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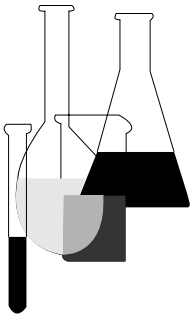
Health Effects Test Guidelines OPPTS 870.1100 Acute Oral Toxicity



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512–0132. This guideline is also available electronically in PDF (portable document format) from EPA's Internet Web site at http://www.epa.gov/opptsfrs/home/guidelin.htm. Also, the Agency has developed, and strongly recommends users to solely use, the software program for performing the Up-and-Down Procedure and calculating the LD50 and confidence interval. The software program (AOT425StatPgm) is available on EPA's Internet Web site at http://www.epa.gov/oppfead1/harmonized.

OPPTS 870.1100 Acute oral toxicity.

(a) **Scope—Applicability**. This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticida Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background**. The source material for this revised harmonized test guideline is OPPTS 870.1100 Acute Oral Toxicity, dated August 1998 and OECD test Guideline 425 Acute Oral Toxicity–Up-and-Down Procedure.

(b) **Purpose**. In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. It provides information on health and environmental hazards likely to arise from short-term exposure by the oral route. Data from an acute study may serve as a basis for classification and labeling. It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance. An evaluation of acute toxicity data should include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

(c) **Definitions**. The definitions in Section 3 of the Toxic Substances Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this test guideline.

Acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours.

Confidence interval (CI) is an interval estimate, a range of values, intended to include the true LD_{50} with a specified degree of confidence.

Delayed death means that an animal does not die or appear moribund within 48 hours, but dies later during the 14-day observation period.

Dose is the amount of test substance administered. Dose is expressed as weight (g, mg (grams, milligrams)) or as weight of test substance per unit weight of test animal (e.g., mg/kg (milligrams/kilograms)).

Dose progression factor, sometimes termed a dose spacing factor, refers to the multiple by which a dose is increased (i.e., the dose progression) when an animal survives or the divisor by which it is decreased when an animal dies. The dose progression factor is recommended to be the antilog of 1/(the estimated slope of the dose-response curve). The default

dose progression factor is recommended to be 3.2 = antilog 0.5 = antilog (1/2).

LD_{50} (median lethal dose), oral, is a statistically derived single dose of a substance 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 substance per unit weight of test animal (mg/kg).

Limit dose refers to a dose at an upper limitation on testing (2000–5000 mg/kg).

Moribund status of an animal refers to being in a state of dying or inability to survive, even if treated.

Nominal sample size refers to the total number of tested animals, reduced by one less than the number of like responses at the beginning of the series, or by the number of tested animals up to but not including the pair that creates the first reversal. For example, for a series where X and O indicate opposite animal outcomes (for instance, X could be dies within 48 hours and O survives) in a pattern as follows: OOOXXOXO, we have the total number of tested animals (or sample size in the conventional sense) as 8 and the nominal sample size as 6. This particular example shows 4 animals following a reversal. It is important to note whether a count in a particular part of the guideline refers to the nominal sample size or to the total number tested. For example, the maximum actual number tested is 15. When testing is stopped based on that basis, the nominal sample size will be less than or equal to 15. Members of the nominal sample start with the (r-1)st animal (the animal before the second in the reversal pair) (see reversal below).

Probit is an abbreviation for the term ‘‘*prob*ability *i*ntegral *t*ransformation’’ and a probit dose-response model permits a standard normal distribution of expected responses (i.e., one centered to its mean and scaled to its standard deviation, *sigma*) to doses (typically in a logarithmic scale) to be analyzed as if it were a straight line with slope the reciprocal of *sigma*. A standard normal lethality distribution is symmetric; hence, its mean is also its true LD_{50} or median response.

Reversal is a situation where nonresponse is observed at some dose, and a response is observed at the next dose tested, or vice versa (i.e., response followed by nonresponse). Thus, a reversal is created by a pair of responses. The first such pair occurs at animals numbered r-1 and r.

Sigma is the standard deviation of a log normal curve describing the range of tolerances of test subjects to the chemical (where a subject is expected capable of responding if the chemical dose exceeds the subject’s tolerance). The estimated *sigma* provides an estimate of the variation

among test animals in response to a full range of doses. See slope and probit.

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 *sigma*, the standard deviation of the underlying test subject tolerances, which are assumed to be normally distributed. See probit and *sigma*.

Stopping rule is used in this guideline synonymously with (1) a specific stopping criterion and (2) the collection of all criteria determining when a testing sequence terminates. In particular, for the main test, stopping rule is used in paragraph (e)(2)(ii) of this guideline as a shorthand for the criterion that relies on comparison of ratios to a critical value.

(d) **Approaches to the determination of acute toxicity**. EPA recommends the Up-and-Down Procedure (UDP) as detailed in this guideline and adopted by the Organization for Economic Cooperation and Development (OECD) as test Guideline 425 (see paragraph (n)(1) of this guideline), to assess acute oral toxicity. This method provides a point estimate of lethality and confidence interval around the LD50. Acute oral toxicity testing may also be performed using the Fixed Dose Method of OECD Guideline 420 (see paragraph (n)(2) of this guideline) or the Acute Toxic Class Method of OECD Guideline 423 (see paragraph (n)(3) of this guideline). These methods assess lethality within a dose range.

(e) **Introduction to the UDP**—(1) **Background**. (i) The concept of the up-and-down testing approach was first described by Dixon and Mood (see paragraphs (n)(4) through (n)(7) of this guideline). In 1985, Bruce proposed to use an UDP for the determination of acute toxicity of chemicals (see paragraph (n)(8) of this guideline). There exist several variations of the up-and-down experimental design for estimating an LD_{50}. This guideline is derived from the UDP of Bruce as adopted by the American Society for Testing and Materials (ASTM) in 1987 (see paragraph (n)(9) of this guideline) and revised in 1990. A study comparing the results obtained with the UDP, the conventional LD_{50} test and the Fixed Dose Procedure (FDP, OECD Guideline 420) was published in 1995 (see paragraph (n)(10) of this guideline).

(ii) The UDP described in this guideline is of value in minimizing the number of animals required to estimate the acute oral toxicity of a chemical. In addition to the estimation of LD_{50} and CI, the test procedure allows the observation of signs of toxicity. The UDP does not provide information about the slope of the dose-response curve.

(iii) The guideline significantly reduces the number of animals used in comparison to the traditional LD_{50} test, which often required at least 30 animals in a test: (A) The stopping rule limits the number of animals

in a test; (B) sequential dosing introduces further efficiencies in animal use; (C) initial dosing is now set to be below the LD_{50} increasing the percentage of animals in which dosing levels will be sublethal and thereby providing some reduction in pain and distress; and (D) the use of a single sex (usually females) reduces the number of animals needed and minimizes the variability in the test population. In addition, the OECD Guidance Document on Humane Endpoints (see paragraph (n)(11) of this guideline) should be followed in order to reduce the overall suffering of test animals used in this type of toxicity test.

(2) **Initial considerations**—(i) **Choice of starting dose and dose progression factor**. All available information on the test substance should be considered by the testing laboratory prior to conducting the study in order to determine if a preliminary estimate of the LD_{50} and the slope of the dose-response curve can be made. Because the method has a bias toward the starting dose, it is essential that initial dosing occur below the LD_{50}. In addition, the UDP performs best when the spacing between doses or dose progression factor is based on an accurate estimate of the slope of the dose-response curve. (See paragraphs (i)(3)(ii) and (m)(1) of this guideline for discussion of dose sequences and starting values.) Initial information may include the identity and chemical structure of the substance; its physical chemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the substance or mixtures; toxicological data on structurally related substances or similar mixtures; and the anticipated use(s) of the substance. For example, data from an *in vitro* cytotoxicity assay can also be useful as one of the tools in setting a starting dose for the *in vivo* assessment of acute oral toxicity (see paragraphs (n)(10) through (n)(12) of this guideline). (A Guidance Document on Using *In Vitro* Data to Estimate *In Vivo* Starting Doses for Acute Toxicity is available (see paragraph (n)(11) of this guideline), and preliminary information suggests that the use of this approach may further reduce the number of animals used for *in vivo* testing (see paragraph (n)(11) of this guideline). Preliminary estimates of the LD_{50} and the dose-response slope will help in selecting a dose progression factor and a starting dose for testing.

(ii) **Default starting dose and dose progression factor**. If no information is available to make a preliminary estimate of the LD_{50} and the slope of the dose-response curve, results of computer simulations have suggested that starting near 175 mg/kg and using half-log units (corresponding to a dose progression of 3.2) between doses will produce the best results. This starting dose should be modified if the substance is likely to be highly toxic. The half-log spacing provides for a more efficient use of animals, and increases accuracy in the prediction of the LD_{50} value. However, for chemicals with large variability (i.e., shallow dose- response slopes), bias can still be introduced in the lethality estimates and the LD_{50} estimate will have a large statistical error, similar to other acute toxicity methods. To correct for this, the main test includes a stopping rule keyed

to properties of the estimate rather than a fixed number of test observations. (See paragraph (i)(3)(iii) of this guideline.)

