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Estimation of acute oral toxicity using the No Observed Adverse Effect Level (NOAEL) from the 28 day repeated dose toxicity studies in rats

Anna Bulgheroni [a], Agnieszka Kinsner-Ovaskainen [a], Sebastian Hoffmann [a], Thomas Hartung [b], Pilar Prieto [a,*]

[a] European Commission Joint Research Centre, European Centre for the Validation of Alternative Methods (ECVAM), Institute for Health and Consumer Protection (IHCP), Via E. Fermi 2749, TP 580, I-21027 Ispra (VA), Italy

[b] Unit for Traceability, Risk and Vulnerability Assessment (TRIVA), Institute for the Protection and Security of the Citizen, European Commission Joint Research Centre, Ispra (VA), Italy

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ABSTRACT

Acute systemic toxicity is one of the areas of particular concern due to the 2009 deadline set by the 7th Amendment of the Cosmetics Directive (76/768/EEC), which introduces a testing and marketing ban of cosmetic products with ingredients tested on animals. The scientific community is putting considerable effort into developing and validating non-animal alternatives in this area. However, it is unlikely that validated and regulatory accepted alternative methods and/or strategies will be available in March 2009. Following the initiatives undertaken in the pharmaceutical industry to waive the acute oral toxicity testing before going to clinical studies by using information from other *in vivo* studies, we proposed an approach to identify non-toxic compounds (LD50 > 2000 mg/kg) using information from 28 days repeated dose toxicity studies. Taking into account the high prevalence of non-toxic substances (87%) in the New Chemicals Database, it was possible to set a NOAEL threshold of $\geqslant$200 mg/kg that allowed the correct identification of 63% of non-toxic compounds, while <1% of harmful compounds were misclassified as non-toxic. Since repeated dose toxicity studies can be performed *in vivo* until 2013, the proposed approach could have an immediate impact for the testing of cosmetic ingredients.

1. Introduction

In view of the 7th Amendment of the Cosmetics Directive (76/768/EEC), which introduced new provisions related to non-animal testing of cosmetics products and ingredients, there is an urgent need to replace the *in vivo* oral acute toxicity testing of cosmetic ingredients (European Commission, 2003). The timelines set by in this new legislation require an immediate ban of finished products from 11th September 2004, and a testing ban of cosmetic ingredients that applied from 11th September 2004, as soon as alternative methods validated by the European Centre for the Validation of Alternative Methods (ECVAM) and adopted in the EU legislation are available, but with a maximum cut-off date of six years (i.e. 11th March 2009), irrespective of the availability of alternative test methods. Moreover a marketing ban of products with ingredients tested on animals will be introduced from 11th March 2009 for all human health effects, with the exception of reproductive toxicity, repeated dose toxicity and toxicokinetics. For the latter health effects a deadline for a marketing ban is foreseen for 11th March 2013 (European Commission, 2003). Therefore, the scientific community is putting considerable effort in developing and validating *in vitro* and *in silico* alternative methods.

The deletion by the Organisation for Economical Cooperation and Development (OECD) of the Test Guideline TG 401 and the introduction in 2002 of three new OECD guidelines based on non-lethal endpoints, i.e. TG 420 (fixed dose procedure), TG 423 (acute toxic class method) and TG 425 (up and down procedure) (OECD, 2001a,b,c), have dramatically reduced the number of animals used for acute oral toxicity assessment. In 2006, the National Toxicology Program (NTP) Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) and ECVAM have completed a validation study of two *in vitro* cytotoxicity assays (NIH, 2006a,b) for the prediction of the starting dose for oral acute toxicity testing according to the OECD Test Guidelines TG 420, 423 and 425. Besides, a large Integrated Project "ACuteTox", funded by the European Commission's 6th Framework Programme, has started in 2005 with the aim to develop and pre-validate a testing strategy to fully replace the acute oral toxicity testing *in vivo* (Clemedson et al., 2006).

Nevertheless, despite all the efforts undertaken in the area of alternative methods, up to now it seems that none of the *in vitro* tests and testing strategies for the prediction of acute oral toxicity, that are currently under development and evaluation, will be ready, validated and regulatory accepted by the deadline that is scheduled for March 2009.

Taking into consideration these restrictions, ECVAM recently proposed to investigate whether for the cosmetic ingredients it would be possible to extrapolate the toxicological information for

* Corresponding author. Fax: +39 0332 785336.
E-mail address: maria.prieto-pilar@jrc.it (P. Prieto).

