Statistical Procedures for Bioassays When the Condition of Similarity Does Not Obtain

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Well-established statistical procedures are available for the analysis of dilution parallel line or slope ratio assays for which the condition of similarity obtains. Research scientists have long been aware that this condition is commonly violated, in log-dose response assays, for example, divergent rather than parallel lines may be obtained. The deviation cannot always be traced to deficient experimental techniques. In fact, as indications of differences in the response processes of the standard and test preparations, or as indications of impure test preparations, such findings may provide the most important inference from an assay experiment and immediately suggest further investigation into the causes of the differences. Statistical procedures have been developed to describe the phenomena in quantitative terms and, especially, to permit potency comparisons. The procedures may also have merit even in dilution assay situations where the condition of similarity may apparently be violated if appreciable differences between the responses of the standard and test preparations result from poorly matched doses.

THE TERM "analytical dilution assay" refers to an assay of a preparation of unknown potency which can be regarded as nothing but a dilution of the standard preparation in a diluent which does not contribute to the response by either chemical or physical properties. In such cases it follows that when response is plotted against log-dose the curves for the two preparations are the same apart from a constant relative displacement parallel to the log-dose axis. Furthermore, as was pointed out by Gaddum (1), the relative potency estimate obtained from an analytical dilution assay should not be dependent on the particular assay circumstances. The estimate should agree with one obtained by, for example, chemical determinations since both procedures should give estimates of the reciprocal of the dilution factor.

When the log-dose response curves are straight parallel lines well-established statistical procedures, such as those described by Bliss (2) and Finney (3), are available for obtaining the relative potency estimate. Estimation procedures for quadratic parallel curves have been described by Bliss (4) and Elston (5). Relatedly, Leaverton (6) has discussed methods, based on techniques described by Lewish (7), for fitting quadratic curves constrained to be strictly monotonic for use in bioassay contexts.

Similar statistical procedures are used if parallel log-dose response curves are obtained—for a particular response—even though the inert diluent assumption is not generally true. Such assays have been termed "comparative assays." Since both types of assay involve comparative experiments, however, there are grounds for using the general term "assay" for either type when distinction is not essential, the qualification "analytical dilution" or just "dilution" being used to distinguish the subclass of assays defined above.

As a prerequisite for estimation of relative potency in the above cases, statistical analyses incorporate tests for the relevance of the assumed mathematical model to the observed phenomena. In particular, an index sensitive to divergence, *i.e.*, to departure from parallelism, can be obtained from the sum of squares for interaction between preparations and log-doses in the analysis of variance. In research, as distinct from routine, bioassay situations it is usually not possible to assert that an unknown preparation is a dilution of the standard preparation; assays may, in fact, be initiated to examine just this question. As isolated from an animal or plant organism, for example, the unknown preparation may consist of a mixture of substances which may affect the response in different degrees. Accordingly, assay research workers, in pharmacology and endocrinology, for examples, have long been aware that assays where divergent rather than parallel lines are obtained are common, although the statistical significance of the divergence term may occasionally be masked because the assay is of low precision.

It should also be remembered that except in the somewhat unusual case that the log-dose response relationship is exactly rectilinear over a wide response range—as distinct from being approximately straight locally—it is an event with probability zero that doses will be so chosen to

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give exactly parallel lines even for an analytical dilution assay.

One early idea on statistical procedures appropriate for divergent line assays was put forward by Ing *et al.* (8), who suggested the computation of an average relative potency determined over a wide range of doses. Later, in Gibbs *et al.* (9), an estimate of the logarithm of the relative potency was computed at the 50% response level as a not strictly valid but nevertheless useful comparison. Neither of these suggested procedures, however, properly incorporate the basic fact that the relative potency is different for different doses or responses. And it was pointed out by Grimshaw and D'Arcy (10) that there was then no adequate method for quantitative assessment in such situations.

Discussions of the above introductory points and related statistical aspects may be found in Thompson (11), Leaverton (6), Cornfield (12), and Finney (13). In Cornfield (12), a procedure is presented for estimating the logarithm of the relative potency as a function of the log-dose in cases when linear log-dose response curves for both preparations can be estimated for each of a number of experimental subjects. Procedures are given here for divergent line assays obtained using the common experimental plan in which only one preparation-dose combination is observed on each of a number of experimental subjects.

SPECIFICATION

Let X_S and X_T denote log-doses of the standard and test preparations, respectively, and let Y denote the response.

Then linear log-dose response lines can be written as,

$$Y = \alpha_S + \beta_S X_S \qquad \text{(Eq. 1)}$$

$$Y = \alpha_T + \beta_T X_T \qquad \text{(Eq. 2)}$$

for the standard and test preparations, respectively.