(iii) **Delayed toxicity**. The method is easiest to apply to materials that produce death within one or two days. The method would not be practical to use when considerably delayed death (five days or more) can be expected.

(iv) **Computation**. Computers are used to facilitate animal-by-animal calculations that establish testing sequences and provide final estimates. The users of this protocol are strongly urged to solely use the Agency-developed software package (AOT425StatPgm) for performing the test and the calculation of the LD 50. The software is available on EPA's Internet Web site at http://www.epa.gov/oppfead1/harmonized.

(v) **Humane practices**. Moribund animals or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed, and are considered in the interpretation of the test results in the same way as animals that died on test. Criteria for making the decision to kill moribund or severely suffering animals, and guidance on the recognition of predictable or impending death are the subject of an OECD guidance document (see paragraph (n)(11) of this guideline).

(vi) **Limit test**. A limit test can be used efficiently to identify chemicals that are likely to have low acute toxicity.

(f) **Principle of the limit test**. The limit test is a sequential test that uses a maximum of 5 animals (see paragraphs (i)(2)(i) through (i)(2)(iv) of this guideline). A test dose of 5000 mg/kg is used. The selection of a sequential test plan increases the statistical power and also has been made to intentionally bias the procedure towards rejection of the limit test for compounds with LD_{50}s near the limit dose; i.e., to err on the side of safety. As with any limit test protocol, the probability of correctly classifying a compound will decrease as the actual LD_{50} more nearly resembles the limit dose.

(g) **Principle of the Main Test**. (1) The main test consists of a single ordered dose progression in which animals are dosed, one at a time, at 48-hour intervals. The first animal receives a dose a step below the level of the best estimate of the LD_{50}. If the animal survives, the dose for the next animal is increased to a factor of one half log times the original dose; if it dies, the dose for the next animal is decreased by a similar dose progression. (Note: 3.2 is the default factor corresponding to a dose progression of one half log unit in the Agency developed software program (AOT425StatPgm). However, this value may be changed. Paragraphs (i)(3)(ii) and (m)(12) of this guideline provide further guidance for choice of dose spacing factor.) Each animal should be observed carefully for up to 48 hours before making a decision on whether and how much to dose the next animal. That decision is based on the 48-hour survival pattern

of all the animals up to that time. (See paragraphs (i)(3)(i) and (i)(3)(v) of this guideline on choice of survival interval.) A combination of stopping criteria is used to keep the number of animals low while adjusting the dosing pattern to reduce the effect of a poor starting value or low slope (see paragraph (i)(3)(iv) of this guideline). Dosing is stopped when one of these criteria is satisfied (see paragraphs (i)(3)(iii) and (k)(2) of this guideline), at which time an estimate of the LD_{50} and a CI are calculated for the test based on the status of all the animals at termination. For most applications, testing will be completed with only 4 animals after initial reversal in animal outcome. The LD_{50} is calculated using the method of maximum likelihood (see paragraphs (k)(2) and (k)(2)(iii) of this guideline.)

(2) The results of the main test procedure serve as the starting point for a computational procedure to provide a CI estimate where feasible. A description of the basis for this CI is outlined in paragraph (k)(3) of this guideline.

(h) **Preparation for testing**—(1) **Selection of animals species**. The preferred rodent species is the rat although other rodent species may be used.

(2) **Single sex selection**. The test is conducted using a single sex in order to reduce variability and as a means of minimizing the number of animals used. Either sex may be used, however, if there is information available indicating differences in sensitivity, the most sensitive sex (usually females) should be tested (see paragraph (n)(11) of this guideline).

(i) Literature surveys of conventional LD_{50} tests show that usually there is little difference in sensitivity between the sexes but, in those cases where differences were observed, females were often slightly more sensitive (see paragraph (n)(10) of this guideline). For chemicals that are direct acting in their toxic mechanism, female rats may 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.

(ii) Occasionally, the results of subsequent testing, for example a subchronic 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 oral 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 doses closer to the LD_{50} in the second test.

(3) **Age and weight ranges**. Healthy young adult animals of commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. At the commencement of its dosing, each animal should be between 8 weeks and 12 weeks old. In order to minimize the contribution of developmental variability to study outcome, 10 weeks, with a range of ± 1 week is recommended if practical. The weight of each animal should fall in an interval ± 20% of the mean initial weight of all previously dosed animals.

(4) **Housing and feeding conditions**. The temperature in the experimental animal room should be 22°C (± 3°C). The relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning. Lighting should be artificial, the sequence being 12 hours light and 12 hours dark. The animals are housed individually. For feeding, conventional rodent laboratory diets may be used with an unlimited supply of drinking water.

(5) **Preparation of animals**. The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to dosing to allow for acclimatization to the laboratory conditions. As with other sequential test designs, care must be taken to ensure that animals are available in the appropriate size and age range for the entire study.

(6) **Preparation of doses**. (i) When necessary, the test substance is dissolved or suspended in a suitable vehicle. The use of an aqueous solution/suspension/emulsion is recommended wherever possible, followed in order of preference by a solution/suspension/emulsion in oil (e.g. corn oil) and then possibly solution in other vehicles. For vehicles other than water the toxicological characteristics of the vehicle should be known. Dosing preparations must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known. Where preparation shortly before administration is not practicable and the stability of the preparation is not known, this will need to be demonstrated analytically.

(ii) Constant concentration should be used in dosing unless there is clear scientific or regulatory justification for not doing so. The maximum dose volume for administration must not be exceeded. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not normally exceed

1 ml/100g of body weight; however, in the case of aqueous solutions, 2 ml/100g body weight can be considered.

(7) **Administration of doses**. (i) The test substance is administered in a single dose by gavage using a stomach tube or a suitable intubation cannula. In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.

(ii) Animals should be fasted prior to dosing (e.g., with the rat, food but not water should be withheld overnight; with the mouse, food but not water should be withheld for 3–4 hours). Following the period of fasting, the animals should be weighed and the test substance administered. The fasted body weight of each animal is determined and the dose is calculated according to the body weight. After the substance has been administered, food may be withheld for a further 3–4 hours in rats or 1–2 hours in mice. Where a dose is administered in fractions over a period of time, it may be necessary to provide the animals with food and water depending on the length of the period.

(i) **The up-and-down testing procedure**—(1) **Choice of limit test and main test**. The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity below regulatory limit doses. Information about the toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance. In those situations where there is little or no information about its toxicity, or in which the test material is expected to be toxic, the main test should be performed.

(2) **Implementation of the limit test**. (i) The Agency has developed dedicated software for performing the test and calculation of test results (see paragraph (e) (2)(iv) of this guideline).

(ii) Dose one animal at 5000 mg/kg. If the animal dies, conduct the main test starting at 175 mg/kg to determine the LD_{50}. If the animal survives, dose two additional animals. If both animals survive, the LD_{50} is greater than the limit dose and the test is terminated (i.e. carried to full 14-day observation without dosing of further animals). If one or both animals die, then dose an additional two animals, one at a time. If an animal unexpectedly dies late in the study, and there are other survivors, it is appropriate to stop dosing and observe all animals to see if other animals will also die during a similar observation period (see paragraph (g)(1) of this guideline for initial observation period). Late deaths should be counted the same as other deaths. The results are evaluated as follows (O=survival and X=death).

(iii) The LD_{50} is less than the test dose (5000 mg/kg) when three or more animals die. If a third animal dies, conduct the main test.

O XO XX

O OX XX

O XX OX

O XX X

(iv) The LD_{50} is greater than the test dose (5000 mg/kg) when three or more animals survive.

O OO

O XO XO

O XO O

O OX XO

O OX O

O XX OO

(v) If a limit test is performed at 2000 mg/kg, animals should be dosed sequentially and testing should be performed on all five animals.

(3) **Implementation of the main test**. (i) The Agency has developed dedicated software for performing the test and calculation of test results (see paragraph (e) (2)(iv) of this guideline).

(ii) Performing the UDP. Single animals are dosed in sequence usually at 48-hour intervals. However, the time interval between dosing is determined by the onset, duration, and severity of toxic signs. Treatment of an animal at the next dose should be delayed until one is confident of survival of the previously dosed animal. The time interval may be adjusted as appropriate, e.g., in case of inconclusive response. The test is simpler to implement when a single time interval is used for making sequential dosing decisions. Nevertheless, it is not necessary to recalculate dosing or likelihood-ratios if the time interval changes midtest. For selecting the starting dose, all available information, including information on structurally related substances and results of any other toxicity tests on the test material, should be used to approximate the LD_{50} as well as the slope of the dose-response curve.

(iii) Choice of starting dose and dose progression. The first animal is dosed a step below the toxicologist's best estimate of the LD_{50}. If the animal survives, the second animal receives a higher dose. If the first animal dies or appears moribund, the second animal receives a lower dose. The same dosing decision pattern is followed for each subsequent animal.

