AN EXPERIMENTAL DESIGN FOR SLOPE-RATIO ASSAYS

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1. Summary. When the response to a drug is a linear function of arithmetic dosage units, the relative potency of two preparations can be computed as a slope-ratio assay. Their dosage-response curves are computed by solving three simultaneous equations to obtain the common intercept a', the slope of the standard, b_1, and the slope of the unknown, b_2. The method is applicable to certain microbiological assays for the vitamins. Usually several unknowns are assayed at one time with a single standard. Their calculation is simplified when such assays meet the following requirements: (1) restriction of treatments to the zone within which the response is related linearly to the dose, (2) equal spacing of doses on an arithmetic scale beginning with the negative control, (3) an equal number (k) of doses of standard and of each unknown and (4) r replicates for each dose of unknown, h' replicates for the negative control and h replicates for each dose of the standard.

2. Method of Analysis. The design and analysis of assays for measuring drug potency has been developed largely about the linear relation between response and the logarithm of the dose of many drugs. An alternative procedure is available when some measure of the response is related linearly to arithmetic dosage units. Recently Finney [5] has applied the technique to microbiological assays of the vitamins. The relationship is also suitable for experiments with toxic agents on micro-organisms, where the length of exposure to treatment is the dose. Since potency is measured from the ratio of the slope of the dosage-response curve for an unknown to that for the standard preparation, Wood [6] has termed the method a "slope-ratio assay."

The validity of quantitative biological assays depends upon a qualitative similarity between the standard and the active agent of the unknown. When the response is related linearly to the log-dose, this is determined by testing the parallelism of the lines fitted separately to the results for the standard and to those for the unknown preparation. If the departure from parallelism is within the sampling error, the combined slope is determined from the data on both preparations and used in computing potency and its error. The analogous test in slope-ratio assays is the convergence of the lines relating response to arithmetic dose at zero content of drug, using drug as a generic term which includes vitamins, poisons and physical agents. When the curves for the standard and the unknown are computed separately, their zero intercept should agree within the experimental error. In assays meeting this requirement, the curves are computed so that they are forced to intersect at zero dose. The curves

$$y_1 = a' + b_1 x_1$$

and

$$y_2 = a' + b_2 x_2$$

are fitted by solving three simultaneous equations to obtain the three statistics, a', b_1 and b_2 which are the best estimates of their respective parameters. Finney [5] has illustrated the technique with data from the microbiological assay of nicotinic acid and given a suitable test for convergence as well as the error of the estimated potency.

The calculation described by Finney is flexible but not adapted for routine use. With certain restrictions in design, the calculation can be reduced to a practicable form for the assay of $(m - 1)$ unknowns against a standard preparation. These restrictions are as follows:

1. Doses both of standard and of unknowns must fall within the range for which some function of the response is related linearly to an arithmetic scale of dosage units with convergence at zero dose.

2. Within this range the doses (x) of standard and of all the unknowns must be spaced similarly and preferably equally on an arithmetic scale, beginning with the negative control $(x = 0)$.

3. The doses of each unknown must match those of the standard in respect to both number (k) and their expected potencies, so far as the latter can be judged in advance. Within an assay group there may be h' replicates of the negative control, h replicates of each dose of the standard and r replicates of each dose of each unknown.

4. Some element of randomization must be introduced within an assay group in respect to the preparation of the tubes, their handling and the reading of the results. Replicates of any given dose or of the negative control must not be prepared together.

3. Computational Procedure. The simplified calculation of potency and its error depends upon substituting the assumed for the actual doses. When spaced equally on an arithmetic scale, they may be coded by using the numbers 1, 2, 3, $\cdots k$, k being equal throughout the assay. The sums of the coded doses, S_1, and of their squares, S_2, are then the same for each preparation and may be entered in the equations for computing the inverse matrix, of which the first three are

$$\begin{array}{lll} & i = 0 & i = 1 \quad i = 2 \\ Nc_{0i} + hS_1c_{1i} + rS_1c_{2i} + \cdots = & 1, & 0, \quad 0, \cdots \\ hS_1c_{0i} + hS_2c_{1i} = & 0, & 1, \quad 0, \cdots \\ rS_1c_{0i} + rS_2c_{2i} = & 0, & 0, \quad 1, \cdots \end{array} \tag{1}$$

