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DRAFT GUIDANCE DOCUMENT ON ACUTE INHALATION TOXICITY TESTING

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INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress or changing assessment practices. The original acute inhalation Guideline 403 was adopted in 1981. Development of an Inhalation Fixed Concentration Procedure (FCP) was considered appropriate, following adoption of the revised Oral Fixed Dose procedure (FDP), OECD Guideline 420. This FCP Guideline will allow the use of a series of fixed concentrations for the determination of acute inhalation toxicity in only one sex (usually females) and is based on the approach suggested originally by the British Toxicology Society (1). Importantly, this approach is based on the observation of clear clinical signs of toxicity and avoids deaths of animals being used as an endpoint. In agreement with the OECD Guidance Document on Humane Endpoints (2) refinements are introduced in order to reduce suffering and distress by the animals and, to the extent feasible, reduce the number of animals used.

2. The FCP allows the classification of substances according to the Globally Harmonised System (GHS) for the classification of chemicals which cause acute toxicity (3).

PURPOSE

3. The purpose of this Guidance Document is to provide additional information for both the regulated community and regulators to assist with the choice of the most appropriate Guideline to enable particular data requirements to be met and the conduct and interpretation of the FCP Guideline 433.

DATA NEEDS

4. Acute inhalation toxicity data are used to satisfy hazard classification and labelling requirements, for risk assessment for human health and the environment, and when estimating the toxicity of mixtures. The provision of either a point estimate of the LC_{50} value or range estimate of the LC_{50} generally meets the acute inhalation toxicity data requirements for classification for all regulatory authorities in the areas of industrial chemicals, consumer products and for many pesticide applications. The data needs of the majority of Member countries can also be met even with the imposition of a limit concentration. However, several countries have a requirement for information on toxicity at concentration levels above the limit concentration. For reasons of animal welfare concern and the practicalities of atmosphere generation, testing of animals in GHS category 5/unclassified ranges (>20 mg/l, vapours; > 5 mg/l dusts/mists and >5000 ppm gases) should only be considered when there is a likelihood that results of such a test have a direct relevance for protecting human or animal health or the environment.

COMPARISON OF GUIDELINES 403 AND 433

OUTLINE OF THE METHODOLOGY

5. Both Guidelines involve the exposure of a single concentration of test substance for a short period of time to healthy young adult rodents by head/nose-only or whole-body exposure, observation for up to 14 days after exposure, recording of body weight and the necropsy of all animals. Animals are exposed to test concentrations that are held as constant as practicably possible.

6. Each animal should be selected from the available animals in a random fashion. In recognition of the fact that most animal suppliers do not indicate littermates, the guidelines do not call for randomising animals from a single litter across exposure groups. Females should be nulliparous and non-pregnant. At the commencement of its exposure, each animal should be between 8 and 12 weeks old and its weight should fall in an interval within ±20% of the mean weight of all previously exposed animals taken on their day of exposure. In order to conform to these age and weight requirements at the start of exposure of each animal, it may be necessary to order animals sequentially as the tests can sometimes take several weeks to complete.

7. **Guideline 433:** The primary endpoint for Guideline 433 is the observation of clear clinical signs of toxicity (termed: 'evident toxicity'). Groups of female animals are exposed for at least four hours to graduated concentrations of the test substance, one concentration being used per group. A sighting study is included for Guideline 433 in order to choose an appropriate starting concentration for a main study and to minimise the number of animals used. Pre-specified fixed concentrations of 0.5, 2, 10 and 20 mg/l for vapours, 0.05, 0.5, 1 and 5 mg/l for dusts/mists and 100, 500, 2500 and 5000 ppm for gases are used both in the sighting study and the main study. For reasons of animal welfare concern, testing of animals in GHS category 5/unclassified ranges (>20 mg/l, vapour; > 5 mg/l dusts/mists and >5000 ppm gases) is discouraged. Groups of animals are exposed in a stepwise procedure, with the initial concentration being selected as that expected to produce some signs of evident toxicity. Further groups of animals may be exposed at higher or lower fixed concentrations, depending on the presence of signs of evident toxicity, until the study objective is achieved; that is, the classification of the test substance based on the identification of the concentration(s) causing evident toxicity, except when there are no effects at the highest fixed concentration.