The dose progression factor should be chosen to be the antilog of 1/(the estimated slope of the dose-response curve) (a progression of 3.2 corresponds to a slope of 2) and should remain constant throughout testing. Thus, when there is no information on the slope of the substance to be tested, a default dose progression factor of 3.2 is used. Using the default progression factor, doses would be selected from the sequence 1.75, 5.5, 17.5, 55, 175, 550, 1750, 5000. If no estimate of the substance's lethality is available, dosing should be initiated at 175 mg/kg. In most cases, this dose is sublethal and therefore serves to reduce the level of pain and suffering. If animal tolerances to the chemical are expected to be highly variable (i.e., slopes are expected to be less than 2.0), consideration should be given to increasing the dose progression factor beyond the default 0.5 on a log dose scale (i.e., 3.2 progression factor) prior to starting the test. Similarly, for test substances known to have very steep slopes, dose progression factors smaller than the default should be chosen. (Paragraph (m)(3) of this guideline relates choice of dose progression to assumed slope and *sigma* and discusses test performance. Paragraph (m)(1) of this guideline includes a table of dose progressions for whole number slopes ranging from 1 to 8 with starting dose 175 mg/kg.)

(iv) Stopping rules. Dosing continues depending on the fixed-time interval (e.g., 48-hours) outcomes of all the animals up to that time. The testing stops when one of the following stopping criteria first is met:

(A) 3 consecutive animals survive at the upper bound;

(B) 5 reversals occur in any 6 consecutive animals tested;

(C) At least 4 animals have followed the first reversal and the specified likelihood-ratios exceed the critical value. (See paragraphs (k)(2)(iv) and (m)(2) of this guideline). Calculations are made at each dosing, following the fourth animal after the first reversal.).

(v) Total number of doses. For a wide variety of combinations of LD_{50} and slopes, stopping rule in paragraph (i)(3)(iii)(C) of this guideline will be satisfied with 4 to 6 animals after the test reversal. In some cases for chemicals with shallow slope dose-response curves, additional animals (up to a total of fifteen tested) may be needed.

(vi) Calculation. When the stopping criteria have been attained, the estimated LD_{50} should be calculated from the animal outcomes at test termination using the method described in paragraphs (k)(1)(i) and (k)(2)(i) of this guideline.

(vii) Humane practices. Moribund animals killed for humane reasons are considered in the same way as animals that died on test. If an animal unexpectedly dies late in the study and there are other survivors at that dose or above, it is appropriate to stop dosing and observe all animals to see if other animals will also die during a similar observation period.

If subsequent survivors also die, and it appears that all dose levels exceed the LD_{50} it would be most appropriate to start the study again beginning at least two steps below the lowest dose with deaths (and increasing the observation period) since the technique is most accurate when the starting dose is below the LD_{50}. If subsequent animals survive at or above the dose of the animal that dies, it is not necessary to change the dose progression since the information from the animal that has now died will be included into the calculations as a death at a lower dose than subsequent survivors, pulling the LD_{50} down.

(j) **Observations**. Animals are observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions and time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed (see paragraph (n)(15) of this guideline). All observations of toxic signs are systematically recorded with individual records being maintained for each animal. Additional observations will be necessary if the animals continue to display signs of toxicity.

(1) **Toxic signs**. Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. The principles and criteria summarized in the Humane Endpoints Guidance Document (see paragraph (n)(11) of this guideline) should be taken into consideration. Animals found in a moribund condition and animals showing severe pain and enduring signs of severe distress should be humanely killed. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

(2) **Body weight**. Individual weights of animals should be determined shortly before the test substance is administered and at least weekly thereafter. Weight changes should be calculated and recorded. At the end of the test surviving animals are weighed and then humanely killed.

(3) **Pathology**. All animals (including those which die during the test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy. All gross pathological changes should be recorded for each animal. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours after the

initial dosing may also be considered because it may yield useful information.

(k) **Data and reporting**—(1) **Data**. Individual animal data should be provided. Additionally, all data should be summarized in tabular form, showing for each test dose the number of animals used, the number of animals displaying signs of toxicity (see paragraph (n)(15) of this guideline), the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and the time course of toxic effects and reversibility, and necropsy findings. A rationale for the starting dose and the dose progression and any data used to support this choice should be provided.

(2) **Calculation of LD_{50} for the main test**—(i) **Maximum likelihood**. The LD_{50} is calculated using the maximum likelihood method, except in the exceptional cases described in paragraphs (k)(2)(ii) and (m)(3) of this guideline. The Agency-developed software program (AOT425StatPgm) available on EPA's Internet Web site at http://www.epa.gov/oppfead1/harmonized should be used to perform this calculation. The following statistical details may be helpful in implementing the maximum likelihood calculations suggested (with an assumed *sigma*). All deaths, whether immediate or delayed or humane kills, are incorporated for the purpose of the maximum likelihood analysis. Following Dixon (see paragraph (n)(5) of this guideline), the likelihood function is written as follows:

$$L = L_1 L_2 \ldots L_n ,$$

where

L is the likelihood of the experimental outcome, given μ and *sigma*, and n the total number of animals tested.

$L_i = 1 - F(Z_i)$ if the ith animal survived, or

$L_i = F(Z_i)$ if the ith animal died,

where

F = cumulative standard normal distribution,

$Z_i = [\log(d_i) - \mu] / sigma$

d_i = dose given to the ith animal, and

sigma = standard deviation in log units of dose (which is not the log standard deviation).

An estimate of the log of the true LD_{50} is given by the value of μ that maximizes the likelihood L (see paragraph (k)(2)(iii) of this guideline).

An estimate of *sigma* of 0.5 is used unless a better generic or case-specific value is available.

(ii) **Special circumstances**. Under some circumstances, statistical computation will not be possible or will likely give erroneous results. Special means to determine/report an estimated LD_{50} are available for these circumstances as described in the following paragraphs (k)(2)(ii)(A), (k)(2)(ii)(B), and (k)(2)(ii)(C). If none of these situations occurs, then the LD_{50} is calculated using the maximum likelihood method.

(A) If testing stopped based on the criterion in paragraph (i)(3)(iii)(C) of this guideline (i.e., a boundary dose was tested repeatedly), or if the upper bound dose ended testing, then the LD_{50} is reported to be above the upper bound.

(B) If all the dead animals have higher doses than all the live animals (or if all live animals have higher doses than all the dead animals, although this is practically unlikely), then the LD_{50} is between the doses for the live and the dead animals. These observations give no further information on the exact value of the LD_{50}. Still, a maximum likelihood LD_{50} estimate can be made provided there is a prior value for *sigma*. The LD_{50} estimate is only as good as the validity of the assumed signa. However, Case 3 as described in paragraph (m)(3)(iii) of this guideline and here is most likely to occur because the dose progression (based on the assumed signma) is too wide. The stopping criterion in paragraph (i)(3)(iii)(C) describes one such circumstance.

(C) If the live and dead animals have only one dose in common and all the other dead animals have higher doses and all the other live animals lower doses, or vice versa, then the LD_{50} equals their common dose. If a closely related substance is tested, testing should proceed with a smaller dose progression.

(iii) **Maximum likelihood calculation**. Maximum likelihood calculation should be performed using a dedicated program developed by and available from EPA (see paragraph (n)(16) of this guideline). If other computer programs are used, the laboratory should take care in handling special cases described in this guideline and the documentation of test performance available on EPA's Internet Web site at http://www.epa.gov/oppfead1/harmonized. Typical instructions for these packages are given in appendices to the ASTM Standard E 1163-87 (see paragraph (n)(9) of this guideline). (The *sigma* used in the BASIC program in (see paragraph (n)(9) of this guideline) will need to be edited to reflect the parameters of the UDP.) The program's output is an estimate of log (LD_{50}) and its standard error.

(iv) **Stopping rule**. The likelihood-ratio stopping rule in paragraph (i)(3)(iii)(C) of this guideline is based on three measures of test progress, that are of the form of the likelihood in paragraph (k)(2) of this guideline,

with different values for μ. Comparisons are made after each animal tested after the sixth that does not already satisfy the criteria in paragraph (i)(3)(iii)(A) or paragraph (i)(3)(iii)(B) guideline. The equations for the likelihood-ratio criteria are provided by following the steps in paragraph (m)(2)(vii) of this guideline. These comparisons are most readily performed in an automated manner and can be executed repeatedly, for instance, by a spreadsheet routine such as that also provided in paragraph (m)(2)(vii) of this guideline. If the criterion is met, testing stops and the LD_{50} can be calculated by the maximum likelihood method.

