Suitability of Histopathology as an Additional Endpoint to the Isolated Chicken Eye Test for Classification of Non-Extreme pH Detergent and Cleaning Products

Association Internationale de la Savonnerie, de la Détergence et des Produits d'Entretien
International Association for Soaps, Detergents and Maintenance Products

E. Cazelle[1], C. Eskes[2], M. Hermann[3], P. Jones[4], P. McNamee[5], M. Prinsen[6], H. Taylor[7], M. Wijnands[6].

[1]Technical & Regulatory Affairs, A.I.S.E. - International Association for Soaps, Detergents and Maintenance Products, Brussels, Belgium; [2]SeCAM, Services & Consultation on Alternative Methods Sagl, Agno, Switzerland; [3]Corporate Scientific Services - Toxicology, Henkel AG & Co. KGaA, Duesseldorf, Germany; [4]Safety and Environmental Assurance Centre, Unilever, Sharnbrook, UK; [5]Central Product Safety, The Procter & Gamble Company, Egham, Surrey, UK; [6]Toxicology and Risk Assessment,TNO Triskelion, Zeist, The Netherlands; [7]Global Regulatory Affairs, Reckitt Benckiser, Hull, UK,

Abstract

A.I.S.E. investigated the suitability of histology as an additional endpoint to the regulatory adopted ICE *in vitro* test method (OECD Test Guideline (TG) 438) to identify non-extreme pH detergent and cleaning products requiring classification as EU CLP / UN GHS Category 1 (serious eye damage). To this aim, 30 non-extreme pH products covering the range of *in vivo* eye irritation classifications, and representing various product categories were tested. Epithelium vacuolation (mid and lower layers) and erosion (at least moderate) were found to be the most relevant histology effects induced by products classified Category 1 *in vivo*. Histology criteria specifically developed for non-extreme pH detergent and cleaning products correctly identified materials classified as Category 1 based on *in vivo* persistent effects and significantly increased the overall sensitivity of the standard ICE prediction model for Category 1 identification (to 75%) whilst maintaining good concordance (73%). In contrast, the EU CLP additivity approach for classification was considerable less predictive with 27% concordance and 100% over-prediction of non-Category 1 products. Use of histology as an additional endpoint in the ICE test method was therefore found suitable to identify EU CLP/UN GHS Category 1 non-extreme pH detergent and cleaning products and to allow better discrimination from Category 2 products.

Background

The UN Globally Harmonized System (UN GHS) for classification (UN, 2013) was adopted in 2008 by the European Union by means of the CLP Regulation on Classification, Labelling and Packaging of substances and mixtures (EC, 2008). It establishes amongst others, new thresholds for the calculation method to be applied from June 2015 and new rules for the classification of mixtures (Figure 1).

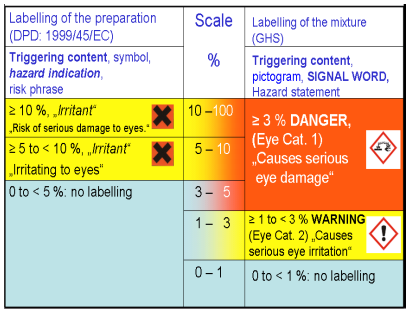

Figure 1. Comparative EU DPD and EU CLP (UN GHS) classification/labelling for eye corrosion/irritation for a mixture containing a R41/Eye Cat. 1 ingredient.

The application of the new EU CLP regulation for mixtures could result in the over-labelling of several detergents and cleaning products that did not up to now require classification according to the current EU Dangerous Preparation Directive (DPD) classification system (EU, 1999) and as corroborated by *in vivo*, *in vitro* and human experience data. Such over-labelling could confuse end-users and lead to underestimation of real risk when this is merited due to trivialisation of labelling and based on the current uses of such products. In order to ensure appropriate product classification the European Detergent Association A.I.S.E. initiated in 2010 a scientific project to investigate the applicability of validated and adopted *in vitro* eye & skin irritation/corrosion methods to reliably classify detergent and cleaning product formulations. The work reported here is part of this programme.

