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Adopted:
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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Avian Acute Oral Toxicity Test

INTRODUCTION

1. This test guideline describes procedures designed to estimate the acute oral toxicity of test chemicals to birds, and it provides three testing options: (1) limit dose test, (2) LD_{50}-slope test, and (3) LD_{50}-only test. The LD_{50}-slope and LD_{50}-only options are sequential testing procedures. The test method selected will depend on whether or not a definitive median dose (LD_{50}) and slope of the dose-response curve are both needed. Sequential testing procedures target the placement of doses and match the precision of the endpoint with the precision required. These sequential procedures were designed to minimise the numbers of birds used. A computer programme – SEquential DEsign Calculator (SEDEC) – is available to aid the placement of doses and estimate the LD_{50}, slope and confidence limits (see paragraph 51).

2. Development of this test guideline began at the SETAC/OECD Workgroup on avian toxicity testing following a workshop held in Pensacola, Florida, United States, in 1994 (1) with subsequent open SETAC and closed OECD Expert Group meetings in Europe and the United States to develop and optimise the sequential testing design. The sequential testing design has been developed with extensive statistical validation (2).

INITIAL CONSIDERATIONS

3. The information required by different hazard assessment schemes may vary considerably. To satisfy these various needs, the following three tests are described:

- **Limit dose test** – This is the preferred test when toxicity is expected to be low and lethality is unlikely at the limit dose. The limit dose must be adequate for assessment purposes, and it is usually 2000 mg/kg-bwt. The needs of some regulatory authorities may necessitate using 2,000 mg/Kg-bwt or the highest environmentally relevant concentration, whichever is higher. Five or ten birds are tested at the limit dose in addition to a control group (Figure 1).
- **LD_{50}-slope test** – This is the preferred test when regulatory or other requirements determine that the slope of the dose-response curve and/or the confidence interval is required in addition to an estimate of the LD_{50}. This is a 3- or 4-stage test with 24 or 34 birds in addition to a control group (Figure 2). [1]

[1] When an estimate of slope is required, a classical dose-response test could be conducted instead (USEPA OPPTS 850.2100). In the interest of animal welfare, test methods which use more animals, such as USEPA OPPTS 850.2100, are generally not preferred.

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This Guideline was adopted by the OECD Council by written procedure on 29 July 2016 [C(2016)103].

- **LD_{50}-only test** – This is the preferred test when regulatory or other requirements determine that only the median lethal dose is required but neither the slope of the dose response curve or the confidence interval for the LD_{50} is required. This may be the appropriate test to estimate a percentile of a species sensitivity distribution of LD_{50}s and to provide information for product labelling purposes. This test has two stages, with 14 birds in addition to a control group (Figure 2).

Before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for the testing of the mixture.

4. The LD_{50}-slope and LD_{50}-only tests consist of stages that are performed sequentially (Figure 2). Stages are defined as periods during an experiment in which birds are dosed simultaneously and observed for a period of time. Thus, the terms sequential test and sequential design are used below to refer to both LD_{50}-slope and LD_{50}-only tests. An explanation of the logic and statistical basis of the design is given in Annex 1.

5. The LD_{50}-slope test design has been shown to have performance properties adequate for hazard identification and/or risk assessment purposes through extensive computer simulation (2). These performance properties include estimating the LD_{50}, confidence intervals, and dose-response slopes.

6. At each stage in either the LD_{50}-slope or the LD_{50}-only test designs, one or more birds are given a single oral dose (mg/kg-bwt) of the test chemical using doses that are expected to include the evolving working estimate of the LD_{50}. Birds are observed for 14 days, but selection of doses for subsequent stages is typically based on observed mortality and toxicity signs after three days. This interval may be reduced if mortality or signs of recovery occurs quickly, or the interval may be extended if delayed mortality is expected or observed.

7. The sequential tests (LD_{50}-slope or LD_{50}-only) can be initiated using information gained from a failed limit dose test (one or more mortalities) or from external information. For compounds of suspected high toxicity, testing may be initiated in Stage 1 where each of four birds is given a different dose, so that doses cover the best available estimate of the LD_{50} (*e.g.,* based on the rodent or other bird species' LD_{50}). Using the outcome of Stage 1, a working estimate of the LD_{50} is determined. The doses are determined for Stage 2 using Maximum Likelihood Estimation (MLE), and at each of ten doses, one bird is dosed. If there is a working estimate of the LD_{50} available from a failed limit dose test, the sequential test may start with Stage 2. The process for the LD_{50}-slope test continues to Stage 3 and possibly to Stage 4 (stopping rules are outlined and detailed in paragraphs 40 through 43).

8. Definitions used in this guideline are given in Annex 2.

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Figure 1: Limit dose test procedure; figure does not include control birds

