Brit. J. Pharmacol. (1964), 22, 392–402.

OBSERVATIONS ON THE APPLICATION OF THE ROBBINS-MONRO PROCESS TO SEQUENTIAL TOXICITY ASSAYS

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(*Received December* 18, 1963)

The Robbins-Monro process is a sequential procedure which can be used in toxicity assays. The principal advantages are maximal economy of drug and animals and immediate availability of an estimate of the LD50 at any stage in the assay. The disadvantages are the need to wait for the outcome in each group of animals before testing the next group and the lack of an accurate method for determining confidence limits. Some practical details of the application of the method are given.

The " up and down " or " staircase " method of obtaining sensitivity data was described by Dixon & Mood in 1948. Brief accounts are also given by Dixon & Massey (1957) and Finney (1952). The principle of the method is that stimulus strength is increased in stepwise fashion until a positive response is obtained. Thereafter the strength of the stimulus is decreased or increased by one step according to whether or not a positive response was obtained at the preceding test. The primary advantage of the procedure over methods for assessment of quantal responses which employ predetermined test levels is that it automatically concentrates testing near the mean. This results in an increase in the accuracy with which the mean can be estimated. Dixon & Mood (1948) suggest that the up and down method requires 30 to 40% fewer observations than the ordinary method of testing groups at previously assigned stimulus levels, for the same degree of accuracy.

Dixon & Mood (1948) also showed that the optimal step size in the up and down method is approximately equal to one standard deviation. They developed procedures for estimating the variance of the mean result obtained. Brownlee, Hodges & Rosenblatt (1953) have since shown that the Dixon-Mood formula for the asymptotic variance is reliable in small samples. The principal disadvantage of the up and down method is that for many purposes it is more time-consuming than the usual methods, since the result of each test must be awaited before the next can be planned. Brownlee *et al.* (1953) described a way of reducing the time taken by breaking up the main series of tests into shorter series run in parallel. This modification results in some loss of precision but can be exploited to check on concomitant variables, by using a different batch of test objects for each sub-series.

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The up and down method has been successfully applied to toxicity assays by Rümke (1959). He discusses the difficulty of assessing confidence limits when the modification of Brownlee *et al.* (1953) is employed. Error limits may be estimated by applying the Dixon-Mood procedure to each sub-series, or by pooling the data and using probit analysis, as suggested by Finney (1952). The up and down procedure has considerable advantages in toxicity assays for which amount of drug and number of animals available are at a premium.

In 1951, Robbins & Monro described a method of performing a sequential process of the type under consideration in which the step size is steadily reduced. This is done in such a way that the sequence of stimulus strengths employed is convergent, tending to the true mean value (see Appendix). When the Robbins-Monro process is employed the stimulus strengths are successively closer approximations to the mean, instead of oscillating regularly in the region of the mean, as in the simple up and down procedure. This can result in an even greater economy of tests (Wetherill, 1963). An estimate of the answer is available after each test; the stimulus strength which would have been employed for the next test can be used as an estimate of the mean. In addition, the Robbins-Monro method has the advantage that several animals may be used for each test. The information available for computation of the next step may then be derived from a group of animals.

In the present work, some practical experiences of the application of the Robbins-Monro process to toxicity assays are described.

METHODS

Female white mice weighing between 12 and 20 g were used. An aqueous solution of the drug employed was injected into the tail vein. Injection volumes were usually kept within the range 0.05 to 0.2 ml. and no mouse was given more than 0.02 ml./g of body weight. Injections were given over 5 to 10 sec, the rate being varied in proportion to the injection volume. No mice given control injections of distilled water or 0.9% saline under these conditions died.

Drugs. *N*-Methylveratramine methiodide was used for the majority of the experiments reported here. This compound had the advantage for the present purpose that mice given a lethal intravenous dose always died within 2 min. No deaths occurred within 24 hr among several hundred mice injected with doses of the compound and surviving more than 2 min.

