

Acute Systemic Toxicity

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Abstract

Use of the test that aimed to identify the single lethal dose of a substance that kills half the animals in a test group (the LD₅₀ test) should finally be discontinued by the end of 2002, after many years of controversy and debate. In its stead are three recently developed alternative animal tests that significantly improve animal welfare: the fixed dose procedure, the acute toxic class method, and the up and down procedure. These tests have already undergone revision, both to improve their scientific performance and, importantly, to increase their regulatory acceptance. They can now be used within a strategy of acute toxicity testing for all types of test substances and for all regulatory and in-house purposes. In vitro cytotoxicity tests could be used (perhaps by mid-2002) as adjuncts to these alternative animal tests to improve dose level selection and reduce (at least modestly) the number of animals used. However, the total replacement of animal tests requires a considerable amount of further test development, followed by validation, which will require at least 10 yr.

Key Words: alternative methods; in vitro cytotoxicity assays; LD₅₀ test; validation

Historical Perspective

Trévan (1927) first introduced a test that aimed to identify the single lethal dose of a substance that kills half the animals in a test group (LD₅₀¹) for testing substances intended for human use (e.g., digitalis and insulin). However, by the 1970s, the test that had become generally accepted as a basis for comparing and classifying the toxicities of chemicals had also gradually become a required test for various regulatory bodies concerned with new drugs, food additives, cosmetic ingredients, household products, industrial chemicals, and pesticides. The test required up to 100 animals, sometimes for each of two species (normally

the rat, but also the mouse when a second species was needed) for each substance tested.

In 1981, the Organisation for Economic Co-operation and Development (OECD¹) incorporated the LD₅₀ test into its new test guidelines. By this time, it was generally agreed that the statistical precision of the lethal dose of a substance identified as killing half the animals in a test group (the LD₅₀ value), together with its confidence intervals and the slope of the dose-mortality curve, which the classical test could provide, were not needed for normal hazard and risk assessment purposes. Hence, the 1981 guideline for acute oral toxicity (OECD 1981a) required the use of only five animals per sex per dose group, with three dose groups per test, which were chosen from sighting studies or from historical data to span the LD₅₀ value. An upper dose level limit of 5000 mg/kg was also introduced and, for essentially nontoxic substances, the concept of a limit test was included, which required only this upper dose level to be tested for substances with LD₅₀ values greater than 5000 mg/kg. OECD also published similar guidelines for acute dermal (OECD 1987b) and inhalation (OECD 1981b) toxicity.

In 1987, OECD 401 was revised, primarily for animal welfare reasons. The test could now be conducted using animals of only one sex, with confirmation that there were no sex differences in the acute toxicity of a test material by testing at just one dose level in the second sex. This change reduced the required number of animals from 30 to 20 and reduced the limit dose to 2000 mg/kg.

In 1984, a working party of the British Toxicology Society proposed a new method for acute oral toxicity testing, which avoided using the death of animals as an endpoint and relied instead on the observation of clear signs of toxicity developed at one of a series of fixed dose levels. This method, which became known as the fixed dose procedure (FDP¹), was assessed in both a national and international validation study, the latter of which was published in 1990 (van den Heuvel et al. 1990). These studies revealed that the FDP is able to provide results that enable substances to be ranked according to the European Union system of classification, in a way broadly compatible with how they would have been allocated by LD₅₀ values derived from classical acute oral toxicity tests. The FDP also provides necessary information on the nature, time to onset, duration, and outcome of signs of toxicity that is required for risk assessment purposes. In so doing, the FDP uses fewer animals than OECD 401, causes less compound-related mortality, and subjects the animals that are used to less pain and distress.

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¹Abbreviations used in this presentation: ATC, acute toxic class; FDP, fixed dose procedure; LD₅₀, lethal dose of a substance that kills half the animals in a test group; OECD, Organisation for Economic Co-operation and Development; UDP, up and down procedure.

The FDP was adopted as an OECD guideline (OECD 1992) but as an alternative to OECD 401, not as a replacement. In 1996, a second alternative method, the acute toxic class method (ATC¹), was adopted (OECD 1996); and it was followed in 1998 by the up and down procedure (UDP¹) (OECD 1998). The ATC also uses the concept of fixed dose levels but retains mortality as a principal endpoint. The UDP, as its name suggests, aims to estimate the LD₅₀ value by testing individual animals sequentially, with the dose for each animal adjusted up or down depending on the outcome for the previous animal.