(3) **Computation of CI**. (i) Following the main test and estimated LD_{50} calculation, it may be possible to compute interval estimates for the LD_{50}. The Agency-developed software program AOT425StatPgm will perform the calculations. Any of these CIs provides valuable information on the reliability and utility of the main test that was conducted. A wide CI indicates that there is more uncertainty associated with the estimated LD_{50}. In this case, the reliability of the estimated LD_{50} is low and the usefulness of the estimated LD_{50} may be marginal. A narrow interval indicates that there is relatively little uncertainty associated with the estimated LD_{50}. In this case, the reliability of the estimated LD_{50} is high and the usefulness of the estimated LD_{50} is good. This means that if the main test were to be repeated, the new estimated LD_{50} is expected to be close to the original estimated LD_{50} and both of these estimates are expected to be close to the true LD_{50}.

(ii) Depending on the outcome of the main test, one of two different types of interval estimates of the true LD_{50} is calculated:

(A) When at least three different doses have been tested and the middle dose has at least one animal that survived and one animal that died, a profile-likelihood-based computational procedure is used to obtain a CI that is expected to contain the true LD_{50} 95% of the time. However, because small numbers of animals are expected to be used, the actual level of confidence is generally not exact (see paragraph (n)(19) of this guideline). The random stopping rule improves the ability of the test overall to respond to varying underlying conditions, but also causes the reported level of confidence and the actual level of confidence to differ somewhat (see paragraph (n)(18) of this guideline).

(B) If all animals survive at or below a given dose level and all animals die when dosed at the next higher dose level, an interval is calculated that has as its lower limit the highest dose tested where all the animals survive and has as its upper limit the dose level where all the animals died. This interval is labeled as ‘‘approximate.’’ The exact confidence level associated with this interval cannot be specifically determined. However, because this type of response would only occur when the dose-response is steep, in most cases, the true LD_{50} is expected to be contained

within the calculated interval or be very close to it. This interval will be relatively narrow and sufficiently accurate for most practical use.

(iii) In some instances, CIs are reported as infinite, through including either zero at the lower end or infinity at the upper end, or both. Such intervals may occur, for example, when the response profile is relatively flat or relatively uncertain.

(iv) Implementing this set of procedures requires specialized computation which is either by use of a dedicated program to be available through the Environmental Protection Agency (EPA) or OECD or developed following technical details available from the EPA or OECD. Achieved coverage of these intervals and properties of the dedicated program are described in a report (see paragraph (n)(16) of this guideline) also available through the EPA. Paragraph (m)(3) of this guideline provides information on choice of dose progression and initial dose level for the UDP and describes test performance under a variety of circumstances.

(l) **Test reporting**. The test report must include the following information:

(1) Test substance:

(i) Physical nature, purity and physicochemical properties (including isomerization);

(ii) Identification data.

(2) Vehicle (if appropriate): Justification for choice of vehicle, if other than water.

(3) Test animals:

(i) Species/strain used;

(ii) Microbiological status of the animals, when known;

(iii) Number, age and sex of animals;

(iv) Rationale for use of males instead of females;

(v) Source, housing conditions, diet, etc.;

(vi) Individual weights of animals at the start of the test, at day 7, and at day 14.

(4) Test conditions:

(i) Rationale for initial dose level selection, dose progression factor and for follow-up dose levels;

(ii) Details of test substance formulation;

(iii) Details of the administration of the test substance;

(iv) Details of food and water quality (including diet type/source, water source).

(5) Results:

(i) Body weight/body weight changes;

(ii) Tabulation of response data by sex (if both sexes are used) and dose level for each animal (i.e., animals showing signs of toxicity including nature, severity, duration of effects, and mortality);

(iii) Time course of onset of signs of toxicity and whether these were reversible for each animal;

(iv) Necropsy findings and any histopathological findings for each animal, if available;

(v) LD_{50} and CI (which the AOT425StatPgm software package uses);

(vi) Statistical treatment of results (description of computer routine used and spreadsheet tabulation of calculations). If other than Agency-supplied software is used, give explanation of now the program was verified against Agency software.

(6) Discussion and interpretation of results.

(7) Conclusions.

(m) **Additional guidance for toxicologists**—(1) **Dosing procedure—dose sequence for main test**. (i) Up-and-down dosing procedure. For each run, animals are dosed, one at a time, usually at 48-hour intervals. The first animal receives a dose a step below the level of the best estimate of the LD_{50}. This selection reflects an adjustment for a tendency to bias away from the LD_{50} in the direction of the initial starting dose in the final estimate (see paragraph (e)(2)(ii) of the guideline). The overall pattern of outcomes is expected to stabilize as dosing is adjusted for each subsequent animal. Paragraph (m)(1)(iii) of this guideline provides further guidance for choice of dose spacing factor.

(ii) Default dose progression. Once the starting dose and dose spacing are decided, the toxicologist should list all possible doses including the upper bound (usually 2000 or 5000 mg/kg). Doses that are close to the upper bound should be removed from the progression. The stepped nature of the UDP design provides for the first few doses to function as a self-adjusting sequence. Because of the tendency for positive bias, in the event that nothing is known about the substance, a starting dose of 175 mg/kg is recommended. If the default procedure is to be used for the main test, dosing will be initiated at 175 mg/kg and doses will be spaced by a factor of 0.5 on a log dose scale. The doses to be used include 1.75,

5.5, 17.5, 55, 175, 550, 2000 or, for specific regulatory needs, 1.75, 5.5, 17.5, 55, 175, 550, 1750, 5000. For certain highly toxic substances, the dosing sequence may need to be extended to lower values.

(iii) In the event a dose progression factor other than the default is deemed suitable, the following Table 1 provides dose progressions for whole number multiples of slope, from 1 to 8. (See paragraph (m)(3) of this guideline for discussion of influence of dose progression on test performance.)

Table 1.—Dose Progressions for UDP

(Choose a Slope and Read Down the Column. All doses in mg/kg body weight)

Slope =	1	2	3	4	5	6	7	8
	0.175*	0.175*	0.175*	0.175*	0.175*	0.175*	0.175*	0.175*
	.....	.....	.....	.....	.....	.....	0.243*	0.233*
	.....	.....	.....	.....	0.28	0.26	.....	.....
	.....	.....	.....	0.31	.....	.....	0.34	0.31
	.....	.....	0.38	.....	.....	0.38	.....	.....
	.....	.....	.....	.....	.....	.....	.....	0.41
	.....	.....	.....	.....	0.44	.....	0.47	.....
	.....	0.55	.....	.55	.....	0.55	.....	0.55
	.....	.....	.....	0.70	.....	0.65	.....	
	.....	.....	.....	.....	.....	.....	0.74	
	.....	.....	.81	.....	.....	.81	.....	.....
	.....	.....	.....	0.98	.....	.....	0.91	0.98
	.....	.....	.....	.....	110	1.19	.....	.....
	.....	.....	.....	.....	.....	.....	1.26	1.31
	1.75	1.75	1.75	1.75	1.75	1.75	1.75	1.75
	.....	.....	.....	.....	.....	.....	2.43	2.33
	.....	.....	.....	.....	2.8	2.6	.....	.....
	.....	.....	.....	3.1	.....	.....	3.4	3.1
	.....	.....	3.8	.....	.....	3.8	.....	.....
	.....	.....	.....	.....	4.4	.....	.....	4.1
	.....	.....	.....	.....	.....	.....	4.7	.....
	.....	5.5	.....	5.5	5.5	.....	5.5	
	.....	.....	.....	.....	7.0	.....	6.5	.....
	.....	.....	.....	.....	.....	.....	.....	7.4
	.....	.....	8.1	.....	.....	8.1	.....	.....
	.....	.....	.....	9.8	.....	.....	9.1	9.8
	.....	.....	.....	.....	11.0	11.9	.....	.....
	.....	.....	.....	.....	.....	.....	12.6	13.1
	17.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5
	.....	.....	.....	.....	.....	.....	24.3	23.3
	.....	.....	.....	.....	28	26	.....	.....
	.....	.....	.....	31	.....	.....	34	31
	.....	.....	38	.....	.....	38	.....	.....
	.....	.....	.....	.....	44	.....	.....	41
	.....	.....	.....	.....	.....	.....	47	.....
	.....	55	.....	55	.....	55	.....	55
	.....	.....	.....	.....	.....	.....	65	.....
	.....	.....	.....	.....	70	.....	.....	74
	.....	.....	81	.....	.....	81	.....	.....
	.....	.....	.....	98	.....	.....	91	98
	.....	.....	.....	.....	110	119	.....	.....
	.....	.....	.....	.....	.....	.....	126	131
	175	175	175	175	175	175	175	175
	.....	.....	.....	.....	.....	.....	243	233
	.....	.....	.....	.....	280	260	.....	.....
	.....	.....	.....	310	.....	.....	340	310
	.....	.....	380	.....	.....	380	.....	.....
	.....	.....	.....	.....	440	.....	.....	410
	.....	.....	.....	.....	.....	.....	470	.....
	.....	550	.....	550	.....	550	.....	550
	.....	.....	.....	.....	.....	.....	650	.....
	.....	.....	.....	.....	700	.....	.....	740
	.....	.....	810	.....	.....	810	.....	.....
	.....	.....	.....	980	.....	.....	910	980
	.....	.....	.....	.....	1100	1190	.....	.....
	.....	.....	.....	.....	.....	.....	1260	1310
	1750	1750	1750	1750	1750	1750	1750	1750
	.....	.....	.....	.....	.....	.....	2430	2330
	.....	.....	.....	.....	2800	2600	.....	.....
	.....	.....	.....	3100	.....	.....	.....	3100
	.....	.....	.....	.....	.....	3800	3400	.....
	.....	.....	.....	.....	.....	.....	.....	4100
	5000	5000	5000	5000	5000	5000	5000	5000