where the total number of observations is $N = h' + kh + kr(m - 1)$. Multiplying the last two rows by $-S_1/S_2$ and adding the products, we have

$$\left\{N - \frac{hS_1^2}{S_2} - (m-1)\frac{rS_1^2}{S_2}\right\} c_{0i} = 1, \qquad -\frac{S_1}{S_2}, \qquad -\frac{S_1}{S_2}, \cdots$$

where the subscript $_1$ refers to the standard and the assay includes $_2$ to $_m$ unknown preparations. Substituting

$$D = NS_2 - hS_1^2 - r(m-1)S_1^2,$$

this leads to the following reciprocal coefficients:

$$\begin{aligned}
c_{00} &= S_2/D \\
c_{0i} &= c_{i0} = -S_1/D, && i = 1, 2, \cdots m. \\
c_{11} &= 1/hS_2 + S_1^2/DS_2 \\
c_{ii} &= 1/rS_2 + S_1^2/DS_2, && i = 2, 3, \cdots m, \text{ and} \\
c_{ij} &= S_1^2/DS_2 && \text{for } i, j = 1, 2, \cdots m, \text{ where } i \neq j.
\end{aligned}$$

The reciprocal coefficients are computed from the sums of the doses and their squares, which are the same for all preparations. The doses are multiplied by the responses observed at each dosage level to obtain $T_i = S(xy_i)$ for any given preparation. For the standard there will be h responses at each dose and for each unknown r responses. Let $T = S(T_i)$ be the sum of these products over all m preparations. The total response for all N observations $S(y)$, including the negative control, the standard, and all the unknowns, is designated as T_y.

Using normal regression theory, the common intercept is computed as

$$a' = c_{00}T_y + c_{0i}T.$$

Substituting the above reciprocal coeficients,

$$a' = (S_2T_y - S_1T)/D. \tag{2}$$

The slope of the standard is computed with the reciprocal coefficients as

$$b_1 = c_{01}T_y + c_{11}T_1 + c_{1i}T - c_{1i}T_1.$$

We may take advantage of the identities

$$c_{01} = -\frac{S_1}{S_2}c_{00} \quad \text{and} \quad c_{1i} = -\frac{S_1}{S_2}c_{0i}$$

to obtain

$$b_1 = (c_{11} - c_{1i})T_1 - \frac{S_1}{S_2}a'$$

reducing to

$$b_1 = \frac{T_1}{hS_2} - \frac{a'S_1}{S_2}. \tag{3}$$

Similarly the slope of each unknown is equal to

$$b_i = c_{0i}T_y + c_{1i}T_1 + c_{ii}T_i + c_{ij}T - c_{ij}\{T_1 + T_i\}$$

where $i, j = 2, 3, \cdots m$ and $j \neq i$. Since $c_{1i} - c_{ij} = 0$, this may be reduced to

$$b_i = \frac{T_i}{rS_2} - \frac{a'S_1}{S_2}, \qquad i = 2, 3, \cdots m. \tag{4}$$

The computation is further simplified if the k doses of all preparations are spaced not only similarly on an arithmetic scale but also at equal intervals. In this case

$$S_1 = k(k + 1)/2 \qquad \text{and} \qquad S_2 = k(k + 1)(2k + 1)/6.$$

Substituting in equations (2), (3) and (4), the common intercept, the slope of the standard and that of each unknown may be computed as

$$a' = \frac{2(2k + 1)T_y - 6T}{N(k - 1) + 3h'(k + 1)} \tag{5}$$

$$b_1 = \frac{3}{2k + 1}\left\{\frac{2T_1}{hk(k + 1)} - a'\right\} \tag{6}$$

$$b_i = \frac{3}{2k + 1}\left\{\frac{2T_i}{rk(k + 1)} - a'\right\}. \tag{7}$$

In computing the slope for each unknown in an assay the only variable is T_i. The intercepts and the slope can be checked by substitution in the equation

$$2Na' + hk(k + 1)b_1 + rk(k + 1)(b_2 + \cdots + b_m) = 2T_y. \tag{8}$$

In terms of coded doses, the potency of an unknown (i) relative to that of the standard ($_1$) is computed as