Several different samples of the compound were used. They varied a little in purity and consequently in potency. Estimates of the relevant parameters from the samples employed, based on a retrospective analysis of the data, are shown in Table 1.

TABLE 1

PARAMETERS FROM SAMPLES OF *N*-METHYLVERATRAMINE METHIODIDE

Values in parentheses are 95% confidence limits

Sample	LD50 in mice (mg/kg)		Standard deviation	
A	9·03	(8·50–9·59)	0·060	(0·041–0·109)
B	9·70	(8·73–10·74)	0·056	(0·035–0·150)
C	9·87	(9·37–11·59)	0·096	(0·055–0·208)
D	8·64	(8·34–9·80)	0·034	(0·019–0·175)

Veratramine hydrochloride and samples of a number of hydrochlorides of the *N*-alkyl derivatives of this alkaloid (Uhle, Krueger & Sallmann, 1960) were used in other experiments. Characteristically, lethal doses of these compounds were effective within 1 hr.

Procedure. The basic procedure for testing a compound of unknown characteristics is as follows:

An advance estimate of the log of the population LD50, μ, and of the population standard deviation, σ, is first made. It may be that no information about these parameters is available from previous experiments or tests on related drugs. In these circumstances two or three experiments on single mice with a range of doses usually serve to give the very rough idea of μ which is required. The advance estimate of σ will be helped by the reviews by Gaddum (1933) and Bliss & Cattell (1943). In three-quarters of the wide variety of toxicity experiments reviewed by these authors, the estimate of σ lies within the range 0.05 to 0.2 on a log scale. In the complete absence of prior information about the behaviour of a new drug, a value of 0.1 provides a reasonable advance estimate for σ.

Let x_1 be the advance estimate of μ, and s the advance estimate of σ. Test a group of animals with the dose of which the log is x_1. Let p_1 be the proportion that die, and calculate $x_2=x_1-\sqrt{2\pi}s(p_1-\frac{1}{2})$. Then test a second group of animals with the dose of which the log is x_2. Similarly $x_3=x_2-\frac{\sqrt{2\pi}\,s}{2}(p_1-\frac{1}{2})$, etc. If the proportion that responds on the nth trial is p_n, then : $x_{n+1}=x_n-\frac{\sqrt{2\pi}s}{n}(p_n-\frac{1}{2})$. If the experiment is concluded after k trials, the best estimate of the LD50, antilog μ, is antilog x_{k+1}.

If the record of the type shown in Table 2 is kept during the experiment, the calculations involved are simple and readily performed with the aid of a table of logarithms and a slide rule. Tables or nomograms relating dose (in mg/kg), weight of animal, and volume to be injected, for drug concentrations 0.1, 0.2, 0.4, 0.8 and 1.0 mg/ml., are of great value in promptly determining the dose to be injected for each animal after it has been weighed.

TABLE 2

SEQUENTIAL TOXICITY ASSAY OF *N*-BUTYLVERATRAMINE

$$x_{n+1}=x_n-\frac{1\cdot253}{n}(p_n-\tfrac{1}{2})$$

* Best estimate of LD50

Step number (n)	Dose (mg/kg)	Log dose	Mice			Log dose (n+1)	Dose (n+1) (mg/kg)
			Tested (m)	Dead (k)	Proportion (p_n)		
1	50·0	1·699	3	3	1·0	1·073	11·83
2	11·8	1·073	3	3	1·0	0·760	5·75
3	5·75	0·760	3	3	1·0	0·551	3·56
4	3·56	0·551	3	0	0·0	0·708	5·11
5	5·11	0·708	3	3	1·0	0·583	3·83
6	3·83	0·583	5	2	0·4	0·604	4·02
7	4·02	0·604	5	2	0·4	0·622	4·19*

An example of the application of the method to the assay of a drug (*N*-butylveratramine) about which no prior information was available is shown in Table 2.