The practical use of these three alternative methods has not been as widespread as the animal welfare community and many toxicologists originally envisaged. In Europe, the FDP and ATC have been used extensively for the notification of new industrial chemical substances under the Dangerous Substances Directive, inasmuch as the methods were incorporated into the European Union Annex V test guidelines (EC 1997). However, in the United States, although they were included in the 1998 revision of the Environmental Protection Agency's Office of Prevention, Pesticides and Toxic Substances (EPA 1998) guidelines, their use has been restricted by the requirements of key legislation governing the classification and risk assessment needs for substances such as consumer products and pesticides. This legislation still requires a point estimate of the LD₅₀, together with slope and confidence intervals, which the FDP and ATC are not able to provide. In addition, this same legislation sometimes requires the use of a limit dose of 5000 mg/kg, and the alternative method guidelines had adopted the lower limit dose of 2000 mg/kg. In Japan also, even in the latest revision of the Japanese Ministry of Agriculture, Fisheries, and Food guidelines for pesticide registration published in 2001, the use of OECD 423 and 425 (but not OECD 420—the FDP) is acceptable.

Controversially, throughout the 1990s, many laboratories continued to use the 1981 version of OECD 401 in addition to underutilizing the alternative tests. This continued use was due not only to the higher limit dose requirement but also to the practical difficulty of knowing which dose level should be used to confirm a lack of sex difference in susceptibility to a test substance.

In an attempt to facilitate the international acceptance of the FDP, ATC, and UDP, and to begin phasing out and eventually deleting guideline 401, the OECD organized a series of expert meetings between 1998 and 2000. Meeting participants drafted revisions to guidelines 420, 423, and 425 and provided a guidance document on the use and interpretation of the alternative methods for toxicologists and regulators to use. In addition, the US Environmental Protection Agency commissioned an Interagency Coordination Committee on the Validation of Alternative Methods (ICCVAM) review of a revised version of the UDP, which a peer review committee initially endorsed in 2000. These revisions have allowed retention of the overall improvements in animal welfare but, more importantly, will allow the alternative methods to be used worldwide for all regu-

latory purposes. Hence, in 2000, the OECD Joint Meeting of the Chemical Committee and Working Party on Chemicals, Pesticides and Biotechnology was able to conclude that OECD 401 could be deleted by linking it to the adoption of the revisions to 420, 423, and 425. At the time these Proceedings were being prepared for publication, it was anticipated that the OECD Council would ratify this conclusion by September 2001, and that the final deletion of the LD₅₀ test would occur 1 yr later to allow a period of training and familiarization with the alternative methods.

Strategy for Acute Oral Toxicity Testing in the "Post-LD₅₀ Test Era"

Because the information the three new methods can provide is not identical, the choice of method must be made on the basis of a clear understanding of the scientific and regulatory purposes for the proposed acute oral toxicity test. One of the criteria to be considered is the type of substance being tested (e.g., whether it is a pesticide, consumer product, or cytotoxic anticancer agent). However, the specific regulatory need (if any) is an especially important consideration (i.e., whether the test is needed for hazard assessment leading to classification and labeling, for consumer risk assessment, or for worker safety). Some tests are required only for "in-house" purposes such as product selection and dose setting for subacute studies.

Details of the three alternative methods are beyond the scope of this presentation; however, they are available on the OECD website (<oecd.org/ehs/test/testlist.htm>). An overall summary is shown in Table 1.

As stated above, the revisions have retained the overall animal welfare benefits of OECD 420, 423, and 425 in comparison with 401. These are summarized in Table 2. Although the choice of method can be made with animal welfare as a major factor, as Table 2 reveals, the numbers of animals used in the three alternatives is very similar and significantly less than with the 1987 version of guideline 401. Similarly, the levels of pain and distress should be controlled uniformly by the requirement to follow the OECD guidance document on humane endpoints, which can also be found on the OECD website (<oecd.org/ehs/test/monos.htm>). The FDP retains its feature of having evident toxicity, rather than mortality, as a principal endpoint. However, even the UDP, which can still allow the derivation of a point estimate of the LD₅₀ and also includes a computational method for obtaining confidence intervals, gives rise to relatively limited mortality because of the introduction of clear stopping rules into the protocol.

Future Strategy for Acute Toxicity Testing—Prospects for Further Reducing, Refining, and/or Replacing

The current status of *in vitro* methods for assessing acute toxicity was reviewed at an international workshop orga-

Table 1 Principles of the three alternative methods

	420 (Fixed dose)	423 (Acute toxic class)	425 (Up and down)
Methodology	Single bolus dose. Young adult rats (one sex) Oral gavage with constant volume or concentration, clinical observations, bodyweight, mortality over 14 days. Necropsy at termination.		
Sighting study	Yes	No	No
Dose levels	Fixed doses of 5, 50 300, 2000 (5000) mg/kg 5 rats per dose level	Fixed doses of 5, 50 300, 2000 (5000) mg/kg 3 rats per dose level	Starting at best estimate of LD ₅₀ (or 175 mg/kg) and using dose progression factor of 3.2, single animals dosed until one of three stopping criteria met
Aim	Identify lowest fixed dose causing evident toxicity	Identify lowest fixed dose causing mortality	Estimate LD ₅₀
Output	Range estimate of LD ₅₀ Signs of acute toxicity Target organ(s)	Range estimate of LD ₅₀ Signs of acute toxicity Target organ(s)	Point estimate of LD ₅₀ with confidence intervals. Signs of acute toxicity Target organ(s)

nized in 2000 by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM 2001a,b). From this review, a process was devised to offer realistic short- and long-term goals for further refining and then replacing the animal studies, at least for acute oral toxicity (the same principles could then be used for acute dermal and inhalation toxicity).