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Table 1
GHS acute toxicity hazard categories and acute toxicity estimate values defining the respective categories.

GHS category	Dose range of LD_{50} values	Human health effect
Category 1	LD_{50} < 5 mg/kg	Fatal if swallowed
Category 2	LD_{50} > 5 mg/kg < 50 mg/kg	Fatal if swallowed
Category 3	LD_{50} > 50 mg/kg < 300 mg/kg	Toxic if swallowed
Category 4	LD_{50} > 300 mg/kg < 2000 mg/kg	Harmful if swallowed
Category 5[a]	LD_{50} > 2000 mg/kg < 5000 mg/kg and LD_{50} > 5000 mg/kg	May be harmful if swallowed not classified

[a] Criteria for category 5 are intended to enable the identification of substances which are of relatively low acute toxicity hazard but which under certain circumstances may present a danger to vulnerable populations. Recognizing the need to protect animal welfare, testing in animals in category 5 ranges is discouraged and should only be considered when there is a strong likelihood that results of such a test have a direct relevance for protecting human health (United Nations, 2007).

the endpoints falling under the 2009 deadline from those tests, which will be still performed *in vivo* until 2013 (i.e. reproductive toxicity, repeated dose toxicity and toxicokinetics).

A similar analysis recently undertaken by the pharmaceutical industry has led to a successful challenge to the requirements for acute toxicity testing and a reduction in animals used (Robinson et al., 2008). In this study the European industry working group represented by 13 pharmaceutical industries and five contract research organisations performed an evidence-based review of acute toxicity studies and assessed the relevance of acute toxicity data in the drug development process. The working group concluded that stand-alone acute toxicity studies should not be required prior to first clinical trials in humans. Instead, information on acute toxicity can be assessed from any short term or dose-escalation studies performed by the clinical route of exposure (e.g. intravenous, oral). These studies, performed at more relevant doses for humans, are already an integral part of drug development (Robinson et al., 2008).

Since the repeated dose toxicity testing of cosmetic ingredients *in vivo* will be allowed until 2013, in the present study we attempted to evaluate whether it might be possible to estimate the oral acute toxicity classes using the no-observed-adverse-effect-level (NOAEL) values obtained from 28 days repeated dose studies in rats.

2. Source and selection of data

The data used in this study were retrieved from the New Chemical Database (NCD), maintained at the Institute for Health and Consumer Protection (JRC, Ispra) (http://ecb.jrc.it) in a security area with authorised access only. New chemicals are all substances that have been notified to the European Authorities from 1981, i.e. from the entry into force of the 6th Amendment to Directive 67/548/EEC (concerned with the classification, packaging and labelling of dangerous substances), that introduced the pan-European notification system. Exemption categories include consumer products pertaining only to pharmaceuticals, cosmetics and foodstuffs. The Directive is not applicable to pesticides, radioactive materials, wastes and substances used in scientific research (European Commission, 1967).

The New Chemicals database contains at that moment circa 7200 new notification dossiers, representing 4773 substances notified in Europe since 1981 (accessed on 27th March 2008).

For the analysis of the prevalence of oral acute toxicity categories, we included all the substances for which LD_{50} values were given in the notification files.

To analyse the relationship between NOAEL values and LD_{50} values, all the compounds for which both the NOAEL obtained from 28 days repeated dose studies in rats and oral LD50 values were available in the submission files, were selected.

3. Prevalence of acute toxicity classes within New Chemicals

The prevalence of the different oral acute toxicity classes was analysed within the new chemicals notified in the NCD.

The European Commission has recently adopted an act which aligns the EU system of classification, labelling and packaging substances and mixtures to the United Nations Globally Harmonised System (GHS). Therefore, we have used the GHS classification system as a basis of our analysis (European Commission, 2007).

The five GHS categories are summarized in Table 1 (United Nations, 2007). From the total number of 4773 chemicals notified, 4219 were included in the analysis, as their notification files contained information on acute oral toxicity (i.e. LD_{50} values).