8. **Guideline 403:** The primary endpoint for Guideline 403 is mortality. Several groups of male and female animals are exposed for at least four hours to graduated concentrations of the test substance, one concentration being used per group. Subsequently, observations of effects and deaths are made. In practice many studies are limit tests at the maximum concentration and use only one group, but for full studies exposure concentrations should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates to produce a concentration mortality curve and permit an acceptable determination of an LC_{50} value. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere a vehicle control group may be used

Animal Welfare Considerations

9. Guideline 433 provides significant improvements in the number of animals used in comparison to Guideline 403, which requires ten animals for a limit test or at least 30 animals for a full study. In addition, it contains a requirement to follow the OECD Guidance Document on Humane Endpoints (2), which should reduce the overall suffering of animals used in this type of toxicity test. Unlike Guideline 403, it has as its endpoint evident toxicity rather than mortality. A sighting study is used to allow selection of the initial concentration level that is expected to produce clear signs of toxicity without causing severe toxic

effects or mortality, so minimising the total numbers of animals used.

10. **Guideline 433:** Groups of five young adult animals of one sex are exposed per step in the main study. Single animals are used per step in the sighting study. Regulatory experience with the oral fixed dose procedure has shown that most tests are likely to be completed with either one or two sighting study steps and one main study step, thus using between 6 and 7 animals. Up to six animals are used in a limit test. Concentrations of test substance that are expected to cause marked pain and distress due to corrosive or severely irritant actions should not be administered.

11. The following estimates of the number of treatment related deaths for tests conducted on substances with LC_{50} values below the limit dose are based on practical experience and statistical modelling.

- **Guideline 433: typically one animal can be expected to die on test.**
- **Guideline 403: the expected number of deaths is typically between 5 and 15.**

12. For both guidelines, careful clinical observations should be made at least twice on the day of exposure or more frequently when indicated by the response of the animals to the treatment, and at least once daily thereafter. Additional observations are made if the animals continue to display evident signs of toxicity. Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Guidance on clinical signs and clear signs of toxicity can be found in Chan and Hayes (4) and Van den Heuvel *et al*. (5). Animals that are moribund and/or suffering severe pain and distress must be humanely killed. Guidance on clinical signs and objective measurements that are indicative of impending death and/or severe pain and/or distress is available in an OECD Guidance Document (2). Humanely killed animals are considered in the interpretation of the results in the same way as animals that died on test. Care should be taken when conducting examinations for clinical signs of toxicity that initial poor appearance and transient respiratory changes resulting from the exposure procedure, are not mistaken for treatment-related effects.

Information Provided by Each Method

13. Test Guideline 433 provides a range estimate of the LC_{50}; the ranges are defined by cut-off values of the new GHS classification system and not as a calculated lower and upper level. The range is inferred from the fixed concentration that produces evident toxicity. Interpretation of the classification achieved under the GHS system (3) is explained in Annex 2 of the Test Guideline 433. In order to bridge the period until the adoption of the GHS in all OECD Member countries a table converting GHS hazard categories to existing EU categories is provided in Annex 1.

14. The results of tests conducted according to Guideline 403 provide a point estimate of LC_{50} with confidence limits on the estimate of the LC_{50} and allow a test substance to be classified according to all the systems in current use.

Limitations of the Methods

15. Guideline 433 has clear advantages over Guideline 403 in terms of animal welfare considerations particularly as it does not use lethality as an end point. Statistical modelling indicated that the test will be completed with considerably fewer deaths and suffering than Guideline 403 and would result in classification outcomes similar to or in a more stringent GHS class than that based on the LC_{50} value, for those substances with a steep (greater than 2) dose response curve for mortality (see Annex 2). Statistical

simulations identified areas where Guideline 433 may have outcomes which result in a more or less stringent classification than that based on the "true" LC_{50} value (as obtained by the Guideline 403). Comparative statistical analysis indicated that the method is likely to perform poorly for chemicals with shallow concentration-response slopes. The study outcome is likely to be influenced by the choice of starting concentration level(s), relative to the "true" LC_{50} value, especially in the case of shallow slopes. Because Guideline 433 uses evident toxicity as an endpoint instead of death, information on toxic effects seen only at levels close to a lethal concentration will not always be obtained.

OPTIMISING THE PERFORMANCE OF THE TEST

16. Guideline 433 provides procedures to assist in selecting the starting exposure concentration, particularly in the event that minimal prior information on the substance itself is available. All available information on the test substance must be made available to the testing laboratory and should be considered prior to conducting the study. Such information will include, for example, the identity and chemical structure of the substance; its physico-chemical properties; the result of any other *in vivo* or *in vitro* toxicity tests on the substance; toxicological data on structurally related substances; the anticipated use(s) of the substance; and the likely regulatory data requirements. This information is necessary to satisfy all concerned that the test is relevant for the protection of human and animal health, to select the most appropriate test to satisfy regulatory requirements and will help in the selection of the starting exposure concentration.

