

## Humane endpoints in toxicity testing

### E. Schlede, W. Diener & I. Gerner

Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), Thielallee 88-92, D-14191 Berlin, Germany [e-mail: e.schlede@bgvv.de]

### Summary

The current use of humane endpoints in the fields of animal testing for acute oral toxicity, skin and eye irritation and corrosion, and skin sensitization is presented. The data used were obtained within the framework of the regulatory activities for the development of new methods, and within the notification procedure for new chemicals in the European Union. It is demonstrated that progress is being made, for example in the recommendation of 'alert clinical signs' indicative of impending death, and the development of *in vitro* tests for skin corrosivity testing on animals. It is obvious that further advancements will be made given the acceptance by the international regulatory agencies of alternative methods that are well validated and approved.

Within the framework of the regulation of chemicals, drugs, pesticides, etc., laboratory animal testing for the assessment of the toxicological properties of a substance is a worldwide requirement. During the last 10–20 years it became obvious that traditional animal testing with the use of large numbers of animals has had to undergo a rigorous reassessment, and animal welfare concerns have been one of the driving forces for this. The use of humane endpoints is a further step in refining and improving toxicological tests. Some examples are demonstrated in acute toxicity testing where the use of humane endpoints will contribute to the avoidance of death and the reduction in severe pain and severe distress for the animals.

### Survey details and results

#### *Acute oral toxicity testing*

The humane killing of animals whose death is anticipated depends on the experience of the responsible staff and on the inherent variability in the onset, duration and severity of the clinical signs of each animal within a test group. The moribund state is defined as a state where death is anticipated as a result of the treatment with a substance. The criteria for making the decision to humanely kill a

moribund animal, or where an impending death is anticipated, are not defined or laid down. Predictions of an impending death are also complicated by the fact that the test substances belong to completely different, and toxicologically often unknown, chemical classes, thus making a standardized recommendation difficult. In order to be able to give recommendations for the impending death of an animal, we are evaluating all the clinical signs that rats showed in multicentre studies on the oral Acute Toxic Class Method (Schlede *et al.* 1992, Schlede *et al.* 1994, OECD 1996). In these studies, 20–30 substances with different chemical structures and different toxicities (the LD<sub>50</sub> values ranged from < 1 mg to > 6000 mg/kg) were tested by 15 laboratories in 6 countries. The onset, duration and severity of the clinical signs in each of the 3942 rats (1128 rats died and 66 rats were killed in a moribund state) were documented in a database containing around 7 million data points. From the results obtained so far, it can be shown that:

- there are large variations in the documentation of clinical signs; e.g. one laboratory reported an incidence of clonic convulsions as 39% and another laboratory as only 9%;

- the 66 moribund animals exhibited 62 different combinations of clinical signs;
- more than 600 different combinations of clinical signs were reported for the 1128 rats that died during the study, and out of them 470 combinations were reported only once;
- in the surviving animals, fewer clinical signs were generally reported and the combinations of clinical signs that were reported only once is lower in comparison with the above-mentioned groups;
- there seems to be no direct relationship between the severity of a single clinical sign in relation to the moribund state, death or survival; e.g. for 'disturbed locomotor activity', animals that died or survived were reported to show both weak and strong, and even severe disturbances in locomotor activity, and one animal was killed after showing a moderate disturbance;
- moribund animals and animals that died during the study had a higher frequency of all forms of convulsions (clonic convulsion, tonic convulsion, tonic-clonic convulsion, saltatory convulsion) than surviving animals. In surviving animals convulsions are almost non-existent (< 1% in comparison to 45% and 28% in the other two groups), e.g. only 10 out of 210 animals that survived had clonic convulsions; and
- lateral recumbency and tremor are observed in surviving animals at distinctly lower rates than in animals that die or that are moribund, the difference being at least 6-fold.

Based on these results, convulsions, lateral recumbency and tremor should be considered as 'alert signs' for the impending death of rats in acute oral toxicity testing. In the ongoing evaluation of clinical signs that precede death we will further investigate, and if possible substantiate, the importance of specific clinical signs as predictors of death, moribund state and survival.

In all acute toxicity tests, the presence or absence of death is used for the classification of substances into categories such as 'very toxic', 'toxic', 'harmful' or 'no classification'.

For example, 75% of new chemicals placed on the market in the European Union are tested but not classified for acute oral and inhalation toxicity. For acute dermal toxicity this rate is 96%. Thus, very few animals die in these sorts of tests. In acute toxicity tests with those substances that have to be classified, death occurs in 30–80% of the animals.

After the completion of our studies on the predictive relevance of clinical signs to survival, death and moribund status, it may be possible to reduce or even avoid death as a criterion for the ranking of hazardous substances.

#### *Skin and eye irritancy and corrosion testing*

Considerations regarding humane endpoints for skin and eye irritancy and corrosion testing have existed for more than 15 years. Formerly, 6 animals were required for each test, but since 1981 this requirement has been reduced to 'at least three animals per test' (OECD 1981) with a further refined approach of the use of a single animal (see OECD 1987, 1992). A test on only a single animal is to be employed when severe irritation, corrosion or marked effects are produced or are anticipated. Depending on the result obtained with the one animal, no further testing may be needed, otherwise 2 additional animals may have to be used. However, these 2 animals are only employed to confirm a 'no effect' result with the first animal. The approach today is to take into account all available existing information, including pH values, structure-activity considerations and *in vitro* tests (OECD 1996). *In vitro* tests for the assessment of skin and eye irritation have still to be approved by the OECD as validated alternatives to animal testing. However, alternatives to skin corrosivity testing on animals (for example, TER and EPISKIN assays) are now validated and will be submitted to the OECD for approval (Fentem *et al.* 1998).

