ECOLOGY AND THERMAL INACTIVATION OF MICROBES IN AND ON INTERPLANETARY SPACE VEHICLE COMPONENTS

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INTRODUCTION

The expression and evaluation of experimental data represent one of the more difficult tasks in all areas of science. The following manuscript, to be published in Applied Microbiology, represents a summary of some of the work done in this laboratory relative to statistical techniques of analysis which has relevance to studies on the thermal inactivation of bacterial spores.

A Comparison of Four Methods Commonly Used to Estimate LD50

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Biologists are often required to determine the potency of a drug or serum, but concentrations are difficult to measure directly by chemical methods, and some toxic products may be of unknown composition. Bioassay is a common technique used to determine the lethal effect of a substance and thus compare the results to a known standard. This technique requires that test animals (rats, pigs, cats, etc.) be injected with several doses of the substance and that the number of survivors at each dose be recorded. The number of subjects at each dose is often held constant, although this is not necessary. These data are used to estimate the mean lethal dose (LD50), or the dose at which 50 percent of the animals survive.

Ten or more methods are used to estimate the LD50. Finney (5) has discussed some of these methods, and he favors the Spearman-Kärber and moving average angle methods for small sample sizes. He states that estimates will, in general, be better by these methods in the face of skewed tolerance distributions.

Two other widely employed methods are the probit analysis (4) and the Reed-Muench method (7). The probit method is often used by workers

with some statistical training, and the Reed-Muench method is widely used by researchers trained in biological sciences. Finney (5) objects to the Reed-Muench method because, among other things, the method lacks an estimate of precision from a single experiment. Brown (2) has given an estimate of variance for the Reed-Muench method so that precision can now be compared.

The present study was designed to compare the results of the four tests -- probit analysis, moving average angle, Reed-Muench, and Spearman-Kärber. Three known tolerance distributions were employed (two symmetrical and one skewed), and a hundred random samples were generated from each. Also, four sample sizes were used, assuming 5, 10, 15, and 20 test subjects at a dose. The four tests were used to estimate LD50 and precision from the three distributions where the known mean and variance could be compared to the estimates. Several sample sizes were used. Conclusions were then drawn about methods and sample sizes.

MATERIALS AND METHODS

Statistical procedures. The tolerance of Y of any one member of a group of experimental animals of a certain species for a specific toxin is defined as that dose which would be just sufficient to cause death; with any smaller dose the animal would survive, and with any greater dose it would die. The natural logarithm x of y has a probability distribution P(x) given by

$$P(x) = \int_{-\infty}^{x} f(t)dt$$

which expresses the probability that the "log tolerance" of a randomly chosen animal does not exceed x. The probability density function of log tolerances, f(t), is generally taken to be the Gaussian or normal density, a symmetric function. The mean and median of any symmetric density coincide; the median of a log tolerance density function is equivalent to the logarithm of the median lethal or effective dose, i.e., ln (LD50). Thus, if Y_o = LD50 and $x_o = \ln (Y_o)$, then

$$P(x_o) = \int_{-\infty}^{x_o} f(t)dt = 0.5$$

An experimental animal chosen at random and administered the dose Y_o consequently has a 50% chance of survival.

The above concept is a reasonable and commonly assumed way of viewing the variation in tolerance to a toxin among the population of all animals of a given species. The existence of such a log tolerance distribution is assumed in this paper.

The four methods used in this study were adapted to the computer. Calculations for the probit are given by Finney (4), and computations used here were those of Daum et al. (3), where the computer calculates the best regression rather than the experimenter choosing values from a graph. The Spearman-Kärber method was written from calculations given by Finney (5), and the Reed-Muench method was computed as given by Reed-Muench (7), with confidence intervals given by Brown (2). Bennett (1) presented the modification of the moving average angle procedure used here, with confidence limits as shown by Harris (6).