17. The efficiency of the test conducted, in terms of reliability and numbers of animals used, is optimised by the choice of a starting concentration close to the lowest concentration producing evident toxicity. When this type of information is not available, advice on the starting concentration level that should be used to minimise the possibility of biased outcome and adverse effects on animal welfare is outlined in the guideline.

18. The limit test is an efficient way to characterise substances of low toxicity when there is sufficient information available indicating that the toxic concentration is higher than the limit concentration. Guideline 433 provides a limit test suitable for the design of the main study (20 mg/l, 5 mg/l or 5000 ppm for vapours, dusts/mists and gases, respectively). A pre-specified fixed concentration of 5 mg/l (actual concentration of respirable substance, or where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration) is used for a limit test conducted according to Guideline 403.

Exposure Conditions

19. It is technically difficult to generate test atmospheres that accurately meet the specified exposure criteria. Therefore, to prevent unnecessary repeat testing, a test atmosphere is considered acceptable for regulatory purposes if the mean of the test atmosphere samples is within ±25% of the concentration tested. When administrating potentially explosive test substances, precautions should be taken to avoid favourable conditions for explosions. During the exposure period, the actual concentrations of the test substance shall be held as constant as practicable and monitored continuously or intermittently depending on the method of analysis. Actual concentrations of the test substance should be measured in the breathing zone of the rats in such a way that the samplings not interfere with the test procedures.

Particle size distribution

20. The particle size distribution of the test aerosol should be determined at least twice during each 4-hour exposure. A range of sampling devices is suitable but the device selected must allow calculation of the mass median aerodynamic diameter (MMAD) (6). Adequate information should be available within the testing facility to demonstrate that such samplers collect an atmospheric sample that is representative of the atmosphere to which the animals are exposed.

USE OF A SINGLE SEX

21. Guideline 433 is conducted using a single sex in order to reduce variability and as a means of minimising the number of animals used. Normally females are used. This is because surveys of conventional LC_{50} tests show that usually there is little difference in sensitivity between the sexes but, in those cases where differences were observed, the available evidence does not clearly suggest that one sex is more sensitive than the other (7). For oral exposure, however, data are available to indicate that females are generally more sensitive than males (8). For systemically toxic chemicals, this may be because female rats have a lower detoxification capacity than males, as measured by specific activity of phase I and II enzymes. However, all available information should be evaluated, for example on chemical analogues and the results of testing for other toxicological endpoints on the chemical itself, as this may indicate that males may be more sensitive than females. Knowledge that metabolic activation is required for a chemical's toxicity can also indicate that males may be the more sensitive sex. In view of this, it is recommended that females should be the preferred sex for use in Guideline 433. Although the use of a single sex (females) also contributes to a further decrease in the use of animals in testing, theoretically this may lead to an oversupply of the other sex (males). However, currently the use of males in experimental animal tests clearly exceeds that of females and, thus, the preference for females in acute toxicity testing may well result in a better overall balance of the use of both genders. Occasionally, the results of subsequent testing, for example a sub-chronic test may raise concerns that the more sensitive sex had not been used. In such cases, and only when considerable differences between the sexes are suspected, it may be necessary to conduct another full acute inhalation toxicity study in the second sex. This is preferable to conducting confirmatory testing in a small group of animals of the second sex as a late satellite to the original test because there is a strong possibility that this would produce results that are difficult to interpret. The impact of conducting a second full test on the overall number of animals used in acute toxicity testing should be small because re-testing is anticipated to be infrequent and the results of the test in one sex, together with data from any subsequent studies, will greatly assist in the selection of starting concentrations closer to the LC_{50} in the second test.