Methodology

Test materials:
Non-extreme pH detergent and cleaning products were chosen from a set of formulations provided by A.I.S.E. members based on the following selection criteria:

- Availability of good quality historical *in vivo* data (i.e., complete Draize and/or Low Volume Eye Irritation Test (LVET) *in vivo* data to derive unequivocal EUCLP/UN GHS classification)
- Sufficient number of EU CLP/UN GHS Category 1 (Cat. 1) formulations
- Distribution of EU CLP/UN GHS Cat. 1, Category 2 (Cat. 2) and Non-Classified (NC) formulations
- Market representative formulations
- Representation of different non-extreme pH product categories, i.e., Hand Dish Wash Liquid detergents (HDWL), Laundry Powder detergents (LP), Laundry Liquid detergents (LL), and All Purpose Cleaners (APC)
- Diversity in composition within each product category

A total of 30 formulations complied with the above selection criteria and could be remade for *in vitro* testing. These formulations represented the various degrees of eye hazards (8 Cat. 1, 14 Cat. 2 and 8 NC according to the EU CLP / UN GHS) and different categories of formulations (17 HDWL, 4 LL, 5 LP, and 4 APC). All 30 formulations were tested blind.

Test methodology: The ICE test method was performed following the OECD Test Guideline 438 (OECD TG 438, 2013a).

Histology evaluation: At termination of the ICE test, i.e. 4 hours after the 10 sec. treatment, the corneas (eyes) were processed for histology evaluation according to the procedures described in Cazelle *et al.*, 2014. Semi-quantitative histological evaluation was performed by a TNO pathologist according to evaluation criteria described in Table 1. Examples of histology observations are provided in Figure 2a-c

Observation	Score (Degree)
Epithelial Erosion	0 (normal); ½ (very slight); 1 (slight); 2 (moderate); 3 (severe)
Epithelial Vacuolization (Squamous, wing and basal cell layers are scored separately)	0 (normal); ½ (very slight); 1 (slight); 2 (moderate); 3 (severe)
Epithelial Necrosis	0 (normal); ½ (very slight); 1 (slight); 2 (moderate); 3 (severe)
Keratocyte Pyknotic Nuclei (Anterior or posterior stroma)	0 (normal); 1 (slight); 2 (moderate)
Stromal Collagen Fibre Bundle Disorder	P (Present)
Endothelial Cell Necrosis	P (Present)

Table 1: Histology observations scoring system as applied to the ICE corneas

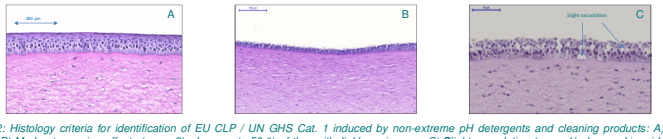

Figure 2: Histology criteria for identification of EU CLP / UN GHS Cat. 1 induced by non-extreme pH detergents and cleaning products: A) Control cornea; B) Moderate erosion effects (score 2) where up to 50 % of the epithelial layer is gone; C) Slight vacuolation (score 1) observed in mid- and low layer, where groups of vacuolated cells or single string of cells with small vacuoles can be seen. In addition, slight erosion (score 1) is also observed where up to three layers (top, mid, low) are gone

Results

Use of histology as an additional endpoint in the standard ICE test method is encouraged in OECD Guidance Document 160 and OECD TG 438. To date no agreed criteria existed on the use of histology observations in the ICE test method for classification purposes. As such a set of defined criteria was developed and tested within this program. Histology criteria for identifying EU CLP/UN GHS Cat. 1 were initially developed based on a 'training set' of 11 HDWLs with existing *in vivo* data (5 Cat. 1 and 6 non-Cat. 1). Histology observations showed that *in vivo* EU CLP/UN GHS Cat. 1 formulations led to the presence of epithelial erosion (≥ 2, moderate) and epithelial vacuolation (≥ ½, very slight) which were generally not observed with the non-Cat.1 formulations. The preliminary histology criteria were further challenged by testing 19 additional non-extreme pH formulations ('Testing set') with existing *in vivo* data and including LL & LP detergents, HDWLs and APCs. Small refinements were made to the preliminary histology criteria (i.e., using 2 out of 3 eyes for consistency with the *in vivo* test) and localization of epithelial vacuolation effects (i.e., mid and lower parts of the epithelium versus top layers). Also, although epithelial necrosis, stromal and endothelial effects were typically not observed for such detergent and cleaning products, criteria taking these effects into consideration have also been proposed based on experience of the testing facility. Final histology criteria for identification of EU CLP / UN GHS Cat. 1 are provided in Table 2.

