Regulatory Toxicology and Pharmacology 65 (2013) 344–365

Contents lists available at SciVerse ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Assessment of the predictive capacity of the 3T3 Neutral Red Uptake cytotoxicity test method to identify substances not classified for acute oral toxicity (LD_{50} > 2000 mg/kg): Results of an ECVAM validation study

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ARTICLE INFO

Article history:
Available online 13 December 2012

Keywords:
Acute oral toxicity
Validation
Cytotoxicity
BALB/3T3 Neutral Red Uptake assay
CLP
Industrial chemicals

ABSTRACT

Assessing chemicals for acute oral toxicity is a standard information requirement of regulatory testing. However, animal testing is now prohibited in the cosmetics sector in Europe, and strongly discouraged for industrial chemicals. Building on the results of a previous international validation study, a follow up study was organised to assess if the 3T3 Neutral Red Uptake cytotoxicity assay could identify substances not requiring classification as acute oral toxicants under the EU regulations. Fifty-six coded industrial chemicals were tested in three laboratories, each using one of the following protocols: the previously validated protocol, an abbreviated version of the protocol and the protocol adapted for an automation platform. Predictions were very similar among the three laboratories. The assay exhibited high sensitivity (92–96%) but relatively low specificity (40–44%). Three chemicals were under predicted. Assuming that most industrial chemicals are not likely to be acutely toxic, this test method could prove a valuable component of an integrated testing strategy, a read-across argument, or weight-of-evidence approach to identify non toxic chemicals (LD_{50} > 2000 mg/kg). However, it is likely to under predict chemicals acting via specific mechanisms of action not captured by the 3T3 test system, or which first require biotransformation *in vivo*.

1. Introduction

Acute oral toxicity studies are performed mainly for classification and labelling in order to assign substances their potential hazard categories and estimate the dose required to cause toxicity (Creton et al., 2010; Seidle et al., 2010). In the EU, the Regulation on Classification, Labelling and Packaging (CLP) (European Commission, 2008) of substances and mixtures entered into force in 2009 to align the previous EU legislation Dangerous Substances Directive (Directive 67/548/EEC) and the Dangerous Preparations Directive (Directive 1999/45/EC), with the Globally Harmonised System of Classification and Labelling (GHS) developed under the auspices of the United Nations (UN, 2011), in order to increase consistency among diverse frameworks used in different jurisdictions and industrial sectors. Formerly, Annex I of Directive 67/548/EEC provided an official EC inventory of classified substances. The same inventory is currently available as Annex VI to Regulation 1272/2008, now transposed as a GHS version, where previous warning terms and risk (R) phrases have been replaced by updated classification categories and hazard (H) statements. According to the CLP Regulation, chemicals are allocated in one of four toxicity categories based on their acute oral toxicity properties: category 1 ($LD_{50} \leqslant$ 5 mg/kg b.w.), category 2 ($5 < LD_{50} \leqslant 50$ mg/kg b.w.), category 3 ($50 < LD_{50} \leqslant 300$ mg/kg b.w.), and category 4 ($300 < LD_{50} \leqslant$ 2000 mg/kg b.w.). Under this EU CLP scheme, the limit dose above which chemicals are not required to have a hazard label for acute oral toxicity is 2000 mg/kg b.w.

According to the cosmetics regulation (European Commission, 2009) it is prohibited in the EU to market cosmetic products and their ingredients that have been tested on animals for most of the human health effects, including acute toxicity. This imposes a great need for the cosmetic industry to have alternative approaches available for safety testing of ingredients of consumer products.

All the accepted methods for determining acute oral toxicity are based on *in vivo* experiments that estimate the LD_{50} value (i.e. the single dose of a substance that can be expected to cause death in

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http://dx.doi.org/10.1016/j.yrtph.2012.11.013

50% of the animals in an experimental group). They include three approved refinement and reduction alternative methods (modifications of the classical LD_{50} test) described in the Organisation for Economical Cooperation and Development (OECD) Test Guidelines (TG). The main endpoint for the Fixed Dose Procedure (FDP, in OECD TG 420) is evident toxicity while the Acute Toxic Class Method (ATC in OECD TG 423) and the Up and Down Procedure (UDP in OECD TG 425) use lethality as endpoint (OECD, 2001a,b,c). FDP and ATC provide an estimated LD_{50} range, whereas UDP gives an LD_{50} point estimate together with confidence interval (Creton et al., 2010).

During a workshop held in 1994 coordinated by the European Centre for the Validation of Alternative Methods (ECVAM; now called EURL ECVAM – the European Union Reference Laboratory for Alternatives to Animal Testing,), it was proposed that the regression equation from the correlation of IC_{50} (the concentration of a substance that causes 50% toxicity *in vitro*) versus LD_{50} from the Registry of Cytotoxicity (RC; Halle, 2003) could be applied to estimate unknown LD_{50} values for a novel chemical from IC_{50} values measured as basal cytotoxicity *in vitro* (Seibert et al., 1996). This estimated LD_{50} would then be used as a starting dose for the *in vivo* experiment (Spielmann et al., 1999) as later recommended by the OECD Guidance Document 24 on Acute Oral Toxicity Testing, particularly in cases where minimal prior information on the chemical is available (OECD, 2001d).

In 2000, an International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, reviewed the implementation of *in vitro* basal cytotoxicity assays in regulatory testing strategies (NIH, 2001). The workshop concluded that no *in vitro* cytotoxicity test (or battery of assays) was available to replace the animal methods. In addition, none of the *in vitro* models had been adequately evaluated for reliability and relevance, leaving their applicability to generating information for acute oral toxicity testing open to further validation.