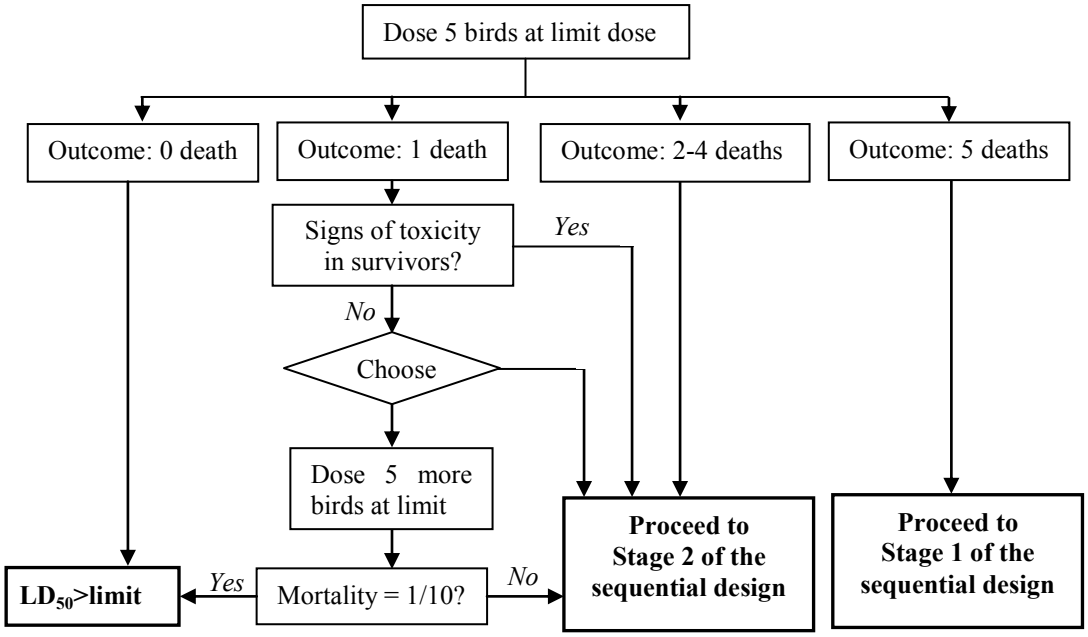
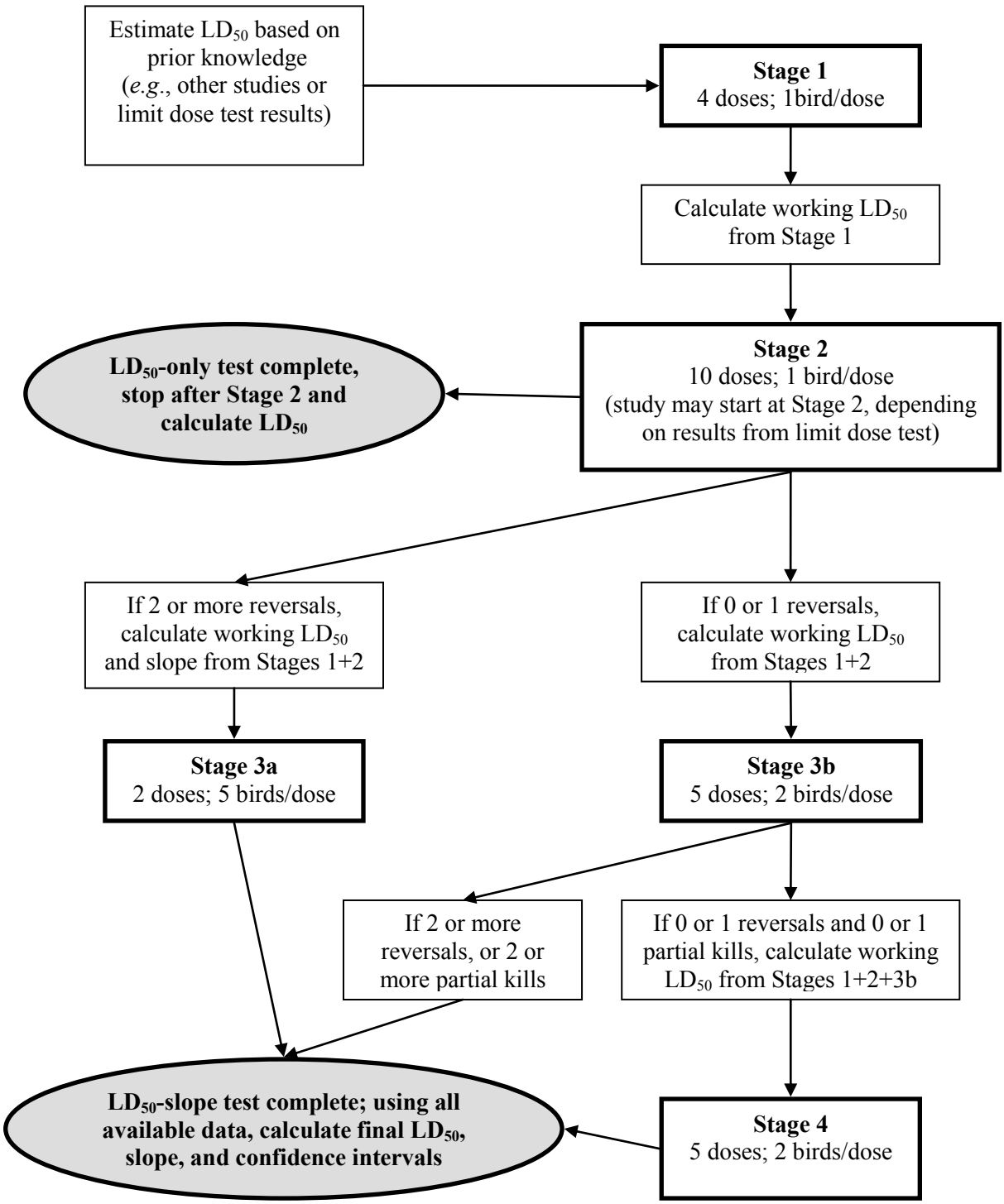


Figure 2: Sequential Design Procedure (LD_{50}-slope and LD_{50}-only tests); figure does not include control birds. Further details on stopping rules are provided in paragraphs 40 through 43.

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Estimate LD_{50} based on prior knowledge (*e.g.*, other studies or limit dose test results)
Stage 1
4 doses; 1bird/dose
Calculate working LD_{50} from Stage 1
Stage 2
10 doses; 1 bird/dose
(study may start at Stage 2, depending on results from limit dose test)
LD_{50}-only test complete, stop after Stage 2 and calculate LD_{50}
If 2 or more reversals, calculate working LD_{50} and slope from Stages 1+2
If 0 or 1 reversals, calculate working LD_{50} from Stages 1+2
Stage 3a
2 doses; 5 birds/dose
Stage 3b
5 doses; 2 birds/dose
If 2 or more reversals, or 2 or more partial kills
If 0 or 1 reversals and 0 or 1 partial kills, calculate working LD_{50} from Stages 1+2+3b
LD_{50}-slope test complete; using all available data, calculate final LD_{50}, slope, and confidence intervals
Stage 4
5 doses; 2 birds/dose

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PRINCIPLE OF THE TEST

9. The test is divided into a number of discrete stages. At each stage, birds are simultaneously given a dose (mg/kg-bwt) of the test chemical into the crop or proventriculus. Depending on the test stage, individual birds may receive different doses or more than one bird may receive the same dose (*i.e.* the dose is replicated within the stage). The recommended strategy for testing chemicals that are unlikely to present a significant hazard is to perform a test with multiple birds dosed at the limit dose. If toxicity is expected, the recommended strategy is to use a sequential design rather than the limit dose approach. Stages 1 and 2 require non-replicated doses, while Stages 3 and 4 require replicated doses. In Stage 1, the range of doses is based on the best available estimate of the LD_{50} (*e.g.*, the rodent LD_{50}). Doses for subsequent stages are determined based on the mortalities observed in all previous stages, so that the estimation of the LD_{50} and the slope of the dose-response curve are carried out simultaneously.

10. After dosing, the birds are observed for a 14-day period in order to measure mortality. It may be necessary to extend the observation period depending on evidence of delayed effects. The staged test design is easiest to apply to chemicals that produce death within a few days. In such cases it is not necessary to wait 14 days before starting the next stage, although observation of all birds should continue. Mortality observed after a shorter interval may be used to determine doses for the following stage (three days may often be used). Birds that are obviously in pain or show signs of distress should be euthanized immediately. The data collected in the first three days of a stage usually supply sufficient information to determine whether birds are likely to recover from effects encountered, or whether additional mortality will occur (2). Calculation of the working estimate on Day 3 of a test stage, allows the test and all dosing to be completed over a shorter time frame. If Day 3 information indicates that further mortality may occur in a test stage, the calculation of the working estimate of the LD_{50} value may be delayed until recovery of the remaining test birds is evident. In some cases it may be necessary to wait for up to 7 days before moving to the next stage. Final calculation is based on the accumulated 14-day outcomes from all stages.

11. Mortality is the primary endpoint in this study and background mortality is presumed to be negligible. Controls are required to monitor the health and husbandry of test birds to ensure that the ability of the study to provide reliable results is not compromised. Procedures and timelines for control birds are discussed in paragraphs 44-45. During the test, animals obviously in pain or showing signs of severe distress should be euthanized immediately. As far as possible the use of humane endpoints as described in OECD Guidance Document No. 19 on the use of humane endpoints will be taken into account when performing this test (10).

VALIDITY OF THE TEST

12. If there is any more than 10% mortality in the control group, the study is considered invalid. No additional control birds should be added during the course of the study, except as otherwise noted in paragraph 25 or 45.

DESCRIPTION OF THE TEST METHODS

Selection of birds

13. Captive bred species with low background mortality and a low propensity to regurgitate are preferred. Frequently used species which fit these requirements are the northern bobwhite quail, *Colinus virginianus* and Japanese quail, *Coturnix japonica* (*Galliform*). The use of wild-caught birds should be avoided if at all possible, owing to animal welfare considerations and disease control.

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14. If it is necessary to test additional species to develop a distribution of species sensitivity, the following species could be used: the mallard duck (*Anas platyrhynchos* (*Anseriform*)), feral pigeon (*Columba livia* (*Collumbiform*)), zebra finch (*Poephila guttata* (*Passeriform*)), and budgerigar (*Melopsittacus undulatus* (*Psittaciform*)) may be used. This list is not intended to limit the recommended species but rather to provide guidance on some relatively robust and laboratory-bred species from different Orders. The background mortality of the chosen species should be demonstrated to be ≤1% in the testing laboratory. The 1% mortality is the background mortality for bobwhite quail, which was the only species used in the TG 223 validation studies.