The frequency of distribution of chemicals within the different oral acute toxicity classes resulted to be significantly unbalanced (Table 2). Only 15 chemicals (0.36%) were classified as "very toxic" (fatal) [2 chemicals in GHS Category 1 (0.05%) and 13 in Cat. 2 (0.31%)], 144 chemicals (3.41%) as "toxic" (Category 3), whereas 9.4% (396 chemicals) were classified as "harmful" (Category 4). The remaining 3664 chemicals (86.8%) fell in Category 5 or did not need to be classified for acute oral toxicity (LD_{50} > 5000 mg/kg).

Table 2
Prevalence of acute oral toxicity within the New Chemicals.

Toxicity class	Number of chemicals	Prevalence
Category 1 LD_{50} < 5 mg/kg b.w.	2	0.05%
Category 2 LD_{50} 5–50 mg/kg b.w.	13	0.31%
Category 3 LD_{50} 50–300 mg/kg b.w.	144	3.41%
Category 4 LD_{50} 300–2000 mg/kg b.w.	396	9.39%
Category 5 LD_{50} 2000–5000 mg/kg b.w. or	2960	70.16%
Not classified LD_{50} > 5000 mg/kg b.w.	704	16.69%

The New Chemicals database, maintained at the Joint Research Centre (consumer products safety and quality unit, formerly European Chemicals Bureau), was accessed on 27th March 2008. Of 7200 notifications since 1981, 4773 substances were found of which 4219 included oral toxicity data.

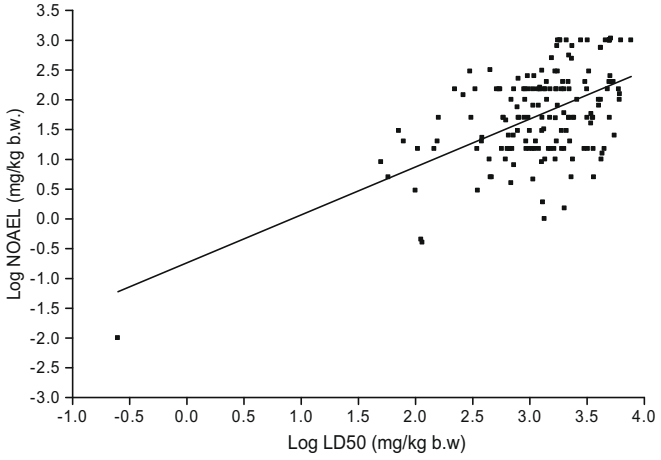

Fig. 1. Regression analysis of substances (n = 166) used in this study. The regression was calculated using LD_{50} values from acute oral toxicity studies and NOAEL obtained from 28 days repeated dose studies in rats (Pearson r = 0.55).

Table 3
Relationship between the oral LD_{50} and NOAEL values grouped using the same dose ranges as in GHS categories.

NOAEL (mg/kg b.w.)	GHS category (LD_{50} mg/kg b.w.)					Total
	GHS 1 (<5)	GHS 2 (5–50)	GHS 3 (50–300)	GHS 4 (300–2000)	GHS 5[a] (>2000)	
<5	**1**	0	3	5	7	16
5–50	0	**0**	7	38	142	187
50–300	0	0	**3**	48	575	627
300–2000	0	0	0	**10**	708	718
>2000	0	0	0	0	**4**	4
Total	1	0	13	101	1436	1552

Bold values are those where acute toxicity of compounds is correctly classified using the NOAEL values using the same does range categories as in GHS.
[a] Since the revised version of the GHS does not recommend testing in animals in category 5 (2000–5000 mg/kg b.w.) and the European classification categories the compounds with $LD_{50} > 2000$ as "not classified", we merged the data with $LD_{50} > 2000$–5000 mg/kg and $LD_{50} > 5000$ mg/kg into one category, labelled here as $LD_{50} > 2000$ mg/kg.

4. Data analysis of the relation between the NOAEL and LD_{50} values

All substances for which both the NOAEL and LD_{50} values obtained from oral rat studies were given were selected for our study, resulting in 1791 eligible chemicals.

To understand the relation between the NOAEL and LD_{50} values, we extracted only those substances (166 compounds in total), for which defined numbers were reported for both endpoints (i.e. data expressed as $>n$ were not included).

