DB-ALM Method Summary n° 37 : Red Blood Cell (RBC) Test - Summary

Eye Irritation

The Red Blood Cell (RBC) test is used to examine ocular irritants by measuring lysis of erythrocytes (haemolysis) and denaturation of released oxyhaemoglobin.

Objective & Application

TYPE OF TESTING	:	Replacement (partial)
LEVEL OF ASSESSMENT	:	Toxic potential

The RBC test was proposed as an alternative to the Draize rabbit eye test (OECD TG 405, 2012; Method B.5 of Annex to Commission Regulation 440/2008/EC, EU 2008; Draize *et al*., 1944) and in fact, in the literature it is reported to be employed to analyse eye irritation potential of surfactants and surfactant-based formulations (Pape *et al.*, 1999).

Lately, it became evident that for testing chemicals for the eye irritation potential, no *in vitro* alternative is known as full replacement of the *in vivo* test, but a strategic combination of several alternative test methods would have to be used for this purpose (ESAC, 2009) and in this context some industried developed the idea to use the RBC test as a part of a test battery for screening purposes (Eskes *et al.*, 2005).

In 2009, an ESAC statement endorsing 4 cytotoxicity/cell-function based *in vitro* assays for eye irritation testing (ESAC, 2009) considered the available evidence regarding the RBC (DB-ALM Protocols No. 37 and No. 99) to be insufficient for a recommendation for regulatory use.

Basis of the Method

The RBC test was developed by Pape and co-workers (1987; 1990) with bovine erythrocytes and by Lewis *et al*. (1993; 1994) using rabbit erythrocytes. It was shown by Pape and co-workers (Pape *et al*., 1987; Pape and Hoppe, 1990*)* that substances, which induce haemolysis, always cause some extent of eye irritation in the Draize rabbit eye test (Eskes *et al*., 2005) and that protein denaturation is a key mechanism for the formation of corneal opacity in the eye and is therefore considered as relevant endpoint. The RBC test, in fact, is a cell-based cytotoxicity assay, which allows assessing membrane damage of erythrocytes (haemolysis) and changes of the oxyhaemoglobin configuration (protein denaturation). To determine haemolysis, the haemoglobin leakage is measured spectrophotometrically. Oxyhaemoglobin denaturation is also analysed spectrophotometrically.
So, both endpoints of the RBC test are measured spectrophotometrically using different wavelengths.

Experimental Description

Biological and Endpoint Measurement:

HAEMOLYSIS: the percentage release of haemoglobin from fresh red blood cells is measured spectrophotometrically

PROTEIN DENATURATION: denaturation of released oxyhaemoglobin (given as denaturation index (DI) in% denaturation) is measured spectrophotometrically

Endpoint Value:

H_{50}: the concentration of a test substance (in mg/l,mol/l or ppm) that induces the lysis of 50% of the red blood cells

Experimental System:

ERYTHROCYTES: mammalian erythrocytes are isolated from fresh blood

Erythrocytes are isolated from fresh blood by centrifugation and a RBC suspension (8 x 10^9 cells/ml) is prepared. Test substances are diluted in PBS. A range finding should be carried out prior to the main assay to ensure adequate concentrations for the dose-response curves (DB-ALM Protocol No. 99). To this end the concentration with the highest amount of denaturation relative to the totally lysed sample (D_{max}) and the concentration, at which denaturation becomes lower than 10% (D_{low}) are determined.

To assess haemolysis, 25 µl of the RBC suspension (8 x 10^9 cells/ml) is added to a range of dilutions (6 to

eight) of each test substance (in a volume of 975 µl). The incubation (10 min at room temperature) is terminated by a centrifugation step and the released oxyhaemoglobin in the supernatant is measured spectrophotometrically (at 541 nm). The maximal lysis (100%) is achieved using distilled water; spontaneous haemolysis (< 5%) is monitored using PBS (negative control). The release caused by each concentration of the test substance is expressed in relative percentage to the maximal lysis and a dose-response curve is prepared. The half-maximal effective concentration (H_{50}) for each substance is calculated.