The fact that, if $\beta_S \neq \beta_T$, the logarithm of the relative potency is a function of the log-dose or the response can now be expressed in any of the three ways *A*, *B*, and *C* described below.

A. Linear Relation Between Equipotent Log-Doses.—If log-doses X_S and X_T give the same response, Y, Eqs. 1 and 2 show that X_S and X_T are related by

$$\alpha_S + \beta_S X_S = \alpha_T + \beta_T X_T$$

that is,

$$X_S = -\frac{(\alpha_S - \alpha_T)}{\beta_S} + \frac{\beta_T}{\beta_S} X_T \qquad \text{(Eq. 3)}$$

B. Log (Relative Potency) as a Linear Function of Log-Dose.—If $\mu(X_T)$ denotes the logarithm of the relative potency at dose X_T, that is, $\mu(X_T)$ is the difference between equipotent log-doses, we have, at log-dose X_T,

$$\mu(X_T) = X_S - X_T$$

so that, from Eq. 3,

$$\mu(X_T) = \frac{(\alpha_S - \alpha_T)}{\beta_S} + \left(\frac{\beta_T}{\beta_S} - 1\right) X_T \qquad \text{(Eq. 4)}$$

C. Log (Relative Potency) as a Linear Function of Response.—If $\mu(Y)$ denotes the logarithm of the relative potency at response Y, $\mu(Y)$ is the difference between equally effective log-doses so that,

$$\mu(Y) = \frac{Y - \alpha_S}{\beta_S} - \frac{Y - \alpha_T}{\beta_T} \qquad \text{(Eq. 5)}$$

$$= -\left(\frac{\alpha_S}{\beta_S} - \frac{\alpha_T}{\beta_T}\right) + \left(\frac{1}{\beta_S} - \frac{1}{\beta_T}\right) Y \qquad \text{(Eq. 6)}$$

Presentation of the relations has been made in the above form to give expressions which are consistent with those obtaining in the usual parallel line assay case when $\beta_S = \beta_T$. In some applications, however, predictions in terms of the test rather than the standard preparation may be of interest. For example, it may commonly be required to estimate the log-dose, X_T, of the test preparation which will give a response equivalent to that obtained with a specified log-dose, X_S, of the standard. The expressions above and their developments below can readily be applied in such cases by simply interchanging the suffixes S and T so that Eq. 3, for example, would give,

$$X_T = -\frac{(\alpha_T - \alpha_S)}{\beta_T} + \frac{\beta_S}{\beta_T} X_S$$

Choice between the various alternative expressions may, therefore, be made according to the practical requirements of particular situations. Care, however, is required in any application because, apart from the simple indication that the test preparation is not a dilution of the standard preparation, the equations in themselves cannot readily be interpreted to give information about modes of action in a context more general than that of the particular assay. Thus, although Eq. 3 represents a calibration relationship between equipotent log-doses, it cannot be assumed that the parameters in such a relation determined from one laboratory species will remain constant for application to another species. In the absence of information about modes of action, therefore, the relationships should preferably be regarded as concise local descriptions of the observed phenomena. The relations are, of course, applicable in repetitions of the original assay circumstances of which accurate and detailed specifications are accordingly desirable. This latter aspect is particularly important for interlaboratory studies, as was recently emphasized by Youden (14).

ESTIMATION

Estimation procedures for the above relationships will first be considered for an assay in a completely randomized design in which one observation is obtained from each of N experimental subjects and r responses are observed at each of n_S log-dose levels of the standard preparation and n_T log-dose levels of the test preparation, so that,

$$N = r(n_S + n_T) \qquad \text{(Eq. 7)}$$

The assumptions will be made that (a) the log-dose response lines for each preparation are straight lines over the range of doses tested; (b) residual errors are normally and independently distributed with population mean zero and variance σ^2, this variance being the same for both preparations.
The following notation will be used:

S refers to the standard and T to the test preparation,
x_{iS} is the ith log-dose value for S, $i = 1, 2, \ldots, n_S$,
x_{jT} is the jth log-dose value for T, $j = 1, 2, \ldots, n_T$,
y_{ikS} and y_{jkT} are the kth response observations at the ith log-dose of S and the jth log-dose of T, $k = 1,2,\ldots,r$, and correspondingly
$\bar{y}_{iS}$ and $\bar{y}_{jT}$ are the mean responses at these log-doses.