The use of a reduction factor (constant/n) produces a sequence of the type required by the Robbins-Monro process. The particular numerator, $\sqrt{2\pi}s=2.506s$, is selected to minimize the variance of the final estimate of μ (see Appendix).

RESULTS

Effect of poor advance estimate of LD50. Use of the "delayed" rule

An experiment with *N*-methylveratramine methiodide is illustrated in Fig. 1,*a*. The true LD50 was known to be approximately 9.03 mg/kg, and the standard

deviation to be about 0.069. Using groups of three mice, the trial was started with a dose of 4.08 mg/kg, 5s away from the LD50. It will be seen that with this advance estimate of the LD50, the results tend to the true value so slowly as to render the method of little practical use.

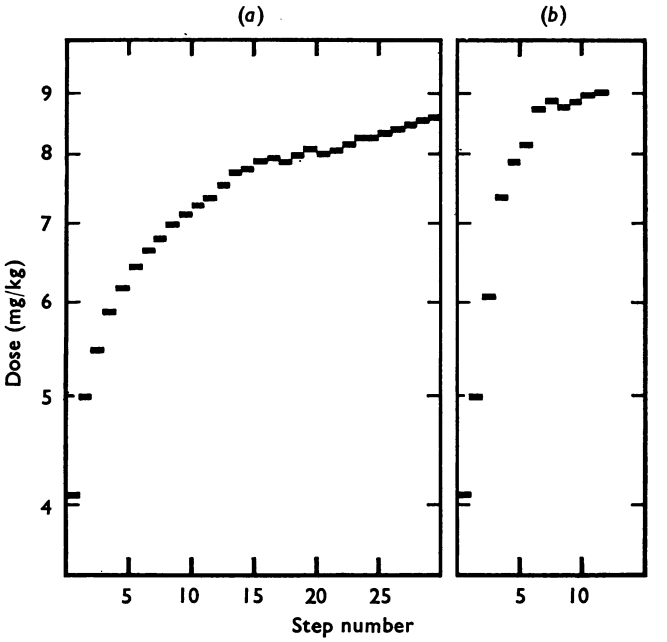

Fig. 1. Sequential toxicity assays of *N*-methylveratramine methiodide (sample B), using groups of three mice. (*a*), Robbins–Monro process applied from the start. (*b*), application of "delayed rule"; reduction factor employed after first mouse killed. See text for further explanation.

In Fig. 1,*b* a similar experiment is illustrated in which a modification was introduced so that a constant step size of $\sqrt{2\pi}s$ was employed until a dose-level was reached at which at least one mouse was killed (see Cochran & Davis, 1963a). The dose was then modified according to the Robbins-Monro process, using the reduction factor $\frac{\sqrt{2\pi}s}{n}$. It will be seen from Fig. 1,*b* that the effect of delaying the introduction of progressive reduction of step size in this way is to expedite greatly the achievement of a good estimate of the LD50. The protocol of the experiment in Fig. 1,*b* is shown in Table 3.

The above modification of using stepwise alterations in dosage, as in the up and down method, until at least one mouse is killed, and then introducing the Robbins-Monro process, will be referred to as the application of the "delayed" rule. Of course, if the initial dose kills all the test animals, introduction of reduction of step size is delayed until the dose has been reduced so that at least one mouse survives.