The linear regression analysis between LD_{50} and NOAEL values of the 166 eligible substances presented in Fig. 1 showed a poor correlation (Pearson $r = 0.55$). Subsequently, the substances were grouped according to the GHS categories for acute oral toxicity, based on the reported LD_{50} (Table 3). The revised version of the GHS does not recommend testing in animals in category 5 (2000–5000 mg/kg b.w.) (United Nations, 2007), and in the recent European Commission proposal there are only 4 categories, and compounds with $LD_{50} > 2000$ are "not classified" (European Commission, 2007). Therefore, we merged the data with $LD_{50} > 2000$–5000 mg/kg and $LD_{50} > 5000$ mg/kg into one category, labelled here as $LD_{50} > 2000$ mg/kg.

Within the 1791 selected substances, for 239 substances the LD_{50} and/or NOAEL values were reported as approximate estimations (e.g. <300 mg/kg). These compounds were excluded from the analysis since they could not be placed in one of the defined categories (e.g. a compound with $LD_{50} > 150$ could fall in cat. 3, 4 or 5). This left 1552 substances for further analysis. The same dose ranges were used to rank the NOAEL values, allowing to present the data in a 5×5 contingency table (Table 3).

As expected, none of the substances had a NOAEL value which was greater than the respective LD_{50}. From the distribution of chemicals shown in Table 3 it is evident that most of the substances have an $LD_{50} > 2000$ mg/kg b.w. (92.5%). Six and a half percent of analysed chemicals fall in the GHS category 4 and the rest (0.9%) in the remaining three GHS categories.

Given the distribution of the substances within the GHS classes we focused on the identification of a threshold for NOAEL values, that enable to discriminate substances not classified as acute oral toxic, i.e. those with an $LD_{50} > 2000$ mg/kg b.w., from the rest. The aim was to set the threshold to minimise false negative results, while still identifying as many non-toxic substances as possible. Analysing the distribution of the NOAEL values in these two classes, i.e. $LD_{50} > 2000$ and $LD_{50} < 2000$, we chose the NOAEL value $\geqslant 200$ mg/kg as a threshold (Table 4). According to this threshold a substance with a NOAEL $\geqslant 200$ mg/kg would be considered as non-toxic after acute oral exposure.

This threshold is suitable to correctly identify 63% (913/1436) of the non-toxic substances ($LD_{50} > 2000$ mg/kg) in our data set. In addition, 15 compounds (i.e. <1%), all being in the harmful class (LD_{50} 300–2000 mg/kg), were misclassified as non-toxic. Thus, setting this threshold the false negative rate was 13% (15/116) and false positive rate was 37% (523/1436). When taking into account the prevalence of the non-toxic substances (87% of new chemicals have $LD_{50} > 2000$ mg/kg), the negative predictive value (NPV) of this approach is 97%, while the positive predictive value (PPV) is 26.5%.

Table 4
The relationship between the NOAEL values (grouped in two classes: >200 and <200 mg/kg b.w.) and the oral LD_{50} (grouped in two classes: >2000 and <2000 mg/kg b.w.).

NOAEL (mg/kg b.w.)	LD_{50} (mg/kg b.w.)		Total
	$\leqslant 2000$[a]	>2000[b]	
<200	**101**	523	624
$\geqslant 200$	15	**913**	928
Total	116	1436	1552

Bold values are those where acute toxicity of compounds is correctly identified using the threshold of NOAEL 200 mg/kg b.w.
[a] Substances falling in GHS categories 1–4.
[b] Substances in GHS category 5 and not classified.

Unfortunately, it was not possible to set any other threshold to further classify all the substances with NOAEL values <200 mg/kg due to the random distribution of the LD_{50} values as shown in Fig. 2A and B, as well as due to the low number of toxic chemicals (Table 1) among new chemicals notified.

5. Conclusions

As opposed to the acute toxicity testing based only on estimation of lethality, repeated-dose toxicity is a more sensitive endpoint, which provides wider toxicological information including in-life clinical signs and histopathology.

On the basis of the results obtained on a set of data, which contained only toxicological information on industrial chemicals and not on compounds with defined biological activity (e.g. pharmaceuticals, veterinary products, pesticides), we can conclude that NOAEL values obtained from 28 days studies in rat could be useful to estimate acute oral toxicity.

Indeed, on the basis of NOAEL values $\geqslant 200$ mg/kg obtained from 28 days repeated dose studies in rat it was possible to correctly identify "non-toxic" substances ($LD_{50} > 2000$ mg/kg b.w.). This approach would miss <1% of the substances which are harmful classifying them as "non-toxic". Notably, this misclassification was only within one category and none of the toxic substances would have been missed. Nevertheless, for all the substances falling in the category NOAEL <200 mg/kg there is still a strong need for an alternative approach, because of the substantial amount of false positives generated (37%, i.e. 523/1436).