Estimates of ln (LD50) obtained by Reed-Muench do not lend themselves to any kind of theoretical investigation as has been made for the probit, Spearman-Kärber, and moving average methods. Regarding the latter two, Finney (5) makes the following recommendations:

1. When the experimenter knows nothing about the value of the LD50 of the toxin question, he must necessarily choose a wide dose range to be sure of bracketing it. In such cases, the moving average method with a span K as large as possible is preferable to the Spearman-Kärber estimate.
2. If the experimenter is confident of bracketing the LD50 within a small range and d/σ is larger than 1.0, the two methods have comparable precision for the same number of animals per dose, n. When $d/\sigma < 1.0$, the precision of the Spearman-Kärber method is markedly superior to that of the moving average method.
3. The experimenter who intends to employ the Spearman-Kärber method is best instructed to use a few animals (small n_1) at each of many (large m) doses, rather than the same total number of animals (N) distributed between fewer doses over the same total range.

Unless n_1 is so small as to reduce the effective precision of the probit estimate on account of a large value of g, the difference between the precisions of Spearman-Kärber and probit applied to data for which each is suitable will then be negligible. Since nothing is known about the behavior of the precision of the Reed-Muench estimate for varying

values of m, n_i, and d/σ, a comparative study of this estimate with those of the other three methods was performed by simulation. The procedure followed is described in the following section. The value m is the number of doses, n_i the subjects per dose, and d the width between doses (i.e. 1 log cycle).

The simulation procedure. Three tolerance distributions were considered for this simulation. One is a $\chi^2(4)$, mean 4, $\sigma = 2\sqrt{2}$, which is skewed. Two normal distributions, which are symmetrical, were chosen also, one with mean = - 10.36161, σ = 2.47060 (normal I), and the other with mean = -10.36161, σ = 6.90780. There are 10 total cases, with subjects per dose being n_i = 5, 10, 15, and 20 for normal I and $\chi^2(4)$. Two sample sizes, n_i = 5, 15, are considered for normal II.

Values for nonsurvivals (r_i) per dose were simulated in the following manner. First, a log tolerance probability distribution F and a set of m doses Y_i were chosen. Then a sample of n_i values of X = ln y were drawn from the chosen F by means of a random number generator in the computer program. The value r_i is the number of values X in this sample which exceed X_i = ln Y_i (corresponding to r_i deaths). This procedure was repeated for each dose level. The resulting set of values r_i was then used to calculate estimates of ln (LD50) by each of the four methods. One hundred sets of randomly chosen samples were generated for the 10 cases.

The normal I case is the ideal situation for a normally distributed ln tolerance. The mean of the ln tolerance distribution (-10.36161) was chosen to be the midpoint of the dosage range, and the σ of the distribution was chosen so that the range of ln dosages was about -10.36161 $\pm$ 3 σ.

The dosages given in Table I are those values assumed to be examined under experimental conditions. In other words, one assumes that the experimenter has chosen six dosages and exactly bracketed the true mean for the normal I case.

The normal II case assumes a mean of -10.36161 and σ = 6.90780, but the dosages were chosen so that the true mean falls at the upper end of the range. This is what might happen when the experimenter tries to bracket the ln LD50 but is off somewhat. The ln tolerance distribution is centered at -10.36161 and has a 3 σ spread from -31.08501 to 10.36179 as compared to the examined range of -20.72322 to -9.21032.

Case three, $\chi^2(4)$, differs from the others in that there exists a minimum lethal dosage with an ln of zero, and the distribution is skewed or asymmetric. The mean and the median values do not coincide. Estimates from the four methods are thus examined when the true distributions are both symmetrical and asymmetrical. The true ln (LD50) = 4.00000. Discussion and results from the simulation procedure are given below.

RESULTS

Estimates were obtained for LD50 by the four methods described above. The means of 100 random sample estimates are presented in Table 2 for each of the 10 cases. Ninety-five-percent confidence limits can be computed by adding and subtracting the factor given in the table. When 100 estimates were not computed for a method and case, then the number used is presented. This was often the case for the probit analysis. An internal computational routine was used to test to see if the regression was

significant (i.e., to test the null hypothesis that $\beta_1 = 0$ in the linear regression). When the regression is not significant, the printout states this result and does not print out estimates.