In response to the workshop recommendations, the NTP Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) and ECVAM conducted a joint validation study of the Neutral Red Uptake (NRU) basal cytotoxicity assay in two standard cell systems: Normal Human Keratinocytes (NHK) and a rodent fibroblast cell line (BALB/3T3). The results of this study showed that the overall accuracy of the NRU basal cytotoxicity assay in BALB/3T3 cells and NHK for correctly predicting each of the GHS acute oral toxicity classification categories was low (31% and 29%, respectively). Of these two test methods, 3T3 NRU is considered to be more cost and time effective (NIH, 2006). Based on the results of this validation study, the OECD has adopted a Guidance Document (OECD GD 129) that describes methods to determine the *in vitro* basal cytotoxicity of test substances using NRU assays and to use these *in vitro* data to determine starting doses for *in vivo* acute oral systemic toxicity tests (OECD, 2010).

The primary aim of the validation study reported here was to assess the predictive capacity of the 3T3 NRU test method to determine if a test chemical correctly falls into one of the two categories: unclassified ($LD_{50} > 2000$ mg/kg b.w.) or classified ($LD_{50} \leqslant 2000$ mg/kg b.w.). The validated test method protocol and the IC_{50}–LD_{50} millimole and weight regression models from the previous NICEATM/ECVAM validation study were used. If the method correctly identifies negatives (unclassified chemicals) it could be used as part of a tiered approach to identify the unclassified chemicals that would not need to be tested further for *in vivo* acute oral toxicity. This validation study was motivated by two assumptions. The first was based on the results of the MEIC programme (Ekwall et al., 1998, 2000), the RC (Halle, 2003), and the NICEATM/ECVAM international validation study (NIH, 2006) that have all shown a correlation of around 60–70% between *in vitro* IC_{50} cytotoxicity data and rat oral LD_{50} values. Furthermore, these studies indicated that the precision of prediction of low systemic toxicity from *in vitro* cytotoxicity test data is much better than the prediction of high systemic toxicity, suggesting that the 3T3 NRU test method may allow discrimination of a large fraction of the EU CLP unclassified compounds ($LD_{50} > 2000$ mg/kg b.w.) without giving false negative results. With regard to the $LD_{50} > 2000$ - mg/kg limit dose, the retrospective analysis of the results of the former NICEATM/ECVAM validation study showed that the 3T3 NRU test method had a high sensitivity of 98%. The set of 72 chemicals tested included pharmaceuticals (42%), pesticides (22%), industrial chemicals (32%) and food additives (4%). Of all the 22 chemicals with an $LD_{50} > 2000$ mg/kg b.w. included in that study, 18 chemicals were identified as false positives, but only one chemical was falsely predicted as a negative (NIH, 2006).

The second assumption arises from an analysis of dossiers from the New Chemical Database (NCD), which showed that most of the EU notified industrial chemicals (ca. 87%) fall into the unclassified group (Bulgheroni et al., 2009). It was assumed that the high prevalence of unclassified chemicals is the same in the whole population of chemicals registered. Thus, it was envisaged that use of the 3T3 NRU test method to identify unclassified substances in a testing strategy, to support a read-across, or as part of a weight-of-evidence approach could potentially reduce the amount of animal testing for acute oral toxicity.

The validation study was sponsored and managed by ECVAM. Fig. 1 illustrates the organisation of the study. A Chemicals Selection Committee was appointed and selected a new set of 56 chemicals.

The study was planned and conducted as a follow-up of the previous NICEATM/ECVAM validation study. The test definition, within and between laboratory reproducibility, transferability (validation modules 1–4, Hartung et al., 2004) and reliability have already been extensively assessed during the NICEATM/ECVAM validation study (NIH, 2006). Therefore, according to the ECVAM's modular approach to validation (Hartung et al., 2004), only one laboratory was required to assess the predictive capacity and applicability domain (modules 5 and 6). Thus, ECVAM awarded the Health and Safety Laboratory (HSL, UK) a contract to test the new set of coded chemicals using the previously validated 3T3 NRU test method protocol.

A secondary goal was to assess whether two variants of the 3T3 NRU test protocol would generate similar data as compared to the validated protocol (for the 56 test chemicals selected) and to thus evaluate to which extent these protocol variants may be useful for identifying negatives. The Institute for Health and Consumer Protection (IHCP) of the Joint Research Centre (JRC, Italy) used an automated version of the 3T3 NRU test method protocol adapted to its robotic testing platform (Bouhifd et al., 2012), and the Institute for *In Vitro* Sciences (IIVS, US) used a less costly abbreviated version of the 3T3 NRU test method protocol that was targeted at resolving acute oral toxicities around the 2000 mg/kg cutoff value. The rationale for the abbreviated version was that, for the purpose of this study, it was not necessary to precisely predict the LD_{50} of chemicals with $LD_{50} < 2000$ mg/kg b.w., but it was only necessary to predict that the LD_{50} was below this cutoff. In effect therefore, less concentrations of a test chemical needed to be tested *in vitro*.

This validation study was completed in 2011 and the report was peer-reviewed by the ECVAM Scientific Advisory Committee (ESAC). The ESAC opinion served as basis for the ECVAM recommendation available on the IHCP website (http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations).

2. Materials and methods

2.1. Selection of testing chemicals

The main chemical selection criterion was to include industrial chemicals, with a statistically justified distribution to analyze dichotomous classifications i.e. chemicals with $LD_{50} > 2000$ mg/