TABLE 3

SEQUENTIAL TOXICITY ASSAY OF *N*-METHYLVERATRAMINE METHIODIDE, USING THE "DELAYED" RULE

Steps 1a to 4a: $x_{n+1}=x_n-0{\cdot}173\ (p_n-\frac{1}{2})$

Steps 1 to 8: $x_{n+1}=x_n-\frac{0.173}{n}(p_n-\frac{1}{2})$

* Best estimate of LD50

Step number (n)	Dose (mg/kg)	Log dose	Mice			Log dose (n+1)	Dose (n+1) (mg/kg)
			Tested (m)	Dead (k)	Proportion (p_n)		
1a	4·08	0·611	3	0	0	0·697	4·98
2a	4·98	0·697	3	0	0	0·784	6·08
3a	6·08	0·784	3	0	0	0·871	7·43
4a; 1	7·43	0·871	3	1	0·333	0·900	7·94
2	7·94	0·900	3	1	0·333	0·914	8·20
3	8·20	0·914	3	0	0	0·943	8·77
4	8·77	0·943	3	1	0·333	0·950	8·91
5	8·91	0·950	3	2	0·667	0·944	8·79
6	8·79	0·944	3	1	0·333	0·949	8·89
7	8·89	0·949	3	1	0·333	0·953	8·97
8	8·97	0·953	3	1	0·333	0·957	9·06*

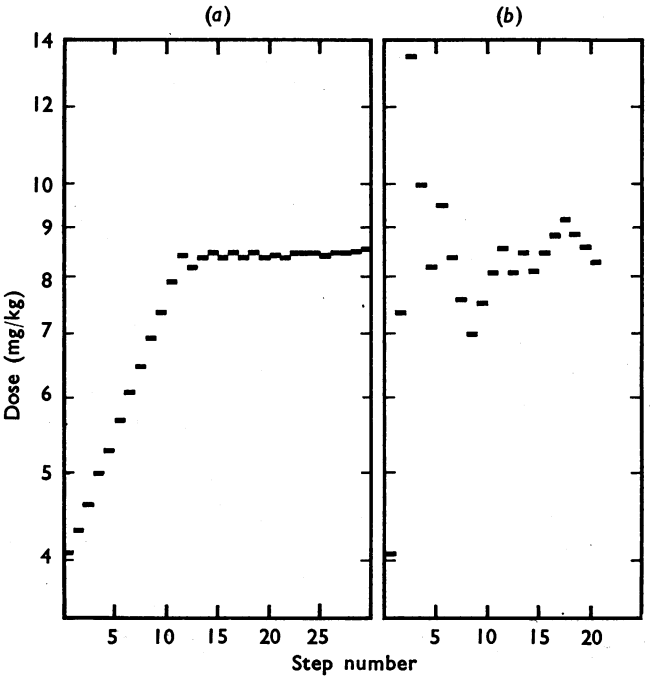

Fig. 2. Toxicity assays of *N*-methylveratramine methiodide (sample D), using single mice sequentially. Effect of poor advance estimates of the standard deviation. (*a*), s approximately one-third of true value. (*b*), s approximately three-times true value.

Effect of poor advance estimate of the standard deviation

The effects of threefold errors in the advance estimates of σ are shown in Fig. 2. In these experiments with *N*-methylveratramine methiodide, the value of σ, the population standard deviation, was thought to be approximately 0.069. In the experiment of Fig. 2,*a* a threefold underestimate was employed, while in that of Fig. 2,*b* a threefold overestimate was used. The delayed rule was applied.

It will be seen that the situation after twelve to fourteen steps was much the same, though thereafter the effect of underestimation (Fig. 2,*a*) was the more desirable. In both cases suspicion of the inaccuracy of the advance estimate arose after as few as four or five steps. Helpful modifications might have been made at this point. The advance estimate of σ could have been increased when no animals had died after four or five steps in the experiment of Fig. 2,*a*. The estimate of σ could have been decreased directly it was apparent that wide oscillation was occurring in the experiment of Fig. 2,*b*.

