: OECD TG 425
- 2010: GD129

**Endocrine Disruptors**
- 2011: OECD TG 456 (EU B.57 - 2014)
- 2012: OECD TG 455 & 457

**Eye Irritation**
- 2011: GD 160
- 2012: OECD TG 460
- 2013: Revised TG 437 & 438

**Regulatory Acceptance of Alternative Methods**

**Chemicals**

**Phototoxicity**
Global outreach: 34 member countries + most countries with relationship

Mutual Acceptance of Data – avoid duplicative testing (reduction)

How to apply the 3Rs principles (recognition, assessment and use of clinical signs as humane endpoints) when performing OECD Test Guidelines.

Test Guidelines on Alternative Methods ((partial) replacement)
http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

Guidance Documents on Alternative methods (e.g. test strategies)
http://www.oecd.org/env/ehs/testing/seriesontestingandassessmenttestingforhumanhealth.htm
Example 1:
Skin Corrosion & Irritation
**In Vitro Skin Corrosion**

- **Validated replacement methods**
  - 1996-1998: TER & EpiSkin™
  - 2000: EpiDerm™ & Corrositex®
  - 2006: SkinEthic RHE™
  - 2009: CellSystems epiCS® (EST-1000)

- **EU acceptance**

- **OECD adoption**
  - 2004: TG 430: TER & TG 431: Human skin models
  - 2006: TG 435: *In vitro* membrane barrier test

⇒ 8 years from start of validation to regulatory acceptance
In Vitro Skin Irritation

1999-2001 Prevalidation study
2002 Optimisation studies
2003-2007 ECVAM Validation Study
EPISKIN™ SIT

2008 Catch-up validation
EpiDerm™ EPI-300-SIT
SkinEthic™ SIT42bis

2009 EU acceptance B.46
2010 OECD TG 439 adopted
2012 Revised TG 439
LabCyte EPI-MODEL24
(Japanese catch-up validation)

⇒ 7 years from start of validation
In EU: full replacement for skin irritation & corrosion

1 in vivo TG
# Skin Irritation & Corrosion Test Guidelines

## Skin Corrosion

<table>
<thead>
<tr>
<th>Human Skin Models</th>
<th>Identifies Cat. 1 / corrosives and able to distinguish GHS sub-categories 1A from a combination of sub-categories 1B-and-1C.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>TER: EU B.40 (2000), OECD TG 430 (2004)</th>
<th>Identifies Cat. 1 / corrosives but not able to distinguish GHS subcategories</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Updates ongoing regarding IATA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Membrane barrier test</th>
<th>Identifies Cat. 1 / corrosives and sub-categories 1A, 1B, 1C. In EU not yet adopted as considered valid only for acids, bases and derivatives.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OECD TG 435 (2006)</td>
<td>Test materials not causing detectable changes in CDS cannot be tested. Updates ongoing regarding IATA</td>
</tr>
</tbody>
</table>

## Skin Irritation

<table>
<thead>
<tr>
<th>RhE models</th>
<th>Identifies Cat. 2 / irritation.</th>
</tr>
</thead>
</table>
OECD Guidance Document on Integrated Approaches to Testing and Assessment (IATA) of skin corrosion & irritation

Aim: combining OECD TG 404, 430, 431, 435 and 439 with the aim to minimize the use of animal testing to the extent possible, while ensuring human safety

- Modules tackled:
  - Human data
  - Animal data
  - In vitro data on skin corrosion and irritation
  - Non-testing data (physico-chemical properties, QSARs, etc)
  - Guidance on weigh-of-evidence analyses

- **Possibility to identify non-hazard effects** if sufficient and appropriate evidence

- Applicability, benefits and limitations of the existing TGs and modules

- Suitability of the OECD TGs for mixtures and preparations

⇒ Guidance Document 203 adopted at OECD WNT meeting in April 2014
  Updates ongoing on TG 404, 430, 431, 435 & 439 accordingly
1. Existing information
   - Existing human data
   - Existing in vivo skin irritation and corrosion data (OECD TG 404)
   - Existing in vitro skin corrosion data (OECD TGs 430, 431 & 435)
   - Existing in vitro skin irritation data (OECD TG 439)
   - Other existing in vivo and in vitro data

2. Physico-chemical properties e.g., pH, acid/alkaline reserve

3. Non-testing methods
   - Substances: (Q)SAR, read-across, grouping and prediction systems;
   - Mixtures: bridging principles and theory of additivity

PART 1
Non-testing
Order may be arranged as appropriate

PART 2
Phases and elements of Weight-of-Evidence approaches

cat. 1 or 1A or 1B/1C§

in vitro skin corrosion test

not corrosive

Cat. 2

in vitro skin irritation test

not irritant

Cat. 1 or 1A or 1B/1C§

in vivo skin irritation/corrosion test

for authorities adopting UN GHS Cat. 3

Cat. 1B or Cat. 1C

in vitro skin irritation or corrosion test in method not adopted by the OECD or in vivo skin irritation/corrosion test

for authorities requiring Cat. 1B vs. Cat. 1C

Cat. 1 or 1A or 1B/1C§

in vitro skin corrosion test

Corrosive

Cat. 2

in vitro skin irritation test

irritant

Cat. 1 or 1A or 1B/1C§

NC

PART 3:
Testing

WoE
where appropriate

C&L or
NC

WoE
where appropriate

C&L or
NC
Example 2:
Skin Sensitization
Skin Sensitisation: Adverse Outcome Pathways