* If lower doses are needed, continue progressions to a lower dose

(2) **Computations for the likelihood-ratio stopping rules**. (i) As described in paragraph (i)(3)(iii) of this guideline, the main test may be completed on the basis of the first of three stopping criteria to occur. In any case, even if none of the stopping criteria is satisfied, dosing would stop when 15 animals are dosed. Tables 2, 4, and 6 in paragraphs (m)(2)(ii), (m)(2)(iii), and (m)(2)(iv), respectively, of this guideline illustrate examples where testing has started with no information, so the rec-

ommended default starting value, 175 mg/kg, and the recommended default dose progression factor, 3.2 or one half log, have been used. Tables 3, 5, and 7 in paragraphs (m)(2)(ii), (m)(2)(iii), and (m)(2)(iv), respectively, illustrate how Tables 2, 4, and 6, respectively, would appear in the dedicated program referenced in paragraph (k)(3)(iv) (see also paragraph (n)(16)).

(ii) The following Tables 2 and 3 show how the main test would stop if 3 animals have survived at the limit dose of 5000 mg/kg. (This example illustrates situations where a limit test was not thought appropriate *a priori*).

Table 2. Example of Stopping Criterion in Paragraph (i)(3)(iii)(A) using 5000 mg/kg.

Stop after animal #6 because 3 animals survive at limit of 5000 mg/kg (#4-#6).

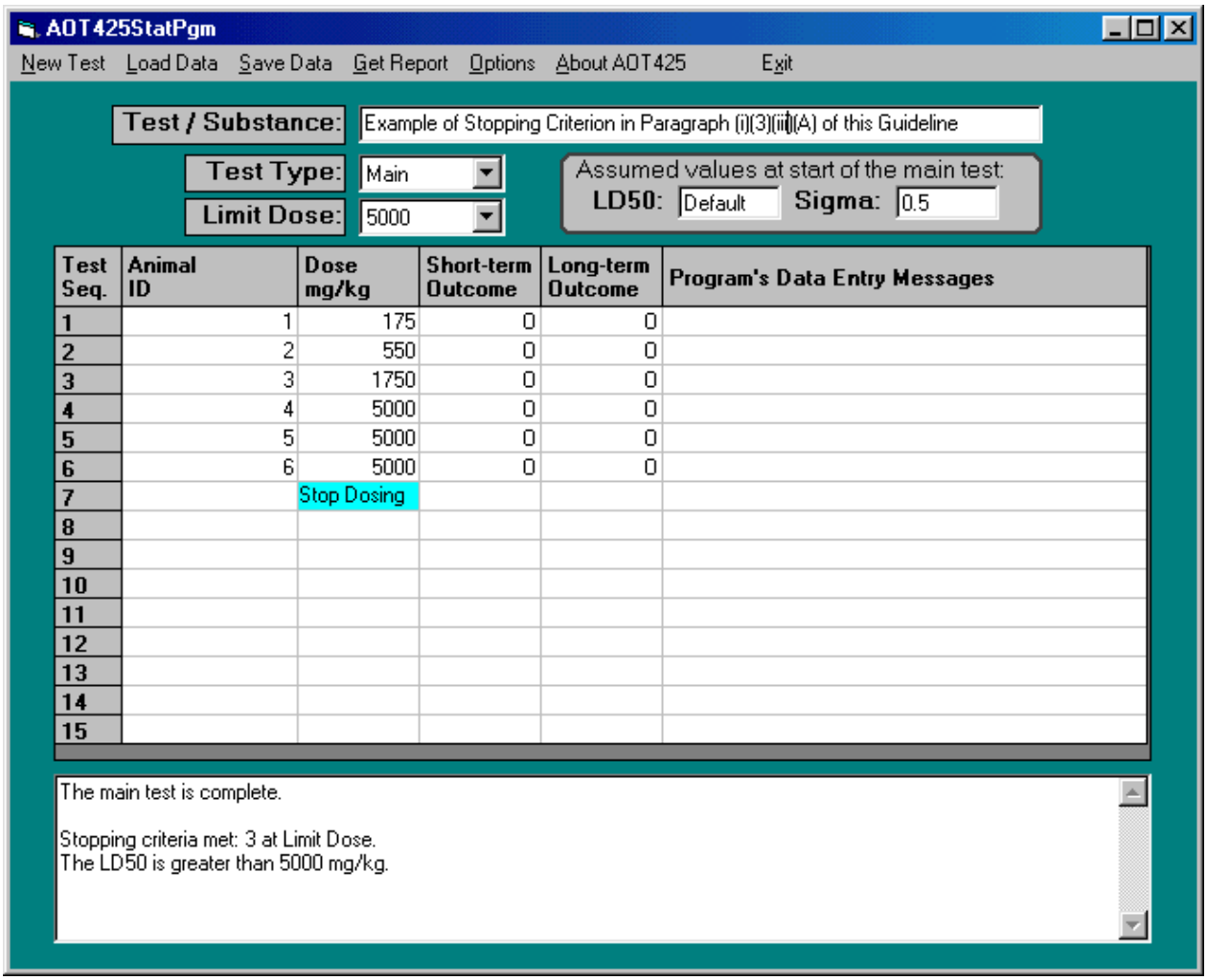
1	2	3	4	5	6	7	8	9	10	11	12
Step	(I)nclude; (E)xclude	Dose	(X)response (O)non-resp. OK	Included in nominal *n*	log10 Dose	LD50 = Prob. of response	#DIV/0! likelihood contribn. (ln *Li*)	LD50 = Prob. of response	#DIV/0! likelihood contribn. (ln *Li*)	LD50 = Prob. of response	#DIV/0! likelihood contribn. (ln *Li*)
1	I	175	O	no	2.2430	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2	I	550	O	no	2.7404	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
3	I	1750	O	no	3.2430	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4	I	5000	O	no	3.6990	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
5	I	5000	O	no	3.6990	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
6	I	5000	O	no	3.6990	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
7	E				-						
8	E				-						
9	E				-	-	-	-	-	-	-
10	E				-	-	-	-	-	-	-
11	E				-	-	-	-	-	-	-
12	E				-				-	-	-
13	E				-				-	-	-
14	E				-				-	-	-
15	E				-	-	-	-	-	-	-
Nominal Sample size =				0							
Actual number tested =				6							

Ignore all calculation cells. No reversal in direction of response.

Maximum Likelihood Calculations cannot be completed. LD50 is greater than 5000 mg/kg.

Calculated maximum likelihood estimate of LD50 = none

Table 3. Example of Stopping Criterion in Paragraph (i)(3)(iii)(A) of this Guideline Using 5000 mg/kg



(iii) The following Tables 4 and 5 show how a particular sequence of 5 reversals in 6 tested animals could occur and allow test completion.

Table 4. Example of Stopping Criterion in Paragraph (i)(3)(iii)(B).