$$J'_i = \frac{b_i}{b_1}. \tag{9}$$

Each J' is converted to original units by multiplying it by the ratio of the dosage intervals, I_s/I_u, the potency being

$$J = \frac{b_u I_s}{b_s I_u}. \tag{10}$$

The variance measuring the distribution of the observations about the m lines may be determined as

$$s^2 = \frac{S(y^2) - a'T_y - b_1T_1 - \cdots - b_mT_m}{N - m - 1}. \tag{11}$$

The variation about the individual lines is assumed not to vary from one preparation to another. This is more likely to be true when the assumed potencies differ but little from those computed from the assay, so that J' differs relatively little from unity.

The confidence limits for potency as estimated from the ratio of the slopes may be computed from Fieller's basic formula [4]. For confidence limits, X_L,

at an appropriate level of significance, such as $P = 0.05$, t is read from the Student-distribution for $N - m - 1$ degrees of freedom and entered with s^2 from equation (11) in the equation

$$(12) \qquad X_L^2(b_1^2 - c_{11}s^2t^2) - 2X_L(b_1b_i - c_{1i}s^2t^2) + (b_i^2 - c_{ii}s^2t^2) \leq 0,$$

where i indicates one of the 2 to m unknown preparations. When solved for 0, the limits may be written

$$(13) \qquad X_L = \frac{b_1b_i - c_{1i}s^2t^2 \pm st\sqrt{(c_{11} - c_{1i})b_i^2 + (c_{ii} - c_{1i})b_1^2 + c_{1i}(b_1 - b_i)^2 - (c_{11}c_{ii} - c_{1i}^2)s^2t^2}}{b_1^2 - c_{11}s^2t^2}$$

where $c_{11} - c_{1i} = 1/hS_2$, $c_{ii} - c_{1i} = 1/rS_2$ and $c_{11}c_{ii} - c_{1i}^2 = \dfrac{(r + h)S_1^2 + D}{rhDS_2}$.

In all critical cases, the exact limits should be computed.

In most slope-ratio assays the individual slopes differ very significantly from zero. Under these circumstances the approximate limits may be computed with reasonable accuracy from the variance of the estimated potency by the familiar formula for the variance of a ratio [1].

$$(14) \qquad \begin{aligned} V(J') &= \frac{b_i^2s^2}{b_1^2}\left\{\frac{c_{11}}{b_1^2} + \frac{c_{ii}}{b_i^2} - \frac{2c_{1i}}{b_1b_i}\right\} \\ &= \frac{s^2}{b_1^4}\{(c_{11} - c_{1i})b_i^2 + (c_{ii} - c_{1i})b_1^2 + c_{1i}(b_1 - b_i)^2\}. \end{aligned}$$

The discrepancies between the approximate and the exact limits are evident from a comparison of equations (13) and (14). When the doses are spaced at equal arithmetic intervals, equation (14) can be reduced to the more convenient form

$$(15) \qquad s_{J'}^2 = \frac{6s^2}{b_1^2(2k + 1)}\left\{\frac{h + rJ'^2}{rhk(k + 1)} + \frac{3(1 - J')^2}{N(k - 1) + 3h'(k + 1)}\right\}.$$

A major limitation to slope-ratio assays is the frequent curvature in the relation between response and arithmetic dosage units. For this reason it is advisable to use routinely four or more doses of each preparation. Occasionally an assay in which there is curvature at the highest dosage level may be salvaged by computing the potencies from the data of the smaller doses. The agreement of a given assay with the postulate upon which it is based may be tested objectively by an analysis of variance, segregating the sums of squares (a) for the agreement of the negative control with the intercept, (b) for the agreement of the individual curves at the intercept, (c) for agreement of the observations with straight lines fitted individually and (d) for the variation among the h replicates of the standard, the h' replicates of the negative control and the r replicates of the unknowns. The calculation of such an analysis is greatly facilitated by the recommended

design. Since it follows the usual pattern, it will not be described here. The procedure has been tested with the data from an experiment on the depth dose of x-rays [2] and has been applied to microbiological assays [3] in papers where the reader will find the technique exemplified.

REFERENCES

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