Tissue Layer	Tissue change triggering a prediction of serious eye damage (Cat. 1)
Epithelium	– erosion ≥ 2 (moderate) in at least 2 out of 3 corneas – and/or vacuolization ≥ ½ (very slight) [wing and/or basal cell layers] in at least 2 out of 3 corneas – or, if erosion ≥ 2 (moderate) is observed in 1 out of 3 corneas AND vacuolization ≥ ½ (very slight)[wing and/or basal cell layer] is observed in at least one other cornea out of the remaining 2 corneas – and/or necrosis ≥ 2 (moderate) is observed in at least 2 out of the 3 corneas
Stroma	and/or pyknotic nuclei ≥ 1 (slight) in at least 2 out of 3 corneas
Endothelium	and/or any damage observed in at least 2 out of 3 corneas

Table 2: Histology criteria for identification of EU CLP / UN GHS Cat. 1 induced by non-extreme pH detergents and cleaning products (to be used in addition to the standard ICE prediction model)

Table 3 provides an overview of the *in vivo*, *in vitro* (ICE with and without histology) and EU CLP additivity approach classifications of the 30 non-extreme pH detergent and cleaning products tested in this programme.

This is translated in Table 4 into predictive capacity values for identification of EU CLP / UN GHS Category 1 non-extreme pH detergent and cleaning products. Detailed are predictive capacity values obtained with: 1) ICE conducted according to the OECD TG 438 protocol/prediction model; 2) ICE conducted according to the OECD TG 438 protocol/prediction model with, in addition, the inclusion of histology; 3) classification based on the EU CLP additivity approach.

The ICE OECD TG 438 standard test method/prediction model resulted in higher concordances (73% versus 27%), and higher specificity (100% versus 0%) as compared to the EU CLP additivity approach. However, a higher under-prediction rate was observed (8 versus 0 under-predictions out of 8). Interestingly, most of the non-extreme pH formulations under-predicted with the standard ICE test method (6 out of 8) were classified EU CLP / UN GHS Cat. 1 *in vivo* based on persistence of effects only, i.e., having tissue effects that did not reverse 21 days after treatment (Table 5).

With the addition of histology, the false negative rate was found to significantly decrease (from 8 to 2 under-predictions out of 8 Cat. 1 formulations), whilst maintaining good concordance (73%) and specificity (73%).

Formulations	Set Designation	Physical State	*In vivo* UN GHS Classification	ICE OECD 438*	ICE + Histology	CLP Additivity Approach
HDWL 1	Training	L	LVET – Cat. 1	No Cat. 1	Cat. 1	Cat. 1
HDWL 2	Training	L	LVET – Cat. 1	No Cat. 1	Cat. 1	Cat. 1
HDWL 3	Training	L	LVET – Cat. 1	No Cat. 1	Cat. 1	Cat. 1
HDWL 4	Training	L	LVET – Cat. 1	No Cat. 1	Cat. 1	Cat. 1
HDWL 5	Training	L	LVET – Cat. 1	No Cat. 1	Cat. 1	Cat. 1
LL 1	Testing	L	LVET – Cat. 1	No Cat. 1	Cat. 1	Cat. 1
LL 2	Testing	L	LVET – Cat. 1	No Cat. 1	No Cat. 1	Cat. 1
LP 1	Testing	S	LVET – Cat. 1	No Cat. 1	No Cat. 1	Cat. 1
APC 1	Testing	L	LVET – Cat. 2A	No Cat. 1	No Cat. 1	Cat. 1
APC 2	Testing	L	LVET – Cat. 2A	No Cat. 1	Cat. 1	Cat. 1
HDWL 6	Testing	L	LVET – Cat. 2A	No Cat. 1	Cat. 1	Cat. 1
HDWL 7	Testing	L	LVET – Cat. 2A	No Cat. 1	Cat. 1	Cat. 1
HDWL 8	Testing	L	LVET – Cat. 2A	No Cat. 1	Cat. 1	Cat. 1
HDWL 9	Training	L	LVET – Cat. 2A	No Cat. 1	No Cat. 1	Cat. 1
HDWL 10	Training	L	LVET – Cat. 2A	No Cat. 1	No Cat. 1	Cat. 1
HDWL 11	Testing	L	LVET – Cat. 2B	No Cat. 1	No Cat. 1	Cat. 1
HDWL 12	Testing	L	LVET – Cat. 2A	No Cat. 1	No Cat. 1	Cat. 1
HDWL 13	Training	L	Draize – Cat. 2A	No Cat. 1	Cat. 1	Cat. 1
HDWL 14	Training	L	Draize – Cat. 2B	No Cat. 1	No Cat. 1	Cat. 1
LL 3	Testing	L	LVET – Cat. 2A	No Cat. 1	No Cat. 1	Cat. 1
LP 2	Testing	S	LVET – Cat. 2A	No Cat. 1	No Cat. 1	Cat. 1
LP 3	Testing	S	LVET – Cat. 2B	No Cat. 1	No Cat. 1	Cat. 1
APC 3	Testing	L	LVET - NC	No Cat. 1	No Cat. 1	Cat. 1
APC 4	Testing	L	LVET - NC	No Cat. 1	No Cat. 1	Cat. 1
HDWL 15	Training	L	LVET - NC	No Cat. 1	No Cat. 1	Cat. 1
HDWL 16	Testing	L	LVET - NC	No Cat. 1	Cat. 1	Cat. 1
HDWL 17	Training	L	Draize - NC	No Cat. 1	No Cat. 1	Cat. 1
LL 4	Testing	L	LVET - NC	No Cat. 1	No Cat. 1	Cat. 1
LP 4	Testing	S	LVET - NC	No Cat. 1	No Cat. 1	Cat. 1
LP 5	Testing	S	LVET - NC	No Cat. 1	No Cat. 1	Cat. 1