15. Birds should be in mature plumage but not in breeding condition. Wild phenotypes are preferred, where possible. Captive-bred birds should be from the same source and breeding population, and when possible, breeding history should demonstrate periodic out-breeding to maintain genetic heterogeneity. If wild phenotypes cannot be used, birds should be selected from a mixture of different phenotypes to limit inbred traits.

16. Birds should be allocated to treatment at random from reproductively quiescent mixed or single sex groups. The use of reproductively quiescent birds may help to minimize differences in susceptibility to acute oral administration of pesticides due to sex of the bird (3, 4). For some test chemicals or chemical classes, evidence of sensitivity due to sex of the birds may exist (5). The frequency of toxicity differences due to sex across all chemicals is uncertain. If sensitivity due to sex is suspected, testing should be performed to allow estimation of the LD_{50} for each sex.

Housing and test conditions

17. This guideline is designed to have single birds per concentration in the early stages of the study. In order to observe the effects and record if/when regurgitation takes place, it is highly recommended that the birds be housed individually at least for the first three days of the study. This makes it possible to observe birds individually and note down details of their behaviour (including regurgitation). For sociable species such as zebra finch it is essential and for other species it is highly recommended that birds be placed in cages next to each other so they have visual and auditive social interactions with each other. Since this study is designed to have birds housed individually, housing conditions should be within optimal limits for the test species and minimum recommended space per bird 3333 cm^2 for pigeon, 2000 cm^2 for mallard, 1000 cm^2 for quail, and 500 cm^2 for budgerigar and zebra finch. Larger cage sizes with more space per bird should be used when required or recommended by regiona lor individual country guidelines and/or regulations for animal welfare[2]. Cage floors should be constructed of mesh, large enough to allow faeces to fall through, but not restrictive to the bird's movements. Pigeons, zebra finches and budgerigars need perches as part of the housing conditions for a healthy environment. Further details on the care of bird species can be found in Felasa 2007 (11).

18. The test environment may be under controlled conditions or at ambient temperature and humidity. Temperatures within the range 15 – 27°C are suitable for quail and duck but should fluctuate as little as possible during the test stages. Ventilation should be sufficient to supply at least ten changes of air per hour. The photoperiod for quail and mallard should be eight hours light and 16 hours dark. For other species it may be necessary to increase the light phase to ten hours. Fresh food and water should be provided *ad libitum*. Commercial gamebird diets and vitamin supplements can be used, but they must be nutritionally appropriate for the species used. Medication should be avoided within 14 days prior to dosing, during dosing and during the observation period. Diets and water should be periodically analysed to check for impurities that may influence the birds' health.

[2] For example the Recommendation of the European Commission (2007/526/EC)

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Preparation of birds

19. Birds should be uniquely identified. Acclimatisation to test conditions and diet prior to dosing should be at least 14 days for cage-reared birds. Normally, wild-caught birds need longer acclimatisation periods. All birds must judged to be in healthy condition and should not be used if greater than 5% of cage-reared or greater than 10% of wild test birds die during the acclimatisation period. If wild birds do not acclimate they should be released close to their place of capture. The use of wild-caught birds should be avoided if at all possible, owing to animal welfare considerations, considerations of protection of wildlife and natural environment, and uncontrolled variables as age, genetic make-up, and health status. Capture, transportation and confinement are associated with extreme stress and may lead to higher mortality of the birds. Cage-reared birds should be approximately the same age.

Preparation and administration of doses

20. The test chemical should be administered in a capsule or dissolved or suspended in a suitable vehicle and then administered by gavage. If the test chemical is dissolved or suspended it is recommended that, whenever possible, the use of an aqueous solution or suspension be considered first, followed by consideration of a solution or emulsion in oil (*e.g.* corn oil), and then by possible dissolution in other vehicles. For vehicles other than water, the toxicity of the vehicle should be known, and it should not cause regurgitation. The dose for each bird is determined based on its body weight measured within 24 hours of dosing.

21. The test chemical is administered in a single dose by gavage. The dosing volume should be kept as low as possible and remain constant with respect to body weight and should not exceed 10 ml/kg body weight. Birds should be fasted for 12 – 15 hours overnight immediately prior to dosing. Fasting overnight prior to dosing for larger birds such as Northern bobwhite and Japanese quail is commended. Shorter fasting periods of two hours are suitable for birds of around 50 g or less. Fasting overnight might be deleterious to birds of small body mass (12).

22. Regurgitation compromises the evaluation of toxicity and must be recorded. The addition of a non-toxic coloured food dye that contrasts with the colour of faeces will allow regurgitation to be more easily recognised (*e.g.,* FD&C Blue#1, Formula 773389, CAS 57-55-6). Regurgitation is a feature of acute oral toxicity testing in birds and may be related to the dosing technique or characteristics of the test chemical. The frequency of regurgitation may be reduced by lowering the dose volume or by changing carriers. It is advised, especially when testing passerine species, to perform some range-finding work to evaluate the propensity of the species to regurgitate. The occurrence or frequency of regurgitation may be reduced by lowering the dose volume, using capsules or by changing carriers. If regurgitation remains a problem, it may be necessary to consider testing another species. If the only remaining option is to perform the study despite the propensity of the species to regurgitate the dose, it may not be possible to determine an accurate LD_{50} value. The only conclusion possible may be that the LD_{50} is above the lowest level at which no regurgitation or mortality occurred.

Observation of birds

23. Birds are observed continuously during the first two hours after dosing for regurgitation and for the onset of clinical signs, on at least three evenly spaced additional occasions during the day light period of exposure for clinical signs, and at least daily thereafter for a total of 14 days. However, the duration of the observation period should not be fixed rigidly. Observations are continued until the progression of clinical symptoms and mortality cease. If the observation period is extended for any stage, it is also extended for all subsequent stages.

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24. Observations made on each individual include regurgitation, signs of intoxication and remission, abnormal behaviour, body weight, mortality and time to death.

25. Observations of deaths that are clearly not treatment related (*e.g.,* physical injury) should be excluded from calculations. If the incidental death occurs in the control group, additional control birds may need to be added.

26. Birds should be weighed before dosing, and then at 3, 7 and 14 days after dosing (or later depending on the duration of the study) to determine weight change. Food consumption should be measured daily until day 3, then for the periods 3-7 and 7-14 days after dosing. At the end of the test, gross pathology should be undertaken on all birds from each treatment group, including controls and birds that did not die during the study, to help identify incidental mortalities and obvious symptoms of toxicity. If no mortalities or clinical signs of significance are recorded for any bird, no necropsy is necessary.

27. During the test, animals obviously in pain or showing signs of distress should be euthanized immediately (10).

PROCEDURE

Limit dose test

28. The limit dose test design consists of dosing five animals simultaneously at the limit dose in addition to sham-dosing the control birds. Control bird procedures and timelines are discussed in paragraphs 44-45. Birds are then observed for 14 days. Figure 1 describes the procedure to be followed according to the mortality observed. Euthanizing a bird to prevent suffering from toxic effects should be considered equivalent to observing a treatment related mortality.

29. If no mortality in the dosed birds occurs for 14 days after dosing, it can be concluded at the 95% confidence level, that the LD_{50} is above the limit dose. The test is completed. A failed limit dose test is one in which it cannot be concluded at the 95% confidence level that the LD_{50} is above the limit dose and one must move to either Stage 1 or Stage 2 of the sequential test.