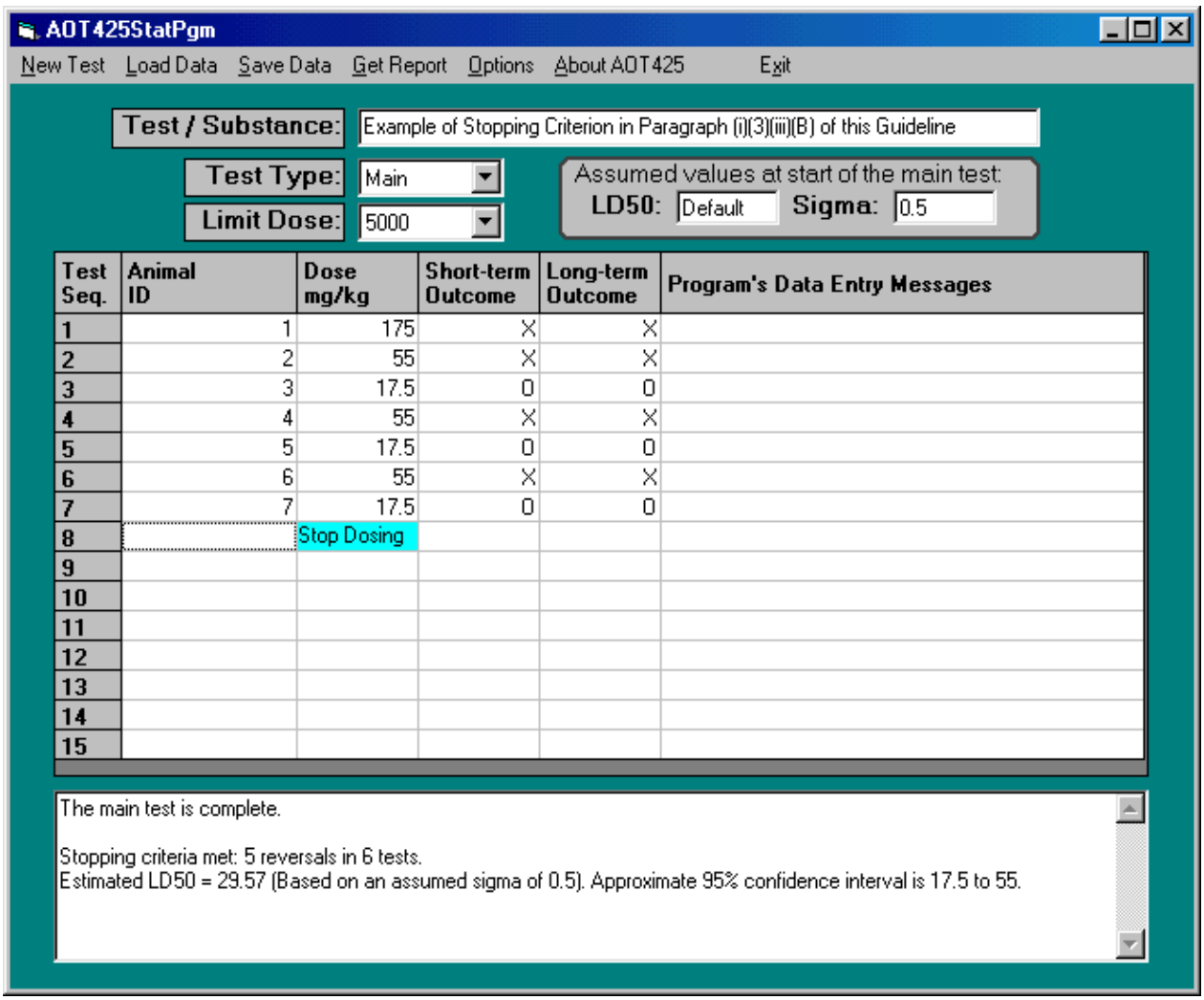
Stop after animal #7 because 5 reversals in 6 consecutive animals tested (#2-#7).

1	2	3	4	5	6	7	8	9	10	11	12
Step	(I)nclude; (E)xclude	Dose	(X)response (O)non-resp. OK	Included in nominal *n*	log10 Dose	LD50 = Prob. of response	31.0 likelihood contribn. (ln *Li*)	LD50 = Prob. of response	12.4 likelihood contribn. (ln *Li*)	LD50 = Prob. of response	77.6 likelihood contribn. (ln *Li*)
1	I	175	X	no	2.2430	0.9335	-0.0688	0.9892	-0.0108	0.7602	-0.2742
2	I	55	X	yes	1.7404	0.6905	-0.3703	0.9020	-0.1031	0.3826	-0.9607
3	I	17.5	O	yes	1.2430	0.3095	-0.3703	0.6174	-0.9607	0.0980	-0.1031
4	I	55	X	yes	1.7404	0.6905	-0.3703	0.9020	-0.1031	0.3826	-0.9607
5	I	17.5	O	yes	1.2430	0.3095	-0.3703	0.6174	-0.9607	0.0980	-0.1031
6	I	55	X	yes	1.7404	0.6905	-0.3703	0.9020	-0.1031	0.3826	-0.9607
7	I	17.5	O	yes	1.2430	0.3095	-0.3703	0.6174	-0.9607	0.0980	-0.1031
8	E				-	-	-	-	-	-	-
9	E				-	-	-	-	-	-	-
10	E				-	-	-	-	-	-	-
11	E				-	-	-	-	-	-	-
12	E				-	-	-	-	-	-	-
13	E				-	-	-	-	-	-	-
14	E				-	-	-	-	-	-	-
15	E				-	-	-	-	-	-	-
Nominal Sample size =				6							
Actual number tested =				7							
Dose-averaging estimator				31.02							
log10 =				1.492							
log-likelihood sums:							-2.2906		-3.2021		-3.4655
likelihoods:							0.1012		0.0407		0.0313
likelihood ratios:									2.4880		3.2378
Individual ratios exceed critical value?				critical=	2.5				FALSE		TRUE
Both ratios exceed critical value?									FALSE		
Calculated maximum likelihood estimate of LD50 =					29.6						

Automated calculation; not relevant to this case.

Final estimate obtained from Maximum Likelihood Calculations

Table 5. Example of Stopping Criterion in Paragraph (i)(3)(iii)(B) of this Guideline.



(iv) Finally, the following Tables 6 and 7 illustrate a situation several animals into a test, where neither the criterion in paragraph (i)(3)(iii)(A) nor the criterion in paragraph (i)(3)(iii)(B) of this guideline has been met, a reversal of response has occurred followed by 4 tested animals, and, consequently, the criterion in paragraph (i)(3)(iii)(C) of this guideline must be evaluated as well.

Table 6. Example of Stopping Criterion in Paragraph (i)(3)(iii)(C).

Stop when LR criterion is first met, here at animal #9.
Check LR criterion starting at animal #6.

Assumed slope	2	sigma =	0.5

Result:	The LR criterion is met

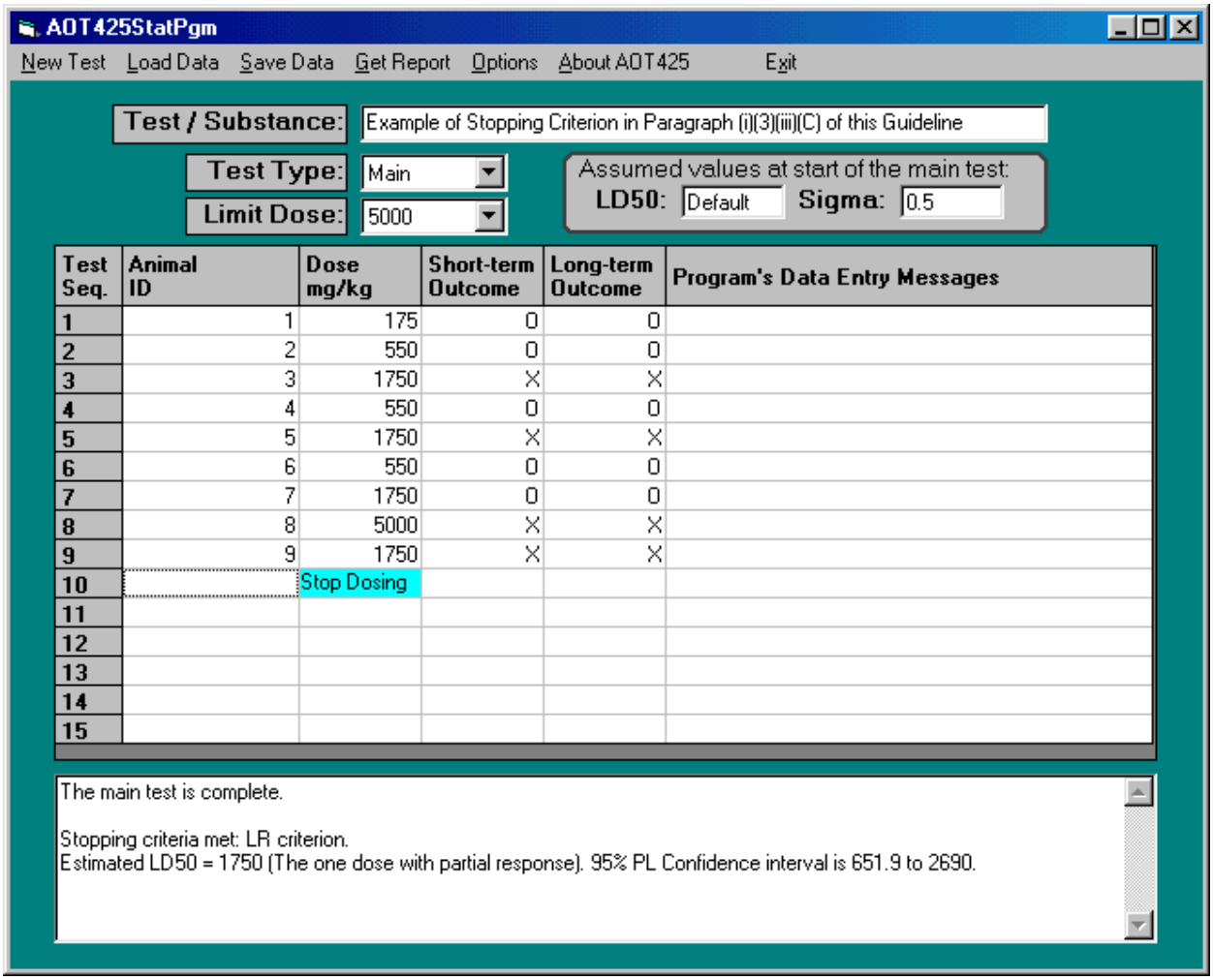
Parameters of convergence criterion	
critical LR	2.5
factor of LD50	2.5