The estimates of variance among LD50 values and the coefficients of variation expressed as a percent are given in Table 3. These results show the expected decrease in variation as the number (n_i) of subjects per dose increases. The coefficient of variation (CV) is satisfactory for the normal I cases, but more than 10% for all $\chi^2(4)$ results. This means that considerable variation occurs when the sampling distribution is skewed and also when the true mean is not centered in the dosage range as in normal II. Although the variance decreases with the increasing subjects per dose, $n_i = 10$ seems to be a practical number per dose to suggest.

Mean results obtained for the methods can be compared to the known means in Table 1. Spearman-Kärber tends to underestimate the mean for the normal I cases, with the other means being close. The confidence intervals for Reed-Muench, moving average angle, and probit analyses in fact include the true mean. For the normal II cases, Reed-Muench and moving average angle methods yield high results, and Spearman-Kärber is very low in its estimates. Only the probit analysis with larger sample size seems adequate. The estimates are closer for the $\chi^2(4)$ cases, with probit analysis yielding higher estimates than the value of 4.00000 and the other three estimates being lower. For $n_i = 10$, only the confidence limits of Reed-Muench and probit analysis include the true mean. The probit analysis was found to be the unbiased estimator for all cases. It also has the minimum variance for cases where $n_i \geq 10$.

DISCUSSION

The results from this simulation study indicate that probit analysis is the most desirable method ($n_i \geq 10$). There are internal tests of consistency, and the mean LD50 estimates are near the true mean (i.e., it is the unbiased minimum variance estimate). In most cases probit analysis is also the method with minimum variance. Using estimates from the other methods for the normal II case might tend to give misleading results. The Reed-Muench method does not have any internal statistical test, so although it generated an estimate for every sample, the LD50 estimates were higher than the true mean more than 90% of the time. All methods performed well for the ideal case where the true mean was centered in the dosage range examined. For the normal I, $\frac{d}{\sigma} \sim 1$ and $\chi^2(4)\ \frac{d}{\sigma} \sim 0.8$.

Another reason for estimation problem was that for normal II, $\frac{d}{\sigma} \sim 0.3$ means that the dosages did not cover the range as they must for the Reed-Muench, Spearman-Kärber, and moving average angle. For normal I, the ($\frac{d}{\sigma} \sim 1$) dosage range did cover the range from all positive and all negative responses, and the plot of estimates was symmetrical about the mean, with only a small difference between estimated and true mean. The variance estimates (Table 3) indicate that there is little improvement above 10 subjects per dose, and this number is suggested as adequate.

Two restraints were placed on the study from the outset in order to control the number of factors to be examined: First, the range of data was set as 10^n, 10^{n+1}, etc. and second, the number of doses was restricted to six. This situation represents the typical type of

experiment used by biologists and microbiologists. More doses would be too expensive, as 10 subjects per dose would require 60 for a single determination.

Thus if it is known that the tolerance distribution is symmetrical and that the doses contain the true mean, then any one of the four methods can be used. Reed-Muench and Spearman-Kärber are about the same in terms of calculation time, but if the data do not go from all positive to all negative, then estimation is poor, as shown by the normal II case. Usually, an experimenter will change his choice of doses and run the experiment again. However, the temptation may be great to use the estimate obtained, which may be far from the true mean. Thus, probit analysis should be used if possible, and certainly if a computer is available. If the experimenter employs fewer than five subjects per dose, however, probit analysis should not be used. For cases similar to normal I, the Spearman-Kärber and Reed-Muench methods give satisfactory results and are easy to compute without the aid of calculators.

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Table 1. Administered dosages for simulation tests

Normal I dose	$\mu = -10.36161$ $\sigma = 2.47060$ ln (dose)	Normal II dose	$\mu = 10.36161$ $\sigma = 6.90780$ ln (dose)	$\chi^2(4)$ dose	$\mu = 4.00000$ $\sigma = 2.82800$ ln (dose)
1×10^{-7}	-16.11806	1×10^{-9}	-20.72322	1×10	0.00000
1×10^{-6}	-13.81548	1×10^{-8}	-18.42064	1×10^{1}	2.30258
1×10^{-5}	-11.51290	1×10^{-7}	-16.11806	1×10^{2}[a]	4.60516
1×10^{-4}[a]	- 9.21032	1×10^{-6}	-13.81548	1×10^{3}	6.90774
1×10^{-3}	- 6.90774	1×10^{-5}	-11.51290	1×10^{4}	9.21032
1×10^{-2}	- 4.60516	1×10^{-4}[a]	- 9.21032	1×10^{5}	11.51290

[a] True mean lies between these two doses.