30. If one treatment related death is observed, and no signs of toxicity are observed in other birds, then five more birds may be dosed at the limit, or at the discretion of the study director and sponsor, the test may proceed to Stage 2 of the sequential design. Additional dosing can begin before the 14-day observation period is complete. If the limit dose test is expanded, and there is only one death and no clinical signs of toxicity are observed in the total of ten birds, then it can be concluded that at the 95% confidence level the LD_{50} is above the limit dose and the toxicity study is completed.

31. If the observed treatment related mortality is one out of five birds and there are signs of toxicity in other birds or if there are two to four mortalities among five birds, or if there are two or more mortalities among ten birds, use the sequential design described below and in Figure 2, starting with Stage 2.

32. If mortality is complete (*i.e.*, all birds have died, including euthanized birds), use the sequential design shown in Figure 2 starting with Stage 1. Additional dosing can begin before the 14-day period of observation is complete.

33. To proceed from the limit dose test to Stage 2 of the sequential design, a working estimate of the LD_{50} is needed to determine doses for Stage 2. Using the working estimate of the LD_{50} from Table 1, where the limit dose was 2000 mg/kg-bwt, the low and high doses can be calculated as described in

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paragraph 40. A method for calculation of the working estimate of the LD_{50} following a failed limit dose test at any limit dose is described in Annex 4.

34. It should be noted that in some circumstances, the LD_{50} cannot be estimated without using doses above the limit dose. Because there are constraints on the use of very high doses of test chemical, it may not always be possible to estimate the LD_{50} for slightly toxic test chemicals.

Table 1: Working estimate of LD_{50} for use in Stage 2 of the sequential design derived from mortality in a limit dose test at 2000 mg/kg-bwt

Mortality (%)	10	20	30	40	50	60	70	80	90
Working Estimate of LD_{50}	3606	2944	2541	2244	2000	1782	1574	1358	1109

LD_{50}-only and LD_{50}-slope tests

Generalities

35. Details of dose determination for each stage of the sequential design are described below. A detailed flowchart of the sequential design for dosed birds is included in Figure 2. A sample timeline for a design with four stages, each with a 14-day observation period, is included in Figure 3. Procedures and timelines for control birds are discussed in paragraphs 44-45.

36. The initial estimate of the LD_{50} can be based on prior knowledge of the toxicity of the test chemical (*e.g.*, mammalian toxicity tests) or on other compounds in the same chemical class. For each stage of the study, *ldose*, *hdose*, and *step* must be recalculated in order to determine the dose sequence for that stage.

37. Maximum Likelihood Estimation (MLE) is a procedure which solves for the values of model parameters that give the greatest likelihood of seeing the data as they were observed. For this test guideline, the parameters determined are the slope and the intercept of the probit model, the values of which are used to calculate the LD_{50}. In order to use MLE it is necessary to have at least two partial kills or at least two reversals (see paragraph 38). If the conditions are met, use of the sequential design will allow the LD_{50} and slope to be estimated by the MLE from the responses in studies at Stages 1 and 2 (if at least two reversals) and at higher stages (if at least two partial kills or at least two reversals).

38. Determination of reversals and partial kills is essential to identifying the next stage of the test correctly. Reversals (*i.e.*, instances when percent mortality is lower at the next higher dose than percent mortality at the given dose) and partial kills (*i.e.,* multiple birds are given a single dose, and mortality is between 0 and 100% at that dose) are used as criteria for continuing to the next stage and stopping (completing) the test. It is recommended to count the number of partial kills first, then, if necessary, count the number of reversals. Examples of hypothetical test outcomes with partials kills and reversals identified are provided in Annex 3.

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Detailed description of the sequential design

For an overview of stopping rules, see paragraphs 7-8 and Figures 1 and 2. For description of the sequential design, see Annex 1. For definitions, see Annex 2.

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Stage 1 *– Four doses equally spaced on a log scale around the initial estimate of the LD_{50}*

39. The calculations for *ldose*, *hdose*, and *step* for Stage 1 are as follows:

(1) Calculate the low and high treatment doses using the following equations where the initial estimate of the LD_{50} is based on LD_{50}'s attained from rodent or other species toxicity tests or it is based on values from Table 1 for a failed limit dose test (Annex 4 contains estimates for LD_{50} when a limit dose other than 2000 mg/kg-bwt was used):

$ldose = dose1 = 0.1414 \times (initial\ \ estimate\ \ of\ \ LD50)$ and

$hdose = dose4 = 7.071 \times (initial\ \ estimate\ \ of\ \ LD50)$

(2) If *hdose* is greater than 3330 then set *hdose* = 3330 (may be set lower if limited by physical constraints) and recalculate the lowest dose as $ldose = hdose / 50$

(3) Calculate: $step = 50^{1/3}$ = 3.679

(4) Calculate the second and third doses:

$dose2 = ldose \times step$ and

$dose3 = dose2 \times step = ldose \times step \times step$

(5) Give one dose (*dose*1, *dose*2, *dose*3, *dose*4) to each of the four birds.

(6) Observe birds for a period (typically three days), and note whether each bird is dead or alive. Compute a "working estimate of the LD_{50}" which is defined here as the geometric mean of the doses that produce a transition from survival to death (see Table 2). This working estimate of the LD_{50} will be used to establish doses for Stage 2.

(7) Continue to observe the birds for eleven additional days (14 days in total).

(8) If Day 3 information indicates that further mortality may occur within this test stage, calculation of the working estimate of the LD_{50} may be delayed until recovery of the remaining test birds is evident. In some cases, it may be necessary to wait up to 14 days before moving to the next stage. If the observation period prior to calculating the working LD_{50} is extended for this stage, it is extended for all subsequent stages. If there is evidence of delayed effects after 14 days, the length of the stage may be further extended.

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Table 2: Calculation of the working LD_{50} computed from the four doses in Stage 1 [a, b]

*dose*1	*dose*2	*dose*3	*dose*4	**Working LD_{50} from Stage 1 data**
O	O	O	O	$(dose4 \times dose5)^{1/2}$
O	O	O	X	$(dose3 \times dose4)^{1/2}$
O	O	X	O	$(dose2 \times dose3 \times dose4 \times dose5)^{1/4} = (dose3 \times dose4)^{1/2}$
O	X	O	O	$(dose1 \times dose2 \times dose4 \times dose5)^{1/4} = dose3$
X	O	O	O	$(dose0 \times dose1 \times dose4 \times dose5)^{1/4} = (dose2 \times dose3)^{1/2}$
O	O	X	X	$(dose2 \times dose3)^{1/2}$
O	X	X	O	$(dose1 \times dose2 \times dose4 \times dose5)^{1/4} = dose3$
X	X	O	O	$(dose0 \times dose1 \times dose4 \times dose5)^{1/4} = (dose2 \times dose3)^{1/2}$
O	X	O	X	$(dose1 \times dose2 \times dose3 \times dose4)^{1/4} = (dose2 \times dose3)^{1/2}$
X	O	X	O	$(dose0 \times dose1 \times dose2 \times dose3 \times dose4 \times dose5)^{1/6} = (dose2 \times dose3)^{1/2}$
X	O	O	X	$(dose0 \times dose1 \times dose3 \times dose4)^{1/4} = dose2$
O	X	X	X	$(dose1 \times dose2)^{1/2}$
X	O	X	X	$(dose0 \times dose1 \times dose2 \times dose3)^{1/4} = (dose1 \times dose2)^{1/2}$
X	X	O	X	$(dose0 \times dose1 \times dose3 \times dose4)^{1/4} = dose2$
X	X	X	O	$(dose0 \times dose1 \times dose4 \times dose5)^{1/4} = (dose2 \times dose3)^{1/2}$
X	X	X	X	$(dose0 \times dose1)^{1/2}$

[a] Survival is represented by O and death by X.