By regression analyses described in standard texts estimates, a_S, b_S, a_T, and b_T, of the regression parameters, α_S, β_S, α_T, and β_T, in Eqs. 1 and 2 are first calculated. Thus, for S, with

$$\bar{x}_S = \frac{1}{n_S} \sum_{i=1}^{n_S} x_{iS} \qquad \text{(Eq. 8)}$$

$$\bar{y}_S = \frac{1}{n_S} \sum_{i=1}^{n_S} \bar{y}_{iS} = \frac{1}{rn_S} \sum_{i=1}^{n_S} \sum_{k=1}^{r} y_{ikS} \qquad \text{(Eq. 9)}$$

so that $\bar{y}_S$ and $\bar{y}_T$ are the means of the mean responses at the individual log-dose values, the estimates are

$$a_S = \bar{y}_S - b_S\bar{x}_S \qquad \text{(Eq. 10)}$$

$$b_S = \frac{\sum_{i=1}^{n_S} (x_{iS} - \bar{x}_S)(\bar{y}_{iS} - \bar{y}_S)}{\sum_{i=1}^{n_S} (x_{iS} - \bar{x}_S)^2} \qquad \text{(Eq. 11)}$$

In practice, of course, b will be calculated using the well-known identity that for any number, n, of pairs (x_i, y_i) with means $\bar{x}$ *and* $\bar{y}$,

$$\Sigma(x_i - \bar{x})(\bar{y}_i - \bar{y}) = \Sigma xy - \frac{1}{n}(\Sigma x)(\Sigma y) \qquad \text{(Eq. 12)}$$

The estimate s^2 of the residual variance σ^2 is calculated by pooling the mean squares for deviations from regression obtained from the two regression analyses. This estimate will have $N - 4$ degrees of freedom and is compounded from the deviations of the mean responses at individual log-dose levels and the deviations between individual responses at each of the log-dose levels, a procedure which is valid under the assumptions (a) and (b) above.

Estimates of the quantities defined in the Eqs. 3, 4, and 6 above can now be calculated as follows.

From Eq. 3, the estimate of the true value of X_S is $\hat{X}_S$ where,

$$\hat{X}_S = -\frac{1}{b_S}(a_S - a_T - b_T X_T) \qquad \text{(Eq. 13)}$$

or equivalently in terms of means as defined in Eqs. 8 and 9,

$$\hat{X}_S - \bar{x}_S = -\frac{1}{b_S} \times \{\bar{y}_S - \bar{y}_T - b_T(X_T - \bar{x}_T)\} \qquad \text{(Eq. 14)}$$

$$= -\frac{1}{b_S}(\bar{y}_S - \hat{Y}_T) \qquad \text{(Eq. 15)}$$

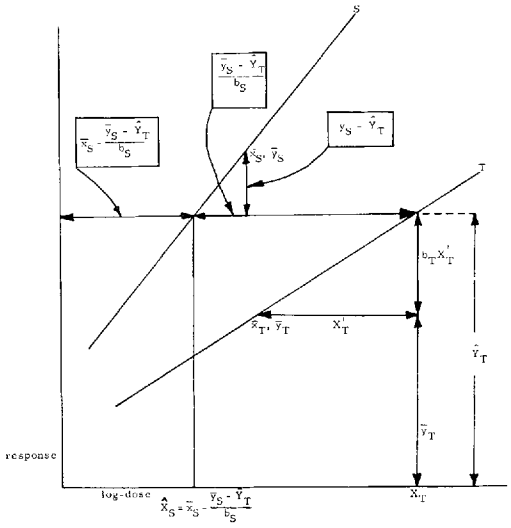

Fig. 1.—The relation between equipotent log-doses.

where

$$\hat{Y}_T = \bar{y}_T + b_T(X_T - \bar{x}_T) \qquad \text{(Eq. 16)}$$

is the response predicted from the line for the test preparation at log-dose X_T. A geometrical construction which illustrates how the above quantities are used to obtain the log-dose estimate $OA = \hat{X}_S$ from the specified value $OB = X_T$ is given in Fig. 1.