Table 2. Estimates of mean LD50's from the 100 random samples

Source	Reed-Muench	Spearman-Karber	Moving Average Angle	Probit
Normal I:[a]				
N = 5	-10.33349 ± 0.16679[b]	-10.04157 ± 0.22781	-10.32695 ± 0.16872	-10.38702 ± 0.17144 (85)[c]
N = 10	-10.35279 ± 0.11628	-10.14980 ± 0.14019	-10.33582 ± 0.11516	-10.33418 ± 0.11434 (99)
N = 15	-10.37336 ± 0.08639	-10.18740 ± 0.11896	-10.65947 ± 0.09176	-10.37356 ± 0.08917 (99)
N = 20	-10.30348 ± 0.07282	-10.09108 ± 0.10266	-10.51842 ± 0.08534	-10.31207 ± 0.07677
Normal II:[d]				
N = 5	-11.80114 ± 0.22472	- 6.91927 ± 0.68838	-11.71748 ± 0.31721 (67)	-11.20921 ± 0.61838 (35)
N = 15	-11.87198 ± 0.13335	- 7.34294 ± 0.39919	-11.70052 ± 0.22833 (77)	-10.07605 ± 0.28324 (79)
$\chi^2(4)$:[e]				
N = 5	3.84638 ± 0.19961	3.56670 ± 0.19442	3.67745 ± 0.18481	4.07458 ± 0.24603 (60)
N = 10	3.90938 ± 0.11934	3.82575 ± 0.13805	3.78520 ± 0.11758	4.08456 ± 0.11577 (88)
N = 15	3.89531 ± 0.08365	3.76780 ± 0.11251	3.57183 ± 0.08910	4.07321 ± 0.08964 (88)
N = 20	3.94329 ± 0.09226	3.75012 ± 0.11571	3.69288 ± 0.42997 (99)	4.12716 ± 0.09766 (90)

a
True mean of distribution sampled = -10.36161.

b
This factor, $\frac{S \cdot t_{\nu,0.975}}{\sqrt{n}}$, when added and subtracted from the mean, gives 95% confidence limits.

c
Figures in parentheses indicate the number of observations when less than 100.

d
True mean of distribution sampled = -10.36161.

e
True mean of distribution sampled = 4.00000.

Table 3. Variance of LD50's estimated from the 100 random samples

Source	Reed-Muench		Spearman-Karber		Moving Average Angle		Probit	
	Variance	CV[a](%)	Variance	CV(%)	Variance	CV(%)	Variance	CV(%)
Normal I:								
N = 5	0.70038	8.1	1.30662	11.4	0.71664	8.2	0.63406 (84)[b]	7.7
N = 10	0.34042	5.6	0.49477	6.9	0.33388	5.6	0.32589 (98)	5.5
N = 15	0.18791	4.2	0.35630	5.9	0.21197	4.3	0.19820 (98)	4.3
N = 20	0.13349	3.5	0.26533	5.1	0.18335	4.1	0.14840	3.7
Normal II:								
N = 5	1.27143	9.6	11.93021	49.9	1.71629 (66)	11.2	2.46171 (34)	14.0
N = 15	0.44770	5.6	4.01184	27.3	1.01886 (77)	8.6	1.09037 (78)	10.4
$\chi^2(4)$:								
N = 5	1.00314	26.0	0.95166	27.4	0.85991	25.2	0.92650 (59)	23.6
N = 10	0.35859	15.3	0.47980	18.1	0.34805	15.6	0.29816 (87)	13.4
N = 15	0.17615	10.8	0.31872	15.0	0.19988	12.5	0.17877 (87)	10.4
N = 20	0.21432	11.7	0.33705	15.5	0.18488	11.6	0.21696 (89)	11.3

[a] Coefficient of variation = $\frac{100\ S}{\bar{x}}$

[b] Figures in parentheses indicate degrees of freedom for variance when less than 99.