* Only Cat 1. versus no-Cat 1 classifications were defined. L = Liquid, S = Solid

Table 3: Overview of the in vivo, in vitro and EU CLP additivity classifications of the 30 non-extreme pH detergent and cleaning products tested in ICE with and without histology

In Vitro Test Method	Concordance	Specificity	Sensitivity	False Positives	False Negatives
ICE	22/30 (73.3%)	22/22 (100.0%)	0/8 (0.0%)	0/22 (0.0%)	8/8 (100.0%)
ICE + histology	22/30 (73.3%)	16/22 (72.7%)	6/8 (75.0%)	6/22 (27.3%)	2/8 (25.0%)
EU CLP additivity approach	8/30 (26.7%)	0/22 (0.0%)	8/8 (100.0%)	22/22 (100.0%)	0/8 (0.0%)

Table 4: Predictive capacity for non-extreme pH detergent and cleaning products of the ICE test method (with and without histology for identifying EU CLP / UN GHS Cat. 1

Formulations	*In vivo* (LVET) EU CLP / UN GHS	Reasons for Cat. 1 classification
LP 1	Cat. 1	CO = 4 in 1/3 animals (days2,3,4 & 7) reversed to CO= 0 at day 21
LL 1	Cat. 1	Persistence of (CO+CR) in 2/6 animals & of CC in 1/6 animals; No severity of effects leading to Cat. 1 classification was observed.
LL 2	Cat. 1	Persistence of CR in 2/3 animals & of (CO+IR+CC) in 1/3 animals; No severity of effects leading to Cat. 1 classification was observed.
HDWL 1	Cat. 1	CO=4 in 1/1 animal (day 4), no data on recovery
HDWL 2	Cat. 1	Persistence of CO in 1/3 animals; No severity of effects leading to Cat. 1 classification was observed.
HDWL 3	Cat. 1	Persistence of CO in 2/3 animals & CC in 1/3 animals; No severity of effects leading to Cat. 1 classification was observed.
HDWL 4	Cat. 1	Persistence of CR in 2/3 animals & of (CO+CC) in 1/3 animals; No severity of effects leading to Cat. 1 classification was observed.
HDWL 5	Cat. 1	Persistence of (CO, CR, CC) in 1/3 animals; No severity of effects leading to Cat. 1 classification was observed.

CO=Corneal opacity, CC= Conjunctival chemosis, CR= Conjunctival Redness, IR= iritis

Table 5. Summary of in vivo effects leading to in vivo EU CLP / UN GHS Cat. 1 classification of non-extreme pH detergent and cleaning formulations

Conclusions

In conclusion, the use of histology as an additional endpoint to the standard ICE test method was shown to be suitable to identify non-extreme pH detergent and cleaning formulations that require EU CLP / UN GHS Category 1 classification. In particular, use of histopathology allowed the identification of EU CLP / UN GHS Category 1 classified *in vivo* due to persistence of effects, and to better discriminate Category 1 from Category 2 products.

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