Although no standardized *in vitro* cytotoxicity assays, with optimized protocols and prediction models, have yet been validated, it appears from the number of studies showing positive correlations between cytotoxicity results *in vitro* and acute toxic effects *in vivo* that the application of such *in vitro* methods does have significant potential. Workshop participants concluded that the approach proposed by Spielmann and colleagues (1999), in which basal cytotoxicity IC₅₀ values (the concentration of a chemical required to kill 50% of cells) are measured in one or more cells or

cell lines using one of several endpoint measurements, could be introduced very quickly to improve the initial dose level selection in the ATC and UDP and to obviate the current need for a sighting study with the FDP. This course of action would result in further modest reductions in animal use. However, before these *in vitro* tests could be introduced into the testing strategy, there would be a need for a guidance document containing test protocol details, supporting information, and worked examples. There would, however, be no need for a validation study.

Workshop participants also concluded that the replacement of animal tests by *in vitro* assays would require much more time to implement. In taking this longer-term view, the development and validation of *in vitro* methods should concentrate on the prediction of human rather than rodent acute toxicity. In addition, the multicenter evaluation of *in vitro* cytotoxicity (MEIC) studies of Ekwall (1999), who investigated the correlation between acute lethal human blood concentrations and basal cytotoxicity in a battery of three human cell lines, are currently some of the most promising. Cytotoxicity assays would be able to give estimations only of the inherent LD₅₀ of a test material; and other *in vitro* assays would be needed to indicate how this potential toxicity would be affected (increased or decreased) by the absorption, distribution, metabolism, and excretion of the compound. In addition, other *in vitro* assays would be needed to provide the additional vital information for risk assessment currently obtained from *in vivo* acute toxicity studies, such as possible target organs, signs of toxicity, and reversibility of the effects.

Even for the more straightforward goal of introducing *in vitro* assays for predicting the inherent LD₅₀, prevalidation and validation studies would be needed, and these studies would require 2 to 3 yr. Hence, total replacement of the animal tests for acute toxicity are unlikely for at least 10 yr (J. Fentem, Unilever PLC, personal communication, 2001). These workshop proposals are summarized in Figure 1.

Table 2 Animal welfare considerations^a

Guideline	No. of animals/test	No. of deaths/test	
401*	≤25	≤12	
420*	5–7	1	Endpoint-evident toxicity
423	Average 7	2–3	
425	6–9	2–3	Stopping criteria to limit number of animals used

^aAll three alternatives contain requirement to follow Organisation of Economic Co-operation and Development Document on Humane Endpoints.

^bIncludes sighting study.

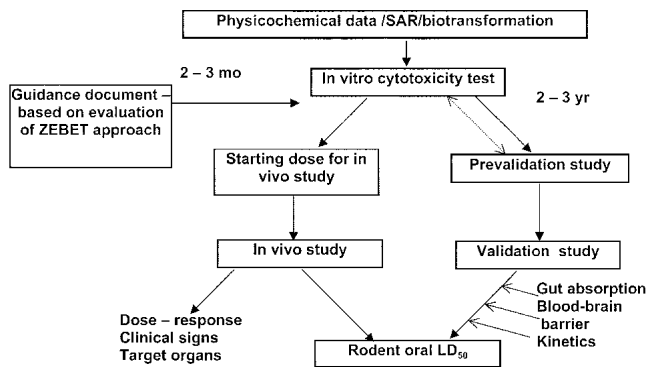


Figure 1 Summarized proposal of the workshop sponsored by the Interagency Coordinating Committee on the Validation of Alternative Methods. SAR, structure activity relationship; ZEBET, National Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments. Adapted from ICCVAM [Interagency Coordinating Committee on the Validation of Alternative Methods] (1) 2001a. Report of the International Workshop on in Vitro Methods for Assessing Acute Systemic Toxicity (NIH publication 01-4499). Research Triangle Park: National Institute of Environmental Health Sciences; and (2) 2001b. Guidance document on using in vitro data to estimate in vivo starting doses for acute toxicity (NIH publication 01-4500). Research Triangle Park: National Institute of Environmental Health Sciences.

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