[b] Even though only four doses (*dose*1 through *dose* 4) are used in the test, calculated values for *dose* 0 and *dose* 5 are used in the table. The values that should be used for these doses are one step up or down from the actual test doses. That is, *dose*0 = *dose*1 / *step* and *dose5* = *dose*4 x *step*. *Dose*0 must be added to the computation of the working LD_{50} when mortality occurs at the lowest test dose, and *dose*5 is added when there is survival at the highest test dose.

Stage 2 *–Ten doses equally spaced on a log scale around the working estimate of the LD_{50} obtained from Stage 1 or from a limit dose test with partial mortality*

40. The calculations of *ldose, hdose* and *step* for Stage 2 are as follows:

(1) Calculate the low and high treatment doses using the following equations where the working LD_{50} is based on Stage 1 results, based on values from Table 1 for a failed limit dose test (Annex 4

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contains estimates for LD_{50} when a limit dose other than 2000 mg/kg-bwt was used), or based on combined limit dose test and Stage 1 results:
$ldose = dose1 = 0.3425 \times working\ LD50$ and
$hdose = dose10 = 2.919 \times working\ LD50$

(2) If *hdose* is greater than 3330 then set *hdose* = 3330 (may be set lower if limited by physical constraints) and recalculate the lowest dose as $ldose = hdose / 8.5$

(3) Calculate: $step = (hdose / ldose)^{1/9}$

(4) Calculate the eight intermediate doses: $dose_i = ldose \times step^{(i-1)}$, for i = 2 to 9

(5) Give each of the ten birds one of the calculated doses.

(6) If an LD_{50}-only test is being conducted, observe the birds for a total of 14 days. Estimate the LD_{50} with the probit model using final data from Stage 1 and Stage 2, and limit dose test data (if available).

(7) If an LD_{50}-slope test is being run, observe the birds for a period (typically three days, longer if a longer period was used in an earlier stage, or longer if delayed mortality occurs), and note whether each bird is dead or alive. The number of reversals are counted (*i.e.,* instances when percent mortality at given dose is lower at the next higher dose than percent mortality at the given dose, see examples in Annex 3), and a probit model is fitted to the combined mortality data from the limit dose test (if available) and Stages 1 and 2 to obtain a working estimate of the LD_{50} and, if possible, the slope. If two or more reversals are observed, proceed to Stage 3a, otherwise proceed to Stage 3b.

(8) Continue to observe the birds for an additional eleven days (14 days in total).

(9) If Day 3 information indicates that further mortality may occur within this test stage, calculation of the working estimate of the LD_{50} may be delayed until recovery of the remaining test birds is evident. In some cases, it may be necessary to wait up to 14 days before moving to the next stage. If the observation period prior to calculating the working LD_{50} is extended for this stage, it is extended for all subsequent stages. If there is evidence of delayed effects after 14 days, the length of the stage may be further extended.

Stage 3a *– (two or more reversals when limit dose test, Stage 1 and Stage 2 data are combined) Two doses equally spaced on a log scale around the working estimate of the LD_{50} obtained from limit dose test (if available) and Stages 1 and 2*

41. The calculations of *ldose, hdose* and *step* for Stage 3a are as follows:

(1) If the estimate of the working slope above is greater than fifteen or less than one, then set the working slope to fifteen or one respectively and recalculate *ldose* and *hdose*. If an estimate of the working slope was not obtained, set the working slope to be 5 for calculation of *ldose* and *hdose*.

(2) Calculate the low and high treatment doses using the following equations where the working LD_{50} and working slope are based on all available data to this point (limit dose test, Stage 1, and/or Stage 2):
$ldose = 10^{(-1.036/\ workingslope)} \times working\ LD50$ and
$hdose = 10^{(1.036/\ workingslope)} \times working\ LD50$

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(3) If *hdose* is greater than 3330, then set *hdose* = 3330 (may be less if limited by physical constraints) and recalculate the lowest dose as $ldose = hdose / 10^{(2.072/\,slope)}$

(4) Give *ldose* to five birds and give *hdose* to five birds, for a total of 10 birds dosed.

(5) Observe the birds for 14 days and note whether each bird is dead or alive. Fit a probit model to the combined mortality data from the limit dose test (if available) and Stages 1, 2 and 3a, in order to obtain final estimates of both the LD_{50} and the slope. The test is completed.

(6) If the probit model cannot be fitted, report this and estimate the LD_{50} as the geometric mean of the doses on both sides of the region where 50% mortality occurs. Alternatively, other interpolation and moving average methods may be used to estimate the LD_{50}.

Stage 3b *– (less than 2 reversals when limit dose test, Stage 1 and Stage 2 data are combined) Five doses equally spaced on a log scale around the working estimate of the LD_{50} obtained from limit dose test (if available) and Stages 1 and 2*

42. The calculations of *ldose, hdose* and *step* for Stage 3b are as follows:

(1) Calculate the low and high treatment doses using the following equations where the working LD_{50} is based on all available data to this point (limit dose test, Stage 1, and/or Stage 2):
$ldose = dose1 = 0.6205 \times working\,LD50$ and
$hdose = dose5 = 1.6113 \times working\,LD50$

(2) If hdose is greater than 3330 then set hdose = 3330 (may be less if limited by physical constraints) and recalculate the lowest dose as $ldose = hdose / 2.6$

(3) Calculate: $step = (hdose / ldose)^{1/4}$

(4) Calculate the three intermediate doses: $dosei = ldose \times step^{(i-1)}$, for i = 2 to 4

(5) Give each of the five doses to two birds, for a total of 10 birds dosed.

(6) Observe the birds for a period (typically three days, longer if a longer period was used in an earlier stage, or longer if delayed mortality occurs) and note whether each bird is dead or alive. Fit a probit model to the combined mortality data from the limit dose test (if available) and Stages 1, 2 and 3b in order to obtain a working estimate of the LD_{50} and slope.

(7) If at least two reversals or at least two partial kills are observed and a working estimate of the slope has been obtained then Stage 4 is not needed. Continue observing the birds until the end of the 14th day and fit a probit model to the combined data from the limit dose test (if available) and Stages 1, 2 and 3b, in order to obtain final estimates for both LD_{50} and slope. The test is completed.

(8) If conditions in step (7) above are not satisfied proceed to Stage 4

(9) Continue to observe the birds for an additional eleven days (14 days in total).

(10) If Day 3 information indicates that further mortality may occur within this test stage, calculation of the working estimate of the LD_{50} may be delayed until recovery of the remaining test birds is evident. In some cases, it may be necessary to wait up to 14 days before moving to the next stage. If the observation period prior to calculating the working LD_{50} is extended for this stage, it is extended for all subsequent stages. If there is evidence of delayed effects after 14 days, the length of the stage may be further extended.