Alternatives to eye testing on animals are still in the evaluation phase because the eye is a complicated organ. The development of *in vitro* tests for effects on eyes should be focused

on specific endpoints, such as the ranking of corneal opacity or of conjunctival lesions. So far, 9 tests have been evaluated and, at present, none of these meet the criteria sufficiently to serve as a replacement to whole animal testing (Purchase *et al.* 1998). However some tests (Spielmann 1997) are acceptable as alternatives by the EU regulatory authorities on condition that the results obtained permit a reliable assessment of severe eye lesions.

We have evaluated the data from skin and eye irritation and corrosion tests from 1040 substances that were submitted within the notification procedure for new chemicals in the EU between 1982 and 1997 and we found the following results:

- 70/1040 substances (6.7%) were corrosive to skin. No animal tests were conducted on 15 of these substances because their effect could be predicted on the basis of their physicochemical properties and/or structure-activity considerations. The remaining 55 substances were tested on either one or 3 animals depending on existing information about them, the year of testing and the current OECD Guideline. It is expected that the use of validated *in vitro* tests for skin corrosivity will lead to animal testing for this endpoint being removed.
- 159/1040 substances (15.3%) were labelled 'risk of serious damage to eyes'.
- 14/159 substances were assessed by alternatives: based on pH value  $\leq 2$  or  $\geq 11.5$  (five substances); structure-activity relationships (four substances); bovine eye test (two substances); HET-CAM test (one substance); EYETEX (one substance); and rabbit isolated eye test (one substance).
- 145/159 substances were assessed by Draize tests using rabbits: 46/145 tests were with a single animal (31.7%); 75/145 tests with 3 animals (51.7%); 24/145 with more than 3 animals per test (16.6%); 41/145 tests were terminated between 10 min and 3 days after the instillation of the substance (28.3%); and 57/145 tests were observed for the full period of 21 days in order to assess the reversibility of the lesions (39.3%).

The above-mentioned *in vitro* tests (Spielmann 1997) are only applied in in-house testing by laboratories with experience of specific chemical classes, and the results obtained from these tests are accepted by the regulatory authorities on a case-by-case basis. Considerations of structure-activity relationships and pH values have been used since 1982, and the use of *in vitro* tests since the beginning of 1990. Thus, several approaches are being used to minimize or avoid animal testing for this endpoint. It is expected that the use of three or even more animals for the assessment of serious eye damage will be terminated in the near future.

Eighty-one out of 1040 substances (7.8%) were labelled 'irritating to skin' and 46/1040 (4.4%) were labelled 'irritating to eyes' and, on average, 3 animals were used in each test. At present, the only achievement has been a reduction in the number of animals used from 6 to 3. The irritation effects ranged from severe to barely perceptible. Substances giving mild or moderate eye effects lead to ambiguous results with all *in vitro* methods, and even animal tests using 18 rabbits did not result in a clear-cut decision. Therefore, the labelling of borderline eye irritancy results becomes a question of toxicological hazard assessment (requiring expert judgment) and is not a question of the number of animals used in a test (OECD 1998). Alternatives to skin testing on animals are most likely to be developed on the basis of structure-activity considerations, and a first step will focus on substances that are not irritant or corrosive to the skin. Given the length of time it took to develop alternatives to skin corrosivity testing, it is expected that no validated alternatives for skin or eye irritancy testing will be available within the next 5 to 10 years.

#### *Skin sensitization*

The use of animals can also be avoided with respect to sensitization. No skin sensitization test should be conducted when a new substance is structurally related to a known skin sensitizer, an example being the group of glycidyl-diethylethers. If a new substance has a

known skin sensitizer in the form of impurities and/or additives at a concentration of  $\geq 1\%$ , then the substance is classified as a skin sensitizer and no animal testing is required. An example is formaldehyde, when it is present as an ingredient or as a result of decomposition from contact with water.

## Discussion

Russell and Burch (1959) proposed the 'Three Rs' of refinement, reduction, and replacement to animal testing. The term 'humane endpoint' should be used to further define the activities of scientists and regulators within the concept of the 'Three Rs'. They are fully aware of the fact that traditional testing using large numbers of animals is, in general, not necessary for meeting the need of human health protection from hazardous substances. However, the goal of using alternatives to animal testing, such as *in vitro* tests and considerations of structure-activity relationships, is often restricted by a limited understanding of the toxicological mechanisms. Thus, progress is slow and advancements are unlikely to be made in the very near future. Alternatives have to be fully validated and must meet human protection requirements to the same degree as, or even more than, animal tests. In addition, alternatives must be approved by the OECD in order to facilitate a worldwide mutual acceptance of the data.

This article has covered the present status of achievements for humane endpoints in acute toxicity testing. Further progress is expected after the implementation of a worldwide harmonization of classification criteria of hazardous substances.

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