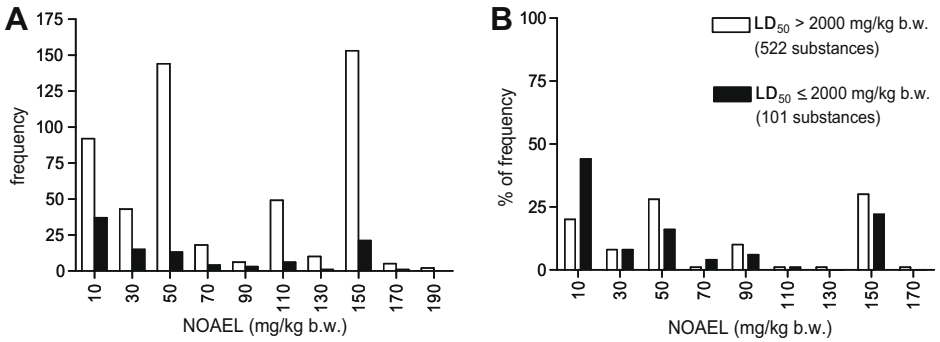

Fig. 2. Distribution of NOAEL values <200 mg/kg b.w. for the substances with LD_{50} < 2000 mg/ml (101 substances) and LD_{50} > 2000 mg/ml (523 substances). The frequency distribution of NOAEL in both categories is random (A) and similar when normalized data (presented as percentages) are compared (B).

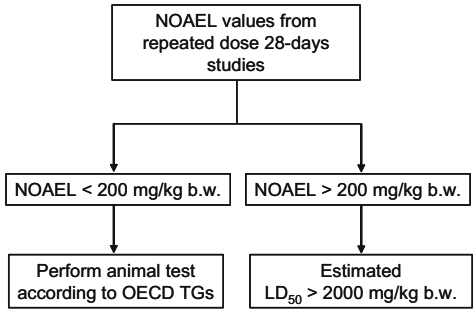

Fig. 3. The scheme illustrates how to apply the proposed threshold approach based on NOAEL to identify compounds with LD_{50} > 2000 mg/kg b.w., which according to the recent E.C. proposal are "not classified" for acute toxicity (European Commission, 2007).

However, taking into consideration the prevalence of "non-toxic" compounds (87%), and the fact that using the threshold of NOAEL $\geqslant$200 mg/kg b.w. it is possible to correctly identify 63% (913/1436) of the non-toxic substances (LD_{50} > 2000 mg/kg) we concluded that with this approach it is feasible to categorise more then half of all the new substances.

It would be worthwhile to perform a similar analysis using information coming from shorter duration repeated dose studies (e.g. dose-range finding studies of 7 days or more), however this information is not available in the New Chemicals Database, which has been the data source for the present study.

The results presented here are of major interest for the cosmetic industry due to the 2009 deadline, which introduces a testing and marketing ban of products with ingredients tested on animals. The approach could filter 55% of substances continuing to potency testing evaluation for classification and labelling only in the remaining cases.

It must be noted that according to the current regulations acute toxicity data are used to select appropriate dosages for the repeated dose studies. However, the *in vivo* acute toxicity testing of cosmetic ingredients will be banned from March 2009 and there are no regulatory accepted alternative methods available so far. A solution would be to derive the starting dosages for the repeated dose studies from *in vitro* cytotoxicity. This is already the case of dose setting for *in vivo* acute oral toxicity, as demonstrated in NICEATM-ECVAM the validation study of the neutral red cytotoxicity assays.

A possible decision tree based on the use of the NOAEL threshold to identify compounds with LD_{50} > 2000 mg/kg b.w. is presented in Fig. 3. According to the recent E.C. proposal (European Commission, 2007) these compounds would be considered as "not classified" for acute oral toxicity.

The proposed approach could have an immediate impact for testing of cosmetic ingredients at least until the 2013 deadline, which might even be postponed according to the legal revision scheduled for 2011 (European Commission, 2003). In the future we are planning to perform the same analysis using specifically data on cosmetic ingredients.

6. Conflict of Interest statement

The authors declare that there are not conflicts of interest.

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