1	2	3	4	5	6		7	8	9	10	11	12
Step	(I)nclude; (E)xclude	Dose	(X)response (O)non-resp. OK	Included in nominal *n*	log10 Dose	Contrib.to DAE	LD50 = Prob. of response	1292.8 likelihood contribn. (ln *Li*)	LD50 = Prob. of response	517.1 likelihood contribn. (ln *Li*)	LD50 = Prob. of response	3232.0 likelihood contribn. (ln *Li*)
1	I	175	O	no	2.2430	0.0000	0.0412	-0.0421	0.1733	-0.1903	0.0057	-0.0057
2	I	550	O	yes	2.7404	2.7404	0.2289	-0.2600	0.5214	-0.7368	0.0620	-0.0640
3	I	1750	X	yes	3.2430	3.2430	0.6037	-0.5046	0.8552	-0.1564	0.2971	-1.2138
4	I	550	O	yes	2.7404	2.7404	0.2289	-0.2600	0.5214	-0.7368	0.0620	-0.0640
5	I	1750	X	yes	3.2430	3.2430	0.6037	-0.5046	0.8552	-0.1564	0.2971	-1.2138
6	I	550	O	yes	2.7404	2.7404	0.2289	-0.2600	0.5214	-0.7368	0.0620	-0.0640
7	I	1750	O	yes	3.2430	3.2430	0.6037	-0.9257	0.8552	-1.9323	0.2971	-0.3525
8	I	5000	X	yes	3.6990	3.6990	0.8800	-0.1279	0.9756	-0.0247	0.6477	-0.4344
9	I	1750	X	yes	3.2430	3.2430	0.6037	-0.5046	0.8552	-0.1564	0.2971	-1.2138
10	E				-	0.0000	-	-	-	-	-	-
11	E				-	0.0000	-	-	-	-	-	-
12	E				-	0.0000	-	-	-	-	-	-
13	E				-	0.0000	-	-	-	-	-	-
14	E				-	0.0000	-	-	-	-	-	-
15	E				-	0.0000	-	-	-	-	-	-
Nominal Sample size =				8								
Actual number tested =				9								
Dose-averaging estimator				1292.78								
log10 =				3.112								
log-likelihood sums:								-3.3894		-4.8270		-4.6260
likelihoods:								0.0337		0.0080		0.0098
likelihood ratios:										4.2104		3.4436
Individual ratios exceed critical value?				critical=	2.5					TRUE		TRUE
Both ratios exceed critical value?										TRUE		

Calculated maximum likelihood estimate of LD50 = 1329.6

Final estimate obtained from Maximum Likelihood Calculations

Table 7. Example of Stopping Criterion in Paragraph (i)(3)(iii)(C) of this Guideline.



AOT425StatPgm

New Test Load Data Save Data Get Report Options About AOT425 Exit

Test / Substance: Example of Stopping Criterion in Paragraph (i)(3)(iii)(C) of this Guideline

Test Type: Main

Limit Dose: 5000

Assumed values at start of the main test:
LD50: Default Sigma: 0.5

Test Seq.	Animal ID	Dose mg/kg	Short-term Outcome	Long-term Outcome	Program's Data Entry Messages
1	1	175	0	0	
2	2	550	0	0	
3	3	1750	X	X	
4	4	550	0	0	
5	5	1750	X	X	
6	6	550	0	0	
7	7	1750	0	0	
8	8	5000	X	X	
9	9	1750	X	X	
10		Stop Dosing			
11					
12					
13					
14					
15					

The main test is complete.

Stopping criteria met: LR criterion.
Estimated LD50 = 1750 (The one dose with partial response). 95% PL Confidence interval is 651.9 to 2690.

(v) Criterion in paragraph (i)(3)(iii)(C) of this guideline calls for a likelihood-ratio stopping rule to be evaluated after testing each animal, starting with the fourth tested following the reversal. Three ‘‘measures of test progress’’ are calculated. Technically, these measures of progress are likelihoods, as recommended for the maximum-likelihood estimation of the LD_{50}. The procedure is closely related to calculation of a CI by a likelihood-based procedure.

(vi) The basis of the procedure is that when enough data have been collected, a point estimate of the LD_{50} should be more strongly supported than values above and below the point estimate, where statistical support is quantified using likelihood. Therefore three likelihood values are calculated: A likelihood at an LD_{50} point estimate (called the rough estimate or dose-averaging estimate in the example), a likelihood at a value below the point estimate, and a likelihood at a value above the point estimate. Specifically, the low value is taken to be the point estimate divided by 2.5 and the high value is taken to be the point estimate multiplied by 2.5.

(vii) The likelihood values are compared by calculating ratios of likelihoods, and then determining whether these likelihood-ratios (LR) exceed a critical value. Testing stops when the ratio of the likelihood for the point estimate exceeds each of the other likelihoods by a factor of 2.5, which is taken to indicate relatively strong statistical support for the point estimate. Therefore two likelihood-ratios (LRs) are calculated, a ratio of likelihoods for the point estimate and the point estimate divided by 2.5, and a ratio for the point estimate and the estimate times 2.5.

(viii) The calculations are easily performed in any spreadsheet with normal probability functions. The calculations are illustrated in Tables 6 and 7 in paragraph (m)(2)(iv) of this guideline, which is structured to promote spreadsheet implementation. The computation steps are illustrated using an example where the upper limit dose is 5000 mg/kg.

(A) Hypothetical example (Tables 6 and 7 in paragraph (m)(2)(iv) of this guideline). In the hypothetical example utilizing an upper boundary dose of 5000 mg/kg, the LR stopping criterion was met after nine animals had been tested. The first ‘‘reversal’’ occurred with the 3rd animal tested. The LR stopping criterion is checked when four animals have been tested following the reversal. In this example, the fourth animal tested following the reversal is the seventh animal actually tested. Therefore, for this example, the spreadsheet calculations are only needed after the seventh animal had been tested and the data could be entered at that time. Subsequently, the LR stopping criterion would have been checked after testing the seventh animal, the eighth animal, and the ninth. The LR stopping criterion is first satisfied after the ninth animal is tested in this example.

(*1*) Enter the dose-response information animal by animal.

(*i*) Column 1. Steps are numbered 1–15. No more than 15 animals may be tested.

(*ii*) Column 2. Place an I in this column as each animal is tested.

(*iii*) Column 3. Enter the dose received by the ith animal.

(*iv*) Column 4. Indicate whether the animal responded (shown by an X) or did not respond (shown by an O).

(*2*) The nominal and actual sample sizes. The nominal sample consists of the two animals that represent the first reversal (here the second and third animals), plus all animals tested subsequently. Here, Column 5 indicates whether or not a given animal is included in the nominal sample.

(*i*) The nominal sample size (nominal *n*) appears in Row 16. This is the number of animals in the nominal sample. In the example, nominal *n* is 8.

(*ii*) The actual number tested appears in Row 17.

(*3*) Rough estimate of the LD_{50}. The geometric mean of doses for the animals in the current nominal sample is used as a rough estimate of the LD_{50} from which to gauge progress. In the table, this is called the ''dose-averaging estimator.'' It is updated with each animal tested. This average is restricted to the nominal sample in order to allow for a poor choice of initial test dose, which could generate either an initial string of responses or an initial string of nonresponses. (However, the results for all animals are used in the likelihood calculations for final LD_{50} calculation below.) Recall that the geometric mean of *n* numbers is the product of the *n* numbers, raised to a power of 1/*n*.

(*i*) The dose-averaging estimate appears in Row 18 (e.g., $(175 * 550 * ... * 1750)^{1/8} = 1292.78$).

(*ii*) Row 19 shows the logarithm (base 10) of the value in Row 18 (e.g., $\log_{10} 1292.8 = 3.112$).

(*4*) Likelihood for the rough LD_{50} estimate.

(*i*) ''Likelihood'' is a statistical measure of how strongly the data support an estimate of the LD_{50} or other parameter. Ratios of likelihood values can be used to compare how well the data support different estimates of the LD_{50}.

(*ii*) In Column 8 calculate the likelihood for Step C's rough LD_{50} estimate. The likelihood (Row 21) is the product of likelihood contributions for individual animals (see paragraph (k)(2) of this guideline). The likelihood contribution for the ith animal is denoted L_i.

(*iii*) Column 7. Enter the estimate of the probability of response at dose d_i, denoted P_i. P_i is calculated from a dose-response curve. Note that the parameters of a probit dose-response curve are the slope and the LD_{50}, so values are needed for each of those parameters. For the LD_{50} the dose-averaging estimate from Row 18 is used. For the slope in this example the default value of 2 is used. The following steps may be used to calculate the response probability P_i.