To determine the denaturation of oxyhaemoglobin, the RBCs are incubated with a 1% solution of the test substance, respectively 0.1% solution of surfactants. After 10 min the supernatant of the samples is measured at 540 nm and 575 nm in a UV/VIS spectrophotometer. The extinction measured at 575 nm is divided by the extinction obtained at 540 nm to get the ratio (R). For oxyhaemoglobin the ratio (R1) is set 1.05 whereas the ratio of the positive control (SDS 3.47 mmol/l) is R2. The difference between R1 and R2 is equal to 100% denaturation. The denaturation index (DI) is calculated as follows:

DI (%) = 100 (R1 – R_i) / (R1 – R2)
R1 = ratio for oxyhaemaglobin (set to 1.05)
R2 = ratio for the internal positive control
R_i = ratio for the irritant (test substance)
In addition, the relationship between haemolysis (H_{50}) and denaturation (DI) is expressed as L/D ratio.

SOPs are available in DB-ALM as Protocols No. 37:" Red Blood Cell (RBC) Test System" and No. 99: "Red Blood Cell (RBC) Lysis and Protein Denaturation".

Data Analysis/Prediction Model

Pape and Hoppe (1990) introduced a prediction model that is based on the ratio between the two endpoints (haemolysis and denaturation). The authors correlated the ocular irritancy obtained after 1 h with a modified Draize rabbit eye test (MIOI) with the L/D ratio. A correlation between these two parameters was found, which led to the following classification for surfactants (Pape and Hoppe, 1990; DB-ALM Protocol No. 37)

In vivo eye irritation	MIOI	*In vitro* L/D ratio
non-irritant	0 - 5	> 100
slightly irritant	> 5 < 15	> 10
moderately irritant	> 15 < 25	> 1
irritant	> 25 < 40	> 0.1
very irritant	> 40	< 0.1

According to Pape and Hoppe (1990).

Lewis *et al*. (1993; 1994) proposed a classification that is based on only one endpoint (haemolysis). The authors correlated the obtained H_{50} values with historical MMTDS obtained by ICI (UK) and categorized the *in vitro* H_{50} values into two classes (non-irritant, irritant). According to Lewis *et al*. (1994), substances with an *in vivo* MMTS > 15 and a H_{50} values < 10 x 10^{-4} were regarded as irritating (no further information is given by the authors).

In Vivo		*In Vitro*	
CLASSIFICATION	**MMTDS**	**RBC H_{50} (x10^{-4} M)**	**CLASSIFICATION**
Non-irritant	0-5	> 100	**Non-irritant**
Minimally irritant	> 5 < 15	10 - 100	
Mild irritant	> 15 < 25	1 -10	**Irritant**
Moderate Irritant	> 25 < 50	< 1	
Severe irritant	> 50 < 80		

Another prediction model was developed by the COLIPA study (Pape *et al*., 1999), using *in vivo* data from the ECETOC Data base (ECETOC, 1992) and from the Eye irritation reference chemical data bank (Bagley *et al*., 1992). The PMs reported above were applied only for surfactants, the COLIPA study introduced a PM suitable for other groups of material, such as ingredients and formulations. Pape *et al*. (1999) reported several values for the coefficients A, B, and C, which are characteristic for certain materials and chemicals. The following equation was used for prediction:
$MMAS = A / [1 + (B \cdot H_{50})^C]$
where A = 110; B = 0.00184; C = 1 for surfactant ingredients
where A = 55; B = 0.00196; C = 1 for surfactant-based formulations
where A = 110; B = 0.00444; C = 1 for surfactants and formulations

Test Compounds

Chemicals, surfactants, surfactant-based formulations have been used.

Modifications

Blood from several species are used for the RBC test. The available SOP are established using bovine, respectively rabbit red blood cells. In addition, various authors performed the test with erythrocytes from other donors, such as rats (Okamoto *et al*., 1990; Sugai *et al*., 1991; Lagarto *et al*., 2006), guinea pigs (Hayashi *et al*., 1994), calves (Pape *et al*., 1987; Kristen *et al*., 1993; Lagarto *et al*., 2006), sheep (Okamoto *et al*., 1999), dogs (Singleton *et al*., 1994), and humans (Singleton *et al*., 1994; Benavides *et al*., 2004; Martinez *et al*., 2006). Lewis *et al*. (1993; 1994) used a prolonged exposure-time of 60 min.

Prediction models are available using one or both endpoints. Some authors employ only the H_{50} value for predicting the irritation potency of a test substance (Lewis *et al*., 1993; Lewis *et al*., 1994), others calculate the ratio between haemolysis and denaturation (Pape *et al*., 1987; Pape and Hoppe 1991; Benavides *et al*., 2004).