Skin Sensitization

Refinement assays Test Guidelines
- Reduced LLNA
  OECD TG 429 (2010) / EU B.42
- LLNA: DA (Daicel Chemical):
  OECD TG 442A (2010) / EU B.50
- LLNA: BrdU-ELISA
  OECD TG 442B (2010) / EU B.51

Validated Test Methods
1. Direct Peptide Reactivity Assay (DPRA)
2. KeratinoSens (activation of antioxidant response element (ARE) through transcription factor Nrf2)
3a. Human Cell Line Activation Test (h-CLAT)
3b. IL-8 Luc assay (Japan)
ARE-Nrf2 Luciferase test method
KeratinoSens™

<2010  Test method developed by industry: activation of the Antioxidant Response Element through the transcription factor Nrf2 - Natsch (2010, Tox. Sci. 113: 274) & Emter et al. (2010, TAP 245: 281)


2012  Independent peer-review conducted by EURL-ECVAM

2014  EURL-ECVAM recommendations

2015  OECD TG 442D adopted in February
⇒ 5-6 years from start of validation
Skin sensitisation ongoing activities

- **Other OECD activities**
  - TG on the DPRA assay → adopted also in Feb 2015 (TG 442C)
  - Draft TG on the h-CLAT assay → under discussions
  - IL-8 Luc assay → under peer-review & OECD consideration
  - OECD Integrated Approaches to Testing & Assessment (IATA, follow-up to GD 88, 2008) → under development

- **Several test strategies proposed**
  - Combine *in vitro, in vivo, in silico & in chemico* data
  - A number of chemicals tested (8 to 145)
  - Different data analyses procedures proposed to assess both hazard & potency
Other developments
**OECD & Adverse Outcome Pathways**

- **Possibility to make an AOP project proposal to the OECD**

Sequential chain of causally linked events leading to an adverse effect

- **AOP knowledge base**
  - Interactive web-based platform for AOP development
  - AOP Wiki: [https://aopkb.org/aopwiki/index.php/Main_Page](https://aopkb.org/aopwiki/index.php/Main_Page)
  - Currently 12 AOPs under OECD review, 3 open for general comments & 29 AOPs under development
OECD GD 211 for describing non-guideline in vitro methods (Dec 2014)

Information to be ideally provided for describing non-guideline in vitro methods

- Harmonize method description & facilitate assessment
- Not prescriptive, allows flexible structure, completeness of information may depend on level of development of in vitro assay
- Novel in vitro assays e.g., high throughput screening, complex models

1. **General information:** Name, developer, status, references…

2. **Test method definition:** Purpose, principle, exposure, quality/acceptance criteria, known limitations & strengths

3. **Prediction model:** Assay responses, data analyses and interpretation

4. **Performances:** Reproducibility, predictive capacity, scope & limitations

5. **Potential regulatory applications**
   Support read-across / Priority setting / Screening purpose / Component of IATA
Applicable to testing approaches subject to regulatory guidance for human and veterinary medicinal products used to support regulatory applications: clinical trial applications, marketing authorisation applications (early screening: no regulatory involvement (in-house validation))
Criteria for regulatory acceptance

1. Formal **method validation** (OECD, ECVAM, ICCVAM)

2. Demonstration that the new or substitute method or testing strategy provides either new data that **fill a recognised gap** or **data that are at least as useful as**, and preferably better than those obtained using existing methods
   → If no formal validation, evaluation on a case-by-case basis by National Control Authorities and/or relevant Working Parties or Expert Working Groups

3. Demonstration of **adequate testing of medicinal products under real-life conditions** (human and veterinary) which can be generated through the “Safe Harbour Process”:
   Period of voluntary submission of data obtained using a new 3R testing approach in parallel with data generated using existing methods. Data generated with 3R approach will be solely used for the purpose of evaluation of novel 3R testing approaches for possible future regulatory acceptance
Regulatory acceptance of 3R approaches

Submission to EMA according to Guideline on Qualification of Novel Methodologies for Drug Development.

In case of veterinary products only, submission according to CVMP guidance for companies requesting scientific advice.

Possible recommendations:

1. Sufficient data → recommended for regulatory acceptance to relevant working parties

2. Need for real-life data collection period (safe harbour provisions)

3. Rejection as considered immature
Conclusions
Validation introduced to facilitate regulatory acceptance
EU strong regulatory request: cosmetics, chemicals, mixtures, biocides

A number of validated assays internationally adopted, however
- Process still long
- Acceptance criteria more stringent than for animal testing
- Tendency to consider animal as a ‘gold standard’ over human effects
- Need for Integrated Approaches to Testing & Assessment & Testing Strategies

Opportunities
- Adverse Outcome Pathways → mechanistic insights
- Complex models: harmonized description for regulatory purposes
- Pharmaceuticals: guidance on regulatory acceptance of 3R methods
Thank YOU!

chantra.eskes@secam-ce.eu