Next from Eq. 4 $M(X_T)$, the estimate of $\mu(X_T)$ is given by

$$M(X_T) = -\frac{1}{b_S}\{a_S - a_T - (b_T - b_S)X_T\} \qquad \text{(Eq. 17)}$$

which may alternatively be written as

$$M(X_T) = \bar{x}_S - X_T - \frac{1}{b_S} \times \{\bar{y}_S - \bar{y}_T - b_T(X_T - \bar{x}_T)\} \qquad \text{(Eq. 18)}$$

$$= \bar{x}_S - \bar{x}_T - \frac{\bar{y}_S - \bar{y}_T}{b_S} + \left(\frac{b_T}{b_S} - 1\right)(X_T - \bar{x}_T) \qquad \text{(Eq. 19)}$$

Third, from Eq. 6 $M(Y)$, the estimate of $\mu(Y)$ is given by

$$M(Y) = -\frac{1}{b_S b_T} \times \{a_S b_T - a_T b_S - (b_T - b_S)Y\} \qquad \text{(Eq. 20)}$$

or

$$M(Y) = \bar{x}_S - \bar{x}_T - \frac{1}{b_S b_T} \times \{\bar{y}_S b_T - \bar{y}_T b_S - (b_T - b_S)Y\} \qquad \text{(Eq. 21)}$$

INTERVAL ESTIMATION OF THE EQUIPOTENT DOSE

By an adaptation of the procedure Fieller (15, 16) for interval estimation from a ratio, a fiducial interval will now be derived for the true value of $\hat{X}_S$ calculated from Eqs. 13 or 14 for a single specified

X_T value. For this, Fieller's argument can be applied to a variate constructed as

$$u = a_S + b_S X_S - a_T - b_T X_T \quad \text{(Eq. 22)}$$

where X_S is the true or population value for the log-dose of the standard preparation corresponding to a log-dose X_T of the test preparation.

Under the stated assumptions, u is a linear combination of the normally distributed variates a_S, b_S, a_T, and b_T and so is itself normally distributed. In virtue of Eq. 3 it is also true that the population or expected value of u is zero. It follows that, if the estimated variance of u is $d_u s^2$, where d_u is a known constant coefficient determined by the construction of u from the original observations, the quantity $u^2/d_u s^2$ is distributed according to the F distribution with 1 and $N - 4$ degrees of freedom.

Hence, if F_c is the tabulated value from this distribution such that

$$P[F \leq F_c] = 1 - \alpha$$

we have

$$P\left[\frac{u^2}{d_u s^2} \leq F_c\right] = 1 - \alpha$$

By Fieller's theorem it now follows that solutions of

$$u^2 - F_c d_u s^2 = 0 \quad \text{(Eq. 23)}$$

which from Eq. 22 is a quadratic equation in the X_S corresponding to a given value of X_T, will give values defining a $100(1 - \alpha)\%$ fiducial interval for the required X_S value.

To obtain d_u, it is first convenient to write u from Eq. 22 in the equivalent form,

$$u = \bar{y}_S + b_S(X_S - \bar{x}_S) - \bar{y}_T - b_T(X_T - \bar{x}_T) \quad \text{(Eq. 24)}$$

in which all the estimates are statistically independent. Hence,

$$V(u) = V[\bar{y}_S + b_S(X_S - \bar{x}_S)] + V[\bar{y}_T + b_T(X_T - \bar{x}_T)]$$

which can be estimated as $d_u s^2$ where

$$d_u s^2 = \left[\frac{1}{n_S} + \frac{(X_S - \bar{x}_S)^2}{r\Sigma(x_S - \bar{x}_S)^2} + \frac{1}{n_T} + \frac{(X_T - \bar{x}_T)^2}{r\Sigma(x_T - \bar{x}_T)^2}\right] s^2 \quad \text{(Eq. 25)}$$

It is now convenient to introduce a more concise notation, wherein a prime is used to denote values "corrected for their means." Thus, we write

$$X_S' = X_S - \bar{x}_S, \quad X_T' = X_T - \bar{x}_T \quad \text{(Eq. 26)}$$

and

$$\Sigma_S' = r\sum_1^{n_S}(x_{iS} - \bar{x}_S)^2, \quad \Sigma_T' = r\sum_1^{n_T} \times (x_{jT} - \bar{x}_T)^2 \quad \text{(Eq. 27)}$$

Then, from Eqs. 24 and 25

$$u = \bar{y}_S - \bar{y}_T + b_S X_S' - b_T X_T' \quad \text{(Eq. 28)}$$

and

$$d_u = \frac{1}{n_S} + \frac{1}{n_T} + \frac{X_S'^2}{\Sigma_S'} + \frac{X_T'^2}{\Sigma_T'} \quad \text{(Eq. 29)}$$

Inserting these values into Eq. 23 and collecting terms then gives the quadratic equation for the unknown X_S' as,

$$AX_S'^2 + 2BX_S' + C = 0 \quad \text{(Eq. 30)}$$

where

$$A = b_S^2 - \frac{F_c s^2}{\Sigma_S'} \quad \text{(Eq. 31)}$$

$$B = b_S\{\bar{y}_S - \bar{y}_T - b_T X_T'\} \quad \text{(Eq. 32)}$$

$$= b_S(\bar{y}_S - \hat{Y}_T) \quad \text{(Eq. 33)}$$

from Eq. 16, and

$$C = (\bar{y}_S - \bar{y}_T - b_T X_T')^2 - F_c s^2\left(\frac{1}{n_S} + \frac{1}{n_T} + \frac{X_T'^2}{\Sigma_T'}\right) \quad \text{(Eq. 34)}$$