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Stage 4 *– Five doses equally spaced on a log scale around the working estimate of the LD_{50} obtained from limit dose test (if available) and Stages 1, 2 and 3b*

43. The calculations of *ldose, hdose* and *step* for Stage 4 are as follows:

(1) Calculate the low and high treatment doses using the following equations where the working LD_{50} is based on all available data to this point (limit dose test, Stage 1, Stage 2, and/or Stage3b):
$ldose = dose1 = 0.6205 \times working\ LD50$ and
$hdose = dose5 = 1.6113 \times working\ LD50$

(2) If hdose is greater than 3330 then set hdose = 3330 (may be less if limited by physical constraints) and recalculate the lowest dose as $ldose = hdose / 2.6$

(3) Calculate: $step = (hdose / ldose)^{1/4}$

(4) Calculate the three intermediate doses: $dosei = ldose \times step^{(i-1)}$, for i = 2 to 4

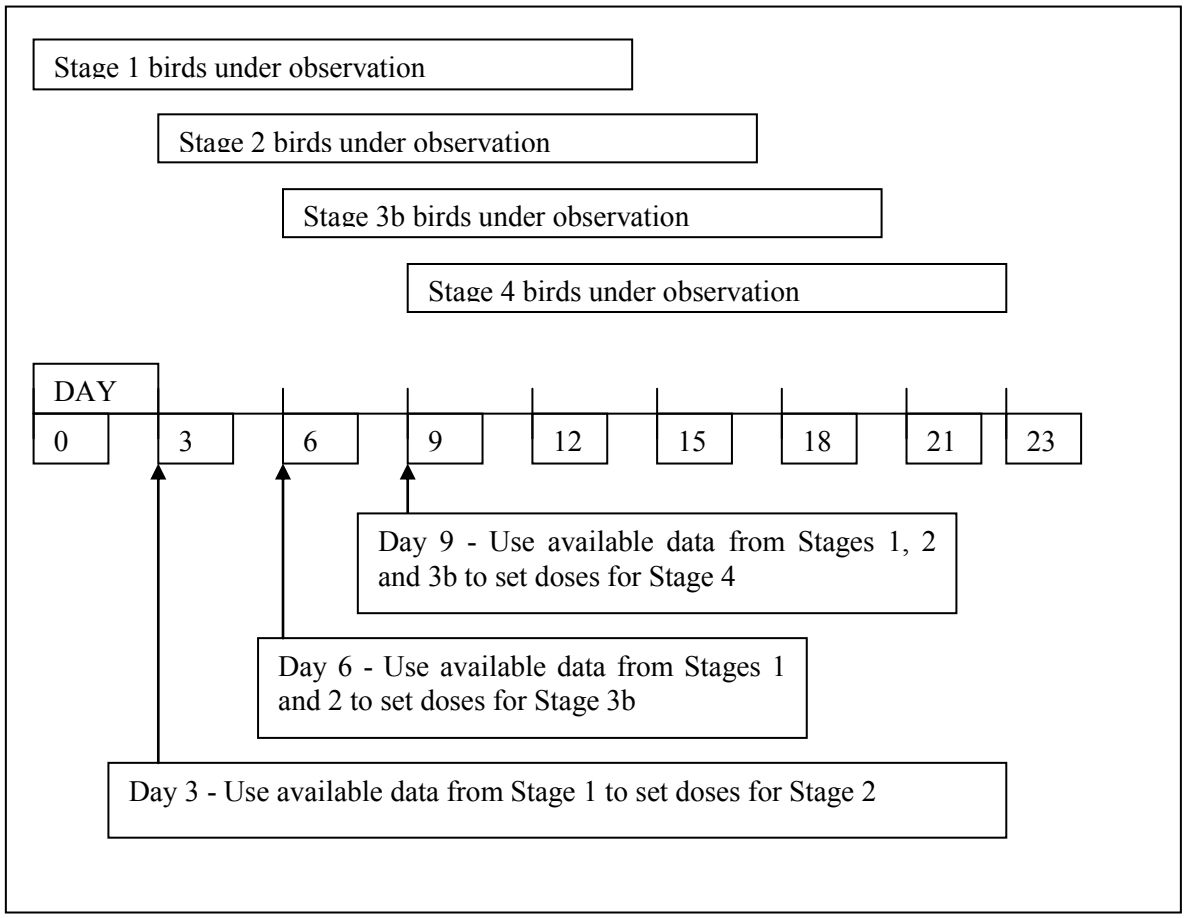
(5) Give each of the five doses to two birds, for a total of 10 birds dosed.

(6) Observe the birds for 14 days and note whether each bird is dead or alive. Fit a probit model to the combined data from the limit dose test (if available) and Stages 1, 2, 3b and 4 in order to obtain final estimates of both the LD_{50} and the slope. The test is completed.

(7) If at the end of Stage 4 no maximum likelihood estimate can be obtained, report this and estimate the LD_{50} as the geometric mean of the doses on both sides of the region where 50% mortality occurs. Alternatively, other interpolation and moving average methods may be used to estimate the LD_{50}.

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Figure 3: Sample timeline for sequential design with four stages (Stages 1, 2, 3b, and 4) with 14 observation days for each stage; figure does not include control birds.



Control birds

44. Five untreated control birds from the same hatch will be included in the test. Control birds will be sham-dosed with the same carrier (or capsule) used with the test chemical, and will be maintained under the same conditions as treated birds. Control birds will be weighed prior to dosing and on days 3, 7, and 14. Sham dosing will be performed on the same day as the first dosing with test chemical (either with the limit dose test if it is performed, and/or with the opening stage (Stage 1 or Stage 2) of the sequential test).

45. There may be circumstances that arise due to the conduct of the test in which an additional five control birds are required:

- if, upon the start of a new stage, birds from a hatch different from the one used to initiate the test are used, control birds from the second hatch must be included on the day that these birds are started on test;
- if there is a difference in dosing volumes that the birds receive across stages, birds must be sham-dosed concurrent with the stage of birds receiving the different dosing volume. This circumstance may occur when it is necessary to increase the highest dose tested and a larger volume is necessary to achieve an appropriate solution/suspension;

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- if an LD_{50}-slope test is being conducted and the time between stage starts is lengthy or changes appreciably (*e.g.*, from three days to 10 days), birds should be sham-dosed and started on test in the middle of the sequential study (*e.g.*, at the start of Stage 2 or Stage 3).

DATA AND REPORTING

Data

46. Individual bird data are reported and summarised in tabular form, showing the dose, number of birds tested, signs of toxicity, death and numbers euthanised for humane reasons, time of death of individual birds, a record of the time to onset and cessation of clinical signs, bodyweights (just prior to dosing and at days 3, 7, 14, and at test termination if longer than 14 days because the observation period was extended due to delayed mortality, food consumption, and gross pathological findings.

Calculation of the LD_{50}, slope, and confidence intervals

47. The calculation methods for estimating the LD_{50} (median lethal dose) are the same for sequential tests as for other types of dose response tests. Certain features of this sequential design are based on the assumption that the underlying form of the dose response curve approximates the probit model, so the use of a probit regression model (with the logarithm of dose as the independent variable) to estimate the LD_{50} is appropriate. For the probit model, the MLE of the Log (LD_{50}) is $-a/b$, where a is the intercept and b is the slope. When the mortality data from all stages of a study are pooled for analysis, other models may also be appropriate if the probit model fails to adequately fit the data.

48. Standard maximum likelihood methods used to fit models to the data can be used only if there are two doses that display partial mortality or there are two reversals in trend somewhere in the observed responses. These conditions are built into the sequential design and only on rare occasions will they not be met. If no maximum likelihood estimate can be obtained, report this and estimate the LD_{50} as the geometric mean of the doses on both sides of the region where 50% mortality occurs. Alternatively, other interpolation and moving average methods may be used to estimate the LD_{50}.

49. Confidence intervals for the LD_{50} can be obtained using Fieller's theorem (6), likelihood ratio methods (7) (8), or by binomial methods (9). Many standard statistical packages have built in routines to estimate the LD_{50} and its confidence interval (*e.g.,* SAS®, LogXact®,).