Effect of the number of animals in the group

The experiments in Fig. 3 were conducted using the delayed rule and a good estimate of σ. In Fig. 3,*a*, *b* and *c*, single mice, groups of three mice and groups of nine mice, respectively, were used. It will be seen that with groups of three, the

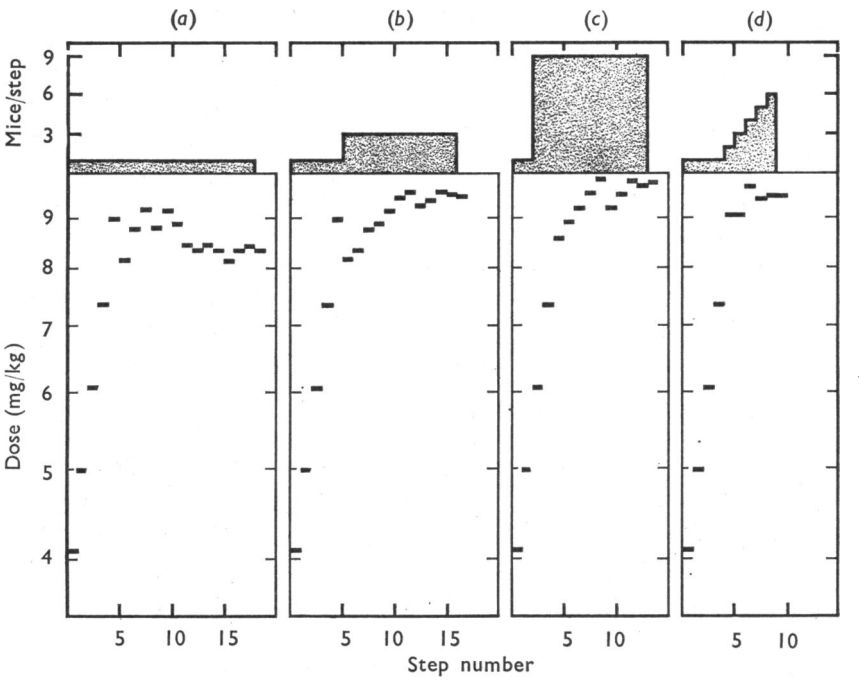

Fig. 3. Sequential toxicity assays using *N*-methylveratramine (sample C). Effect of altering m, the number of mice per group.

dose level tended more rapidly towards a good estimate of the LD50 than with the single mice. Little further advantage appears to be gained by increasing the group size to nine.

In the experiments of Fig. 3,*d*, there appears to be some advantage in progressively increasing the size of the groups. Computations by Cochran & Davis (1963b) indicate that in experiments involving twenty to fifty animals the standard error of the log LD50 depends primarily on the product of the number of animals per step and the number of steps (see also Appendix), provided that the first guess at log LD50 is within 2σ of the correct value. If he is reasonably sure that this is so, the experimenter may save time by increasing the number of animals per step. With a starting point that is further out, a larger number of steps with fewer animals per step gives superior estimates.

The form of the reduction factor, $\frac{\sqrt{2\pi}s}{n}$

It has been shown (see Appendix) that, if the log of the "just fatal" dose is normally distributed, the optimal form for the factor by which the step size is reduced is $\sqrt{2\pi}\frac{\sigma}{n}=2.506\frac{\sigma}{n}$, where σ is the population standard deviation and n the step number. If, on the other hand, the tolerance follows the logistic distribution, then the optimum factor is $\frac{4\sqrt{3}}{\pi}\cdot\frac{\sigma}{n}=2.205\frac{\sigma}{n}$

As the former factor was used in the present work it was of some interest to confirm that the variance of estimates of μ determined experimentally, approximated reasonably closely to that which would be expected if the distribution was normal. For this purpose twenty sequential trials were conducted, each of which had six steps with one mouse per step. The experiments were performed in three sets, four trials on the first day, and eight on each of two subsequent days. The drug used was *N*-methylveratramine methiodide (sample A in the first two sets, sample B in the third set). The initial estimates of μ and σ were derived from a preliminary probit assay, and each trial was initiated with a dose approximately one standard deviation greater than the LD50. The delayed rule was not used.