1. Calculate the base-10 log of dose d_i (Column 6).

2. For each animal calculate the z-score, denoted Z_i (not shown in the table), using the formulae

$sigma = 1 / slope$,

$Z_i = (\log_{10}(d_i) - \log_{10}(LD_{50})) / sigma$

For example, for the first animal (Row 1),

$sigma = 1 / 2$

$Z_1 = (2.243 - 3.112) / 0.500 = -1.738$

3. For the ith dose the estimated response probability is

$P_i = F(Z_i)$

where F denotes the cumulative distribution function for the standard normal distribution (i.e., the normal distribution with mean 0 and variance 1).

For example (Row 1),

$P_1 = F(-1.738) = 0.0412$

The function F (or something very close) is ordinarily what is given for the normal distribution in statistical tables, but the function is also widely available as a spreadsheet function. It is available under different names, for example the @NORMAL function of Lotus 1-2-3 (see paragraph (n)(19) of this guideline) and the @NORMDIST function in Excel (see paragraph (n)(20) of this guideline). To confirm that you have used correctly the function available in your software, you may wish to verify familiar values such as $F(1.96) \approx 0.975$ or $F(1.64) \approx 0.95$.

(*iv*) Column 8. Calculate the natural log of the likelihood contribution ($\ln(L_i)$). L_i is simply the probability of the response that actually was observed for the ith animal:

Responding animals: $\ln(L_i) = \ln(P_i)$

Non-responding animals: $\ln(L_i) = \ln(1 - P_i)$

Note that here the natural logarithm (ln) is used, whereas elsewhere the base-10 (common) logarithm was used. These choices are what are ordinarily expected in a given context.

The steps above are performed for each animal. Finally:

Row 20: Sum the log-likelihood contributions in Column 8.

Row 21: Calculate the likelihood by applying the exp function applied to the log-likelihood value in Row 20 (e.g., exp(-3.389) = $e^{-3.389}$ = 0.0337).

(*5*) Calculate likelihoods for two dose values above and below the rough estimate. If the data permit a precise estimate, then one expects the likelihood should be high if the estimate is a reasonable estimate of the LD_{50}, relative to likelihoods for values distant from this estimate. Compare the likelihood for the dose-averaging estimate (1292.8, Row 18) to values differing by a factor of 2.5 from that value (i.e., to 1292.8*2.5 and 1292.8/2.5). The calculations (displayed in Columns 9–12) are carried out in a fashion similar to those described above, except that the values 517.1 (=1292.8/2.5) and 3232.0 (=1292.8*2.5) have been used for the LD_{50}, instead of 1292.8. The likelihoods and log-likelihoods are displayed in Rows 20–21.

(*6*) Calculate likelihood-ratios. The three likelihood values (Row 21) are used to calculate two likelihood-ratios (Row 22). A likelihood-ratio is used to compare the statistical support for the estimate of 1292.8 to the support for each of the other values, 517.1 and 3232.0. The two likelihood-ratios are therefore:

LR1 = [likelihood of 1292.8] / [likelihood of 517.1]

= 0.0337 / 0.0080

= 4.21

and

LR2 = [likelihood of 1292.8] / [likelihood of 3232.0]

= 0.0337 / 0.0098

= 3.44

(*7*) Determine if the likelihood-ratios exceed the critical value. High likelihood-ratios are taken to indicate relatively high support for the point estimate of the LD_{50}. Both of the likelihood-ratios calculated in paragraph (m)(2)(viii)(A)(*6*) of this guideline (4.21 and 3.44) exceed the critical likelihood-ratio, which is 2.5. Therefore the LR stopping criterion is satisfied and testing stops. This is indicated by a TRUE in Row 24 and a note at the top of the example spreadsheet that the LR criterion is met. Determination of the point estimate and CI is carried out separately.

(B) [Reserved]

(3) **Performance of the UDP**. This section addresses choice of dose progression and initial dose level for the UDP and describes the performance of the test under a variety of circumstances. A companion document titled ''Toxicology Summary: Performance of the Up-and-Down Procedure'' provides assistance to the user in interpretation of the test results and is available on the ICCVAM web site at http://iccvam.niehs.nih.gov/methods/udpdocs/udprpt/udp_ciprop.htm. The statistical methods applied will depend upon the case into which the test response patterns fall (see Table 8 in paragraph (m)(3)(iii) of this guideline.

(i) Adjusting the dose progression and initial dose. For optimum performance of the UDP, the dose progression used should be based on an accurate prior estimate of *sigma*. The following two cases describe the outcome when an accurate estimate of *sigma* is not available. In addition, to account conservatively for any bias in the LD_{50} estimate, it is essential that dosing be initiated below the actual LD_{50}.

(A) Assumed *sigma* << true *sigma*: When the assumed *sigma* (i.e., the *sigma* on which the dose progression is based) is much smaller than the true *sigma* of the actual test population, the estimated LD_{50} may be ''biased'' in the direction of starting dose. For example, if the starting dose is less than the true LD_{50} of the test population, the estimated LD_{50} will generally be below the true LD_{50}. Also, if the starting dose is greater than the true LD_{50} of the test population, the estimated LD_{50} will tend to be greater than the true LD_{50}. To minimize the chance of overestimating the LD_{50} due to this bias, the UDP guideline recommends a choice of starting dose just below the assumed LD_{50}.

(B) Assumed *sigma* >> true *sigma*: If the assumed sigma on which the dose progression is based is much larger than the true *sigma* of the test population, the median estimated LD_{50} can be much larger or much smaller than the true LD_{50} depending on the starting dose. In this case, the LD_{50} can be estimated only within a range. (This is Case 3 described below.)

(ii) CI. Coverage of the CI is the probability that a calculated CI encloses the true LD_{50} for an experimental sample. Because the profile likelihood method is approximate, coverage of the CI does not always correspond to its nominal value. For example, coverage falls below 95% for populations with shallow slopes and is better than 95% for populations with steep slopes. In addition, the width of the CI is limited by the dose progression chosen. Generally, no type of CI would be more narrow than the dose progression.

(iii) Response Patterns. Data gathered under the UDP fall into one of five animal response patterns. The five types of animal response patterns, referred to as Case 1 through Case 5 in the following Table 8, can

be distinguished for the purpose of describing the performance of the UDP. These cases can be distinguished by looking at the experimental outcome (survival or death) as reflected in the AOT425StatPgm Data Grid or Report windows (see paragraph (n)(18) of this guideline). In considering these cases, note that doses can be repeated more than once in the course of sequential dosing.

Table 8.—Outcomes of the UDP: Cases and Confidence Intervals

Case #	Definition of Case	Approach Proposed	Possible Findings
1	No positive dose-response association. (1a) All animals tested in the study responded, or (1b) none responded, or (1c) the geometric mean dose is lower for animals that responded than for animals that did not respond.	LD_{50} cannot be calculated. CI not applicable.	Possible inferences: (1a) LD_{50} < lowest dose; (1b) LD_{50} > highest dose; (1c) reverse dose-response curve; unlikely test outcome. In case 1b, the highest dose tested is equivalent to a limit dose.
2	Multiple partial responses. One or more animals responded at a dose below some other dose where one or more did not respond. The conditions defining Case 1 do not hold. (The definition of Case 2 holds if there are 2 doses with partial responses, but holds in some other cases as well.)	Maximum likelihood estimate and profile likelihood computations of CI are straightforward.	The LD_{50} can be estimated and its CI calculated.
3	No intermediate response fractions. One or more test doses is associated with 0% response and one or more is associated with 100% response (all of the latter being greater than all of the former), and no test doses are associated with a partial response.	Lower bound = highest test dose with 0% response. Upper bound = lowest test dose with 100% response.	High confidence that the true LD_{50} falls between the two bounding doses. Any value of LD_{50} between highest dose with 0% response and lowest dose with 100% response is equally plausible.
4	One partial response fraction, first subcase. An intermediate partial response is observed at a single test dose. That dose is greater than doses associated with 0% response and lower than doses associated with 100% response.	The LD_{50} is set at the single dose showing partial response and its CI is calculated using profile likelihood method.	The LD_{50} can be estimated and its CI calculated.
5	One partial response fraction, second subcase. There is a single dose associated with partial response, which is either the highest test dose (with no responses at all other test doses) or the lowest test dose (with 100% response at all other test doses).	The LD_{50} is set at the dose with the partial response. A profile likelihood CI is calculated and may be finite or infinite.	The true LD_{50} could be at the boundary of the testing range with more or less confidence.

(n) References. The following references should be consulted for additional background material on this test guideline.

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E.P.A. under Contract 68–W7–0025, Task Order 5-03. Currently available at web site: http://iccvam.niehs.nih.gov/methods/udpdocs/udprpt/udp_ciprop.htm

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