50. It should be recognized that it is often possible to calculate the slope and confidence intervals for the LD_{50} from the results of the LD_{50}-only test. However, due to the small number of birds and lack of replication at any given dose, these estimates may be unreliable. Thus, the slope and confidence intervals for the LD_{50} should be reported only for the LD_{50}-slope test. Some measure of goodness of fit of the regression model (*e.g.,* Pearson goodness of fit, likelihood ratio Chi-square) should be reported for each dose-response test. Reporting of a measure of goodness of fit is optional for the LD_{50}-only test.

51. A Microsoft® Excel workbook called SEquential DEsign Calculator (SEDEC) is available on the OECD website to aid in dose selection and analysis of studies performed in accordance with OECD TG 223. SEDEC requires Microsoft® Excel for Windows 2000 or later versions to be installed on the user's computer.

Report

52. The report should contain the following minimum information to confirm compliance with the Guideline and test results:

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test chemical

- source, lot number, limit date for use, if available
- stability of the test chemical, if known.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;

- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

test method and system

- test type
- test species, source, strain, age, weight, health
- description of test method

conduct of test

- test groups and design (number of treatments and replicates, individual or group caging)
- acclimation and assignment procedures (duration, randomisation)
- dose method (gavage/capsule, carrier/solvent, volume/bird as % body weight)
  housing conditions (type, size, pen materials, floor covering, temperature, humidity, photoperiod, light intensity)
- food and water (availability, identification, source, composition, calorific value, results of contaminant analysis)
- frequency, duration and method of observations (health/mortality, body weight, food consumption)
- description of statistical methods

results of test

- mortality (time to death, clinical symptoms, calculation of LD_{50}, slope and confidence limits, if appropriate)
- onset and cessation of clinical signs (in minutes)
- gross pathological examination
- individual bodyweight data
- food consumption data

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ANNEX 1

GENERAL DESCRIPTION, RATIONALE AND STATISTICAL BASIS OF THE SEQUENTIAL DESIGN

Statistical Background

1. The philosophy underlying tolerance distributions is that an individual bird will die if it receives a dose above a certain value but will survive if the dose is equal to or less than this value. The specific value is called a tolerance and is assumed to be fixed for an individual bird, but to vary among birds. Thus if we have a population of birds we can speak of a distribution of tolerances, or a tolerance distribution.

2. In order to estimate the tolerance distribution from a sample of birds we fit a statistical model. If we assume that the tolerances follow a normal distribution we fit a probit model that takes the form:

$$\text{Probit}(p) = \alpha + \beta * \log(d)$$

Where: 'p' is the probability that the tolerance of an individual bird is less than dose d – *i.e.*, the probability that a bird receiving dose d will survive.

α and β are parameters representing the intercept and slope of a straight line relationship between probit (p) and log(d).

3. The probit model is fitted to test data in order to obtain estimates of the parameters α and β which we call a and b respectively. The estimate of the mean of the tolerance distribution of the population of birds (called the LD_{50}) can then be determined from the equation:

$$\text{Estimate } [\log(LD_{50})] = -a/b$$

and the variance of the tolerance distribution can be estimated by:

$$\text{Variance} = 1/b$$

4. Once the tolerance distribution has been estimated (i.e. once estimates of α and β have been obtained) percentiles of the distribution can be computed (dose levels corresponding to specific values of p).

5. Methods for estimating LD_{50}, slope and confidence intervals for a variety of different experimental outcomes are recommended in (2). The slope is estimated if there are at least two partial kills and/or reversals.

6. This design consists of several stages, in each of which a number of birds is dosed simultaneously and observed for a period of time. The advantage of such a design is that information

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gathered in earlier stages, notably the working estimates of LD_{50} and slope of the dose response curve, is used to help design the next stage, in particular placing of doses. The generic design is flexible with respect to the number of stages, number of doses per stage, and number of birds per dose. The specific design described here has been established by means of computer simulations and consists of four birds in the first and ten birds in later stages, while the number of doses and the number of birds per dose vary between stages.

7. At the start of the test it is assumed that an initial estimate of the LD_{50} is available. In practice this estimate may be obtained using knowledge of the chemistry and toxicity of similar active ingredients or from the results of tests on other bird species or on mammals. Alternatively, the initial estimate can be derived from a limit dose test. The first three stages of the sequential design differ considerably from each other. The first is a 'ranging' stage designed to confirm and improve an initial guess at the LD_{50}. The second is designed to more accurately locate the LD_{50}. The third stage and any subsequent stages are intended to provide estimates of the confidence interval and slope of the dose response curve or to further reduce uncertainty in the estimate of the LD_{50}.

8. Because of the way that the dosing of stages is staggered, working estimates of the LD_{50}, and slope are recalculated repeatedly. Each time the recalculations are performed, the number of groups with partial mortality or the number of reversals in mortality may change. Working estimates may be obtained 3, 6 and 9 days after the start of the test, but in each stage observations are continued until day 14. At the conclusion of the study, final estimates of LD_{50} and slope (and confidence intervals, if appropriate) are determined from the combined set (*i.e.*, from all stages) of 14-day assessments.

9. In Stage 1, four doses are equally spaced on a log scale around the initial estimate of the LD_{50}. In computer simulations it was found that four doses and a very large ratio of high to low dose gave an adequate working estimate of the LD_{50} and preserved birds for later stages where they were more useful in estimating the slope. A working estimate of the LD_{50}, computed as the geometric mean of the transition doses, has several advantages. First, it can provide a reasonable estimate given very little information, and second, it can be adapted to provide a working estimate of the LD_{50} even when there is mortality at the lowest dose or survival at the highest dose.

10. The purpose of Stage 2 is to refine the estimate of the LD_{50} and to obtain an initial estimate of the slope. To permit this stage to give a final LD_{50} estimate for the LD_{50}-only test and a working estimate for the LD_{50}-slope test, the extreme doses are placed at those points corresponding to one and 99 percent kill when a slope of five is assumed for a normal tolerance distribution with a mean equal to the LD_{50} and a standard deviation equal to 1/slope. As justification for using a value of five for slope, a review of the Environmental Protection Agency ECOTOX database [http://www.ipmcenters.org/Ecotox/index.cfm] suggests that, for the historical record of pesticide tests submitted to the Agency, the modal slope for avian acute tests is five.

11. The purpose of Stages 3a and 3b is to refine and improve the working estimates of LD_{50} and slope obtained from Stage 2 so that it may be possible to obtain confidence intervals. Stage 3a is the preferred design where there are >1 reversals indicating that we have good information on the LD_{50} and slope. Stage 3b is the preferred design where there are <2 reversals indicating that information on the LD_{50} and slope is poor.

12. In Stage 3a, limits are placed on the slope estimates because maximum likelihood estimates using small numbers of observations may give extreme (probably inaccurate) slope estimates. Half of the ten birds are given a lower dose corresponding to 15% kill and half are given the upper dose corresponding to 85% kill. The rationale for placing doses at the 15^{th} and 85^{th} percentiles follows from a statistical concept

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called D-optimality (8). This form of optimality gives the best simultaneous estimates of LD_{50} and slope (in the sense that the widths of confidence intervals for LD_{50} and slope are simultaneously minimised).

13. In Stage 3b the slope is assumed to be five, and the extreme doses are placed at responses corresponding to 15% and 85% kill. In addition to the fitting of a probit model the number of reversals and number of partial kills are counted to permit a decision regarding the stability of the LD_{50} estimate obtained at this stage. If the number of reversals is two or more and/or the number of partial kills is two or more then the study can be stopped.

14. Stage 4 is similar to Stage 3b and is carried out to permit slope estimation when the Stage 3b estimate is unstable or confidence intervals are very wide. No testing continues after Stage 4.