The analysis of variance of the log LD50 in the trials was as follows:

	Degrees of freedom	Sum of squares	Mean square
Between sets	2	0.005567	0.002783
Within sets	17	0.025465	0.001498

The "between sets" mean square is rather higher than that of the "within sets," due to day-to-day variation and to variation between samples A and B of the drug. The "within sets" mean square is the appropriate figure for a check on the theoretical formula for the variance of the LD50 from a single experiment.

On the assumption that the distribution is normal, the asymptotic formula for the variance of the LD50 (on the log scale) is $\frac{\pi\sigma^2}{2nm}$, where n is the number of steps

and n the number of mice per step (see Appendix). The estimate of σ, taken from the preliminary probit trial, was 0.0689. Substituting, the asymptotic formula gave an expected variance of 0.001242, as against the experimental real error of 0.001498. The agreement is remarkably good, considering that the asymptotic formula tends to underestimate the variance, and that it assumes that the best step size was used, that in fact used being merely an estimate of the best size derived from the probit assay.

It therefore seems that the assumption that the tolerance distribution is normal is reasonable.

DISCUSSION

Sequential assays using the Robbins-Monro process

In order to gain some practical experience of the method a number of sequential toxicity assays were performed using veratramine and related compounds. The actual toxicity estimates will be reported elsewhere (Hawkins, Uhle & Krayer, unpublished).

The Robbins-Monro method has the inherent disadvantage of all sequential procedures in that it is necessary to await the answer to one set of tests before proceeding to the next. In practice it was found that no real waste of time was involved.

During tests of the intravenous toxicity of a drug like *N*-methylveratramine methiodide, the animals could be assessed as either dead or surviving within 2 min of the injection and no problem arose. With the aid of tables of dose against weight of animals and a slide rule, and use of a protocol like those shown in Tables 2 and 3, computation of the doses for a group of animals took very little time. The calculations and the weighing and injection of a group of mice took between 10 and 20 min. An assay with a reasonable number of steps could easily be performed in half-a-day.

With drugs like veratramine itself, where the effect could only be assessed an hour after injection, an assay of about eight steps took all day. In these circumstances it was found convenient to perform two or three assays in parallel with different compounds. By the time a group of animals from each assay had been dealt with, the results from the first group were available, and the next step in the first assay could be assessed. With drugs which take longer to kill, it may take 24 hr before results for a group of animals can be assessed. In this case the simplest procedure is to inject a group of animals each day. It may be a week or 10 days before the result of a given assay is available, but if a series of drugs is being tested no real loss of experimental time is incurred.

The conditions under which the method was tested using the veratramine derivatives were not optimal. The initial estimates of the standard deviation were poor, the amount of drug available was often small, and the delayed rule was not employed. Even with these adverse factors, it appeared possible to obtain reasonable estimates of the LD50's of the compounds using small numbers of animals, as few as fifteen or twenty mice, as would be expected on theoretical grounds (Cochran & Davis, 1963a, b). Considerable economy in the amount of drugs used resulted.

Against this economy of animals and drugs must be discounted the paucity of information available for determining accuracy and confidence limits by the classical methods. The Robbins-Monro process has the effect of grouping the test dose-levels close to the LD50, resulting in a relative increase in accuracy of determination of the positional parameter. At the same time the amount of data relating to test dose-levels some distance from the mean is drastically reduced, and consequently the slope of the dose/mortality curve is poorly estimated. If, for example, probit analysis is employed, three or four cycles of calculation may be required before even a reasonable approximation to the slope of the curve is obtained, and the error limits of the slope constant are wide. The increased accuracy of determination of the positional parameter compensates for this in the final calculation of the confidence limits for the LD50, but the actual computations are tedious. The data are often not susceptible to treatment by simplified methods such as that of Litchfield & Wilcoxon (1949).

In practice, a certain amount of skill is acquired in modifying the initial estimates of μ and σ, on the basis of a few results, perhaps with single mice. The ease with which these adjustments can be made contrasts with the usual type of toxicity assay, with which the operator is committed to prior estimates of the LD50 and dose-interval which, if inaccurate, will render the assay imprecise.