A modified cell-free HD assay is carried out in the MHW/NIHS between-laboratory validation study (Hatao *et al*., 1999) (for more information a corresponding method summary is available).

Discussion

According to Pape *et al*. (1987), the RBC test is an inexpensive, rapid assay with reliable results and a

good reproducibility. Red blood cells from several animal species are easily available (e. g animal blood from slaughterhouse), while human blood is available from blood banks. Some differences between the size and the stability of the red blood cells are observed between species (Pape *et al*., 1999) and have to be taken into account using the RBC assay. According to Lagarto *et al*. (2006), the predictive capacity of the assay was better using bovine than rat RBCs. The assay is limited to water-soluble or water-dispersible substances. In addition, Eskes *et al*. (2005) mentioned that the RBC test can be used for coloured substances, but the membrane damage (haemolysis) has to be determined by another alternative method, since some colours might interfere with the wavelength used for the haemolysis measurement.

Status

The RBC assay showed a good between-laboratory reproducibility (Brantom *et al*., 1997; Pape *et al*., 1999; Ohono *et al*., 1994; Okamoto *et al*., 1999) and within-laboratory repeatability for the assessment of surfactants or surfactant-based formulations (Brantom *et al*., 1997; Pape *et al*., 1999). A good correlation (correlation coefficient 0.945) between the results on haemoglobin denaturation (H_{50}) for 18 substances from two different validation studies (EC/HOME and the COLIPA validation study) was reported by Pape *et al*. (1999).

The RBC assay was evaluated **in validation studies in Europe, the United States and Japan**. From these studies (EC/HOME, COLIPA, CTFA, Japanese between-laboratory validation, and IRAG) it was concluded that the assay has its best performance with surfactants and surfactant-based formulations (Balls *et al*., 1995; Brantom *et al*., 1997; Pape *et al*., 1999; Gettings *et al*., 1996; Ohono *et al*., 1994; Okamoto *et al*., 1999).

IRAG (working group 4: cell-cytotoxicity assays) concluded that for a proper assessment of the assay additional data is required from other classes of substances than surfactants (Harbell *et al*., 1997). The Management Team of the EC/HOME validation study stated that the RBC assay alone cannot serve as complete substitute for the Draize test (Balls *et al.*, 1995).

The RBC assay has been included in a preliminary evaluation applying reference substances from different classes of substances in order to improve regulatory acceptance of the assay (Balls *et al*., 1999; Brantom *et al*., 2000; Eskes *et al*., 2005). Brantom *et al*. (2000) mentioned that the results for surfactants obtained in this study with the RBC assay showed a similar ranking to the *in vivo* Draize MMAS.

Eskes *et al*. (2005) pointed out that the assay is routinely used by industry as part of a test battery for screening purposes. Furthermore, the authors recommend the RBC assay in combination with other alternative methods based on different endpoints to improve the prediction of ocular irritating substances.

In 2005, ECVAM initiated a retrospective evaluation for the following four *in vitro* methods: the NRR assay, the RBC test, the FLT, and the SM. The final evaluation of DB-ALM Protocols 37 and 99 both concerning the Red Blood Cell Haemolysis method done by the Validation Management Team indicates that **" ... the available evidences are not sufficient to support a recommendation for a regulatory use consideration (ESAC, 2009)."**

Abbreviations & Definitions

COLIPA: European Cosmetic, Toiletry and Perfumery Associations
CTFA: Cosmetic, Toiletry and Fragrance Associations
EC: European Commission
ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals)
EC/HO: European Commission/British Home Office
ESAC: ECVAM Scientific Advisory Committee
EU: European Union
FLT: Fluorescein Leakage Test
HD: Haemoglobin Denaturation
IRAG: Interagency Regulatory Alternatives Group
L/D: Lysis/Denaturation
MMAS: Modified MAximum Score
MHW/NIHS: Japanese Ministry of Health and Welfare/ National Institute of Health Sciences
MIOI: Mean Indices of Ocular Irritation
MMTDS: Maximum Mean Total Draize Score
NRR: Neutral Red Release
OECD: Organisation for Economic Co-operation and Development
PBS: Phosphate Buffered Saline
PM. Prediction Model
RBC: Red Blood Cell
SM: Silicon Microphysiometer
SOP: Standard Operating Procedures
TG: Test Guideline
UV-VIS: ultraviolet-visible

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