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ANNEX 2

DEFINITIONS

Acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a test chemical, or multiple doses given within 24 hours.

Dose is the amount of test chemical administered. Dose is expressed as weight (g, mg) or as weight of test chemical per unit weight of test animal (*e.g.* mg/kg-bwt, where bwt is 'body weight' of the test animal).

LD_{50} (median lethal oral dose), is a statistically derived single dose of a test chemical that can be expected to cause death in 50 per cent of animals when administered by the oral route. The LD_{50} value is expressed in terms of weight of test chemical per unit weight of test animal (mg/kg-bwt).

Limit dose refers to a dose at an upper limitation on testing.

Probit is an abbreviation for the term "probability integral transformation" and a probit dose-response model permits a standard normal distribution of expected responses (*i.e.*, one centred to its mean and scaled to its standard deviation) to doses (typically in a logarithmic scale) to be analyzed as if it were a straight line with slope the reciprocal of the standard deviation. A standard normal lethality distribution is symmetric; hence, its mean is also its true LD_{50} or median response.

Slope (of the dose-response curve) is a value related to the angle at which the dose response curve rises from the dose axis. In the case of probit analysis, when responses are analyzed on a probit scale against dose on a log scale this curve will be a straight line and the slope is the reciprocal of the standard deviation of the underlying test subject tolerances, which are assumed to be normally distributed.

Stage (in a sequential design) refers to a period during an experiment in which a number of birds are dosed simultaneously, and observed for a period of time. Provisional estimates of LD_{50} and slope from the previous stage may influence the design of the current stage. Likewise the data resulting from the current stage may influence the design of succeeding stages.

ldose is the lowest dose used during a particular stage (mg/kg-bwt).

hdose is the highest dose used during a particular stage (mg/kg-bwt).

step is the multiplication factor used in calculating individual doses.

Initial estimate of the LD_{50} is an estimate made prior to the start of the study and around which the four doses in Stage 1 are arranged.

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Working estimate of LD_{50} and slope are provisional estimates made before the end of a stage and which are used to determine doses in following stages. Typically working estimates are based on observations from all stages that have been started and made three days after the start of the current stage.

Final estimates of LD_{50} and slope are made after the study has been stopped. They are based on observations from each completed stage, normally 14 days or longer.

Partial kills can occur when the same dosage is given to multiple birds. A partial kill occurs when some birds die and some birds survive at a given dose. Partial kills are sometimes called partial responses.

Reversals are analogous to partial kills for non-replicated single birds receiving a dose and occur in consecutive pairs of birds when lower doses result in a kill while higher doses result in survival. This is likely to occur in proximity to the LD_{50}. So for example, if zero represents survival and one represents kill and responses are listed in order of increasing dose, then sequences such as 0001011111 and 000110111 both exhibit a single reversal, and 0001010111 and 00011010011 both exhibit two reversals. Further examples are provided in Annex 3.

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ANNEX 3

DETERMINATION OF THE NUMBER OF PARTIAL KILLS AND REVERSALS

Reversals (*i.e.*, instances when percent mortality at given dose is higher than percent mortality at the next higher dose) and partial kills (*i.e.,* multiple birds are given a single dose, and mortality is between 0 and 100% at that dose) are used as criteria for continuing to the next stage and stopping (completing) the test. It is recommended to count the number of partial kills first, then, if necessary, count the number of reversals. A reversal following a partial kill is counted as both a reversal and a partial kill.

Example of hypothetical test outcomes with partials and reversals identified are provided below. In these examples, doses increase from left to right, partials are noted by an asterisk (*), and reversals are noted in bold with the cells shaded.

Example 1. Stage 2 ($n_{birds} = n_{stage1} + n_{stage2} = 4 + 10$, number of unique doses = 14)
0 partials, 2 reversals, go to Stage 3a.

Number of mortalities	0	0	0	0	0	1	**0**	1	1	**0**	1	1	1	1
Number of tested individuals	1	1	1	1	1	1	**1**	1	1	**1**	1	1	1	1
Percent (%) mortality	0	0	0	0	0	100	**0**	100	100	**0**	100	100	100	100

Example 2. Stage 2 ($n_{birds} = n_{stage1} + n_{stage2} = 4 + 10$, number of unique doses = 12, the lowest and highest doses for Stage 1 and 2 were the same, the minimum and maximum limits (66.6 and 3330 mg/kg-bwt))
0 partials, 1 reversal, go to Stage 3b.

Number of mortalities	0	0	0	0	0	1	**0**	1	1	1	1	2
Number of tested individuals	2	1	1	1	1	1	**1**	1	1	1	1	2
Percent (%) mortality	0	0	0	0	0	100	**0**	100	100	100	100	100

Example 3. Stages 1, 2, and 3b ($n_{birds} = n_{stage1} + n_{stage2} + n_{stage3b} = 4 + 10 + 10$, number of unique doses = 19)
2 partials, 3 reversals, test completed

Number of mortalities	0	0	0	1	**0**	0	0	1	1	**0**	2	1	1	2	**0**	1	1	1	1
Number of tested individuals	1	1	1	2	**1**	2	1	2	1	**1**	2	1	1	2	**1**	1	1	1	1
Percent (%) mortality	0	0	0	50*	**0**	0	0	50*	100	**0**	100	100	100	100	**0**	100	100	100	100

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Example 4. Stages 1, 2, and 3b ($n_{birds} = n_{stage1} + n_{stage2} + n_{stage3b} = 4 + 10 + 10$, number of unique doses = 19)
1 partial, 2 reversals, test completed

Number of mortalities	0	0	0	0	0	0	0	1	1	**0**	2	1	1	2	**0**	1	1	1	1
Number of tested individuals	1	1	1	2	1	2	1	2	1	**1**	2	1	1	2	**1**	1	1	1	1
Percent (%) mortality	0	0	0	0	0	0	0	50*	100	**0**	100	100	100	100	**0**	100	100	100	100

Example 5. Stages 1, 2, and 3b ($n_{birds} = n_{stage1} + n_{stage2} + n_{stage3b} = 4 + 10 + 10$, number of unique doses = 19)
1 partial, 1 reversal, go to Stage 4

Number of mortalities	0	0	0	0	0	1	1	2	1	1	2	1	1	2	**0**	1	1	1	1
Number of tested individuals	1	1	1	2	1	2	1	2	1	1	2	1	1	2	**1**	1	1	1	1
Percent (%) mortality	0	0	0	0	0	50*	100	100	100	100	100	100	100	100	**0**	100	100	100	100

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ANNEX 4

CALCULATION OF A WORKING LD_{50} WHEN MORTALITY OCCURS IN A LIMIT DOSE TEST

Table 1 of the guideline provides working estimates of the LD_{50} that can be used to calculate doses for Stage 2 of the sequential test if a limit dose test has been performed and partial mortality has been observed among the test birds. However, this table is useful only if the limit dose was 2000 mg/kg-bwt. If an alternative limit dose has been used, a working estimate of the LD_{50} can be determined as:

$$LD_{50} = 10^{\left(\frac{probit_p - 5}{5} + \log_{10}(limit\ dose)\right)}$$

where p is the observed proportion of dead, and $probit_p$ is one of the values from the Table 3. For this calculation, the slope is assumed to be 5.

Table 3: Probit values for different proportions dead

Mortality (proportion)	1/10	2/10 or 1/5	3/10	4/10 or 2/5	5/10	6/10 or 3/5	7/10	8/10 or 4/5	9/10
Probit	3.72	4.16	4.48	4.75	5.00	5.25	5.52	5.84	6.28

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