Design of assays and assessment of error

It should be possible to design, in terms of number of animals per group and number of groups, a procedure which will give maximal accuracy in minimal time for a given expenditure of animals and drug. In some cases no information about the compound will be available, in others well-informed estimates of μ and σ may be obtainable. The best design will vary according to how much is known about the drug at the start (see Appendix).

This type of development is best performed using electronic computers (Wetherill, 1963 ; Cochran & Davis, 1963a, b) rather than in the laboratory, and an approach to the problem along these lines is in progress. The other aspect of the method which is being studied is the development of a simple method for obtaining approximate estimates of confidence limits for LD50 values determined using the Robbins-Monro process.

I am grateful to Dr J. L. Hodges, Jr., and Professor P. B. Dews for drawing to my attention the possibility of applying the Robbins-Monro process to toxicity assays, to Dr F. C. Uhle for the veratramine derivatives, and to Professor W. G. Cochran who has given me invaluable advice, assistance and encouragement at all stages of the investigation. The work was supported by Grant HE-02205 from the U.S. Public Health Service and by funds from the Eugene Higgins Trust.

APPENDIX

Robbins & Monro (1951) proposed a method of making experiments at stimulus levels $x_1, x_2, \ldots x_n$, chosen according to the rule:

$$x_{n+1}=x_n-a_n(y_n-\alpha)$$

where y_n is the observed response to stimulus x_n, and α is the required response level. In the case of a toxicity assay α would be 0.5, corresponding to 50% mortality.

If the sequence $\{a_n\}$ is of the form c/n where c is any positive constant, then x_n is a consistent estimator of the required response-level, though not necessarily the most efficient estimator.

The *efficiency* of the sequence of doses $\{x_n\}$ depends on the choice of x_1, the nature of the function which represents the distribution of "just fatal" doses, and the sequence $\{a_n\}$. Results by Robbins & Monro (1951), Chung (1954) and Hodges & Lehmann (1956) showed that as n tends to infinity, x_n becomes normally distributed with mean, μ, and a variance which may be expressed as u/n. Clearly, optimal efficiency is obtained when u is minimized. Hodges (1953, personal communication) and Cochran & Davis (1963b) have demonstrated that, if a_n=constant/n, this is achieved for a normal tolerance distribution by making the sequence $\{a_n\}=\left\{\frac{\sqrt{2\pi}\,\sigma}{n}\right\}=\left\{\frac{2.506\,\sigma}{n}\right\}$, where σ is the standard deviation of that distribution. The corresponding a_n for a logistic distribution is $\left\{\frac{4\sqrt{3}\,\sigma}{\pi n}\right\}=\left\{\frac{2.205\,\sigma}{n}\right\}$. In practice σ is not known accurately, only an advance estimate, s, being available. The efficiency of $\{x_n\}$ depends on the choice of s.

The limiting or asymptotic variance of the final estimate of μ is u/n. From the work of Hodges & Lehmann (1956), it follows that the value of u/n is given by $\frac{\pi\,\sigma^2}{2mn}=\frac{1.571\,\sigma^2}{mn}$ for a normal tolerance distribution, where m is the number of animals tested at each step. The value for a logistic distribution is $\frac{12\sigma^2}{\pi^2 mn}=\frac{1.216\sigma^2}{mn}$.

It is of considerable interest to note that, in theory, when σ is a known quantity the asymptotic variance depends directly on mn, the total number of animals used, rather than on the number of steps. Cochran & Davis (1963b) have shown that in practice the closeness to the theoretical variance which is achieved is very dependent on how well the initial estimates of μ and σ are selected. Optimal experimental design is achieved when values of m and n are chosen giving due consideration to how much error there is likely to be in the initial estimates of the parameters μ and σ

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