22. Guideline 403 is conducted using animals of both sexes.

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Annex 1

Conversion of Classifications for LC_{50} by inhalation using GHS and EU classification systems

	Classify EU Category		
GHS Classification	**Vapours**	**Dusts/Mists**	**Gases**
Class 1	**T+**	**T+**	**T+**
Class 2	**T**	**T+**	**T**
Class 3	**H**	**T**	**T**
Class 4	**H**	**H**	**H**
Class 5/unclassified	**U**	**U**	**U**

T+ = very toxic
T = toxic
H = harmful
U = unclassified

Annex 2

STATISTICAL BASIS FOR ESTIMATING ACUTE INHLATION TOXICITY BY OECD GUIDELINE 433

INTRODUCTION

1. This document describes the statistical strengths and limitations of Guideline 433 for accurately determining a point estimate of the LC_{50}, confidence limits around the point estimate of LC_{50}, and information on the dose-effect response. In this context, a concentration-response curve applies to the estimation of lethality and a concentration-effect response applies to the estimation of the change in the variety and distribution of all other types of toxicological signs with the change in exposure concentration.

2. The statistical basis for all test methods is that lethality is a quantal response. Its measurement will give rise to a frequency distribution of responses reflecting the composite tolerances of the test population upon exposure to graded concentrations of the test chemical. In practice, most chemicals give rise to an approximately lognormal distribution of deaths versus dose, skewed toward hypersensitivity. When this frequency population is transformed to a logarithmic abscissa, a (symmetric) normal distribution generally results that can be characterised by two parameters, the median and the standard deviation, SD. The median is the dose at which 50% of the animals are killed by the test chemical and is called the LC_{50}. Not all animals will react in the same way to the chemical and thus SD represents the square root of the variance of the test populations' response to the chemical. The dose-response curve is sigmoidal in nature and represents the cumulative response of the test animals to the chemical. The inflection point of this sigmoidal curve coincides with the LC_{50} for the test population.

3. What follows is a brief description of the mathematical and biological principles underlying the acute inhalation FCP method followed by a listing of how this test estimates or does not estimate the specific parameters mentioned above.

Principles Underlying The Test Method

4. The Fixed Concentration Procedure (FCP) is a method for assessing acute inhalation toxicity that involves the identification of a dose level that causes evidence of non-lethal toxicity (termed *evident toxicity*) rather than a dose level that causes lethality. *Evident toxicity* is a general term describing clear clinical signs of toxicity following exposure to the test substance, such that an increase to the next highest fixed concentration would be expected to result in the development of severe toxic signs and probably mortality.

5. Underpinning the FCP is a belief that the toxic profile of a substance can be characterised with sufficient reliability for most regulatory situations without the need for the identification of a lethal concentration. That is, observations made at non-lethal concentrations will allow substances to be ranked, or classified, according to their acute toxicity, provide information to aid concentration level selection for repeat exposure studies and provide hazard data for use in a risk assessment.

6. Fixed concentration levels of 100, 500, 2500 and 5000 ppm for gases, 0.5, 2, 10 and 20 mg/l for vapours and 0.05, 0.5, 1 and 5 mg/l for dusts/mists (as required by the GHS classification scheme) and rules for the sequential procedure were adopted following a rigorous analysis using a statistical model (1). The analysis predicted the classification outcome (according to the EU scheme and the lethality-based GHS), numbers of animals used and number of substance-related deaths using a number of FCP design options for substances with a range of LC_{50} values and dose response slopes for lethality. On the basis of this analysis, the design of the FCP was optimised with respect to classification performance and animal welfare.

7. The statistical modelling showed that the FCP produces either classification outcomes similar to or in a more stringent GHS class than that based on the LC_{50} value, for those substances with a steep (greater than 2) dose response curve for mortality (1). For substances with a relatively shallow (2 or less) dose response curve there is an increasing probability the FCP will produce a more or less stringent classification than that based on the LC_{50} value; however, this is largely a consequence of the relatively close concentrations at which testing is conducted to comply with the GHS, rather than problems with the FCP. The influence of the choice of starting concentration on the classification outcome, which can be a problem with sequential procedures, is negligible.

Point Estimate of LC_{50}

8. The FCP is not designed to determine a point estimate of LC_{50}. However, an approximate LC_{50} range can be inferred from the classification outcome. The ability of the FCP to correctly classify (*i.e.*, assign to an LC_{50} range) is discussed above.

Confidence Limits on the Estimate of LC_{50}

9. The FCP is not designed to determine a point estimate of LC_{50}, or confidence limits on the estimate of the LC_{50}.

Concentration-Effect Curve

10. Since lethality is not the preferred endpoint for the FCP, information on toxicological effects seen only at concentration levels close to a lethal concentration will not always be available.

REFERENCES TO ANNEX 2

(1) Stallard, N. and Whitehead, A. (2002). Statistical evaluation of the FCP for inhalation studies (manuscript in preparation).