By the usual formula for the roots of a quadratic equation the lower and higher limits, X_{SL} and X_{SH}, of the fiducial interval are then given by

$$X_{SL}', X_{SH}' = \frac{-B \mp \sqrt{B^2 - AC}}{A} \quad \text{(Eq. 35)}$$

that is,

$$X_{SL} = \bar{x}_S - \frac{1}{A}\{B + \sqrt{B^2 - AC}\},$$

$$X_{SH} = \bar{x}_S - \frac{1}{A}\{B - \sqrt{B^2 - AC}\} \quad \text{(Eq. 36)}$$

In practice, the computations can be simplified by first calculating the quantity $\hat{X}_S' = \hat{X}_S - \bar{x}_S$ from the point estimate $\hat{X}_S$ of the equipotent dose. Then, substituting $-b_S\hat{X}_S'$ for $\bar{y}_S - \bar{y}_T - b_T X_T'$ in Eqs. 32 and 34 leads to an expression for the interval as,

$$X_{SL}', X_{SH}' = \frac{1}{A}\left[b_S^2 X_S' \mp \sqrt{F_c s^2\left\{A\left(\frac{1}{n_S} + \frac{1}{n_T} + \frac{X_T'^2}{\Sigma_T'}\right) + \frac{b_S^2\hat{X}_S'^2}{\Sigma_S'}\right\}}\right] \quad \text{(Eq. 37)}$$

By analogy with the usual calculations for parallel line assays [Finney (3)], an approximate formula, which is often sufficiently accurate, can now be easily obtained. For this we note that the quantity s^2/Σ_S' in Eq. 31 is the variance of b_S, and, if b_S^2 is very much larger than its variance, *i.e.*, if the square of the coefficient of variation of b_S is very small, we have, from Eq. 31

$$A = b_S^2\left(1 - \frac{F_c s^2}{b_S^2\Sigma_S'}\right) \simeq b_S^2$$

As an empirical working rule, following Finney (3), it may be suggested that the approximation will give sufficiently accurate results if $A/b_S^2 > 0.95$, or, equivalently, if $20\,F_c < b_S^2\Sigma_S'/s^2$. In such cases it can easily be checked that the fiducial interval defined above becomes,

$$X_{SL}, X_{SH} = \hat{X}_S \mp \sqrt{\frac{F_c s^2}{b_S^2}\left(\frac{1}{n_S} + \frac{1}{n_T} + \frac{X_T'^2}{\Sigma_T'}\right)} \quad \text{(Eq. 38)}$$

TABLE I.—FOUR-POINT ASSAY OF LUTEINIZING HORMONE IN SWINE PITUITARY TISSUE BY ASCORBIC ACID DEPLETION METHOD

	Standard (N.I.H.)		Test	
	0.4 mg.	1.6 mg.	0.0625 mg.	0.2500 mg.
	77	55	64	60
	81	45	71	54
	80	47	70	54
	78	52	80	61
	80	48	72	54
Total	396	247	357	283
Mean	79.2	49.4	71.4	56.6

The fiducial intervals calculated as above apply when the $\hat{X}_S$ value corresponding to only one X_T value is required. Commonly, however, the procedure may be required for an unspecified number of X_T values. The theoretical treatment by Scheffé (17) then indicates that the interval should be calculated by substituting for F_c, as defined above, the value $4F_c'$ where F_c' is the tabulated value from the F-distribution with 4 and $N - 4$ degrees of freedom such that,

$$P[F \leq F_c'] = 1 - \alpha$$

EXAMPLE

The data in Table I were obtained from a four-point assay of luteinizing hormone (LH) in swine pituitary tissue [Melampy and Hendricks (18)] by the ascorbic acid depletion (AAD) method. The responses are in units of mcg. AAD/100 mg. rat ovary tissue.

When the doses are, as here, conveniently chosen so that the ratio of the higher to the lower dose is the same for both preparations, the log-dose transformations can be chosen to give a log-dose metameter which takes simple integral values. Thus, in Table I, where the dose-ratio is 4, the transformations from doses z_S and z_T to metameters x_S and x_T such that,

$$x_S = \frac{1}{\log 4}\{\log z_S - \log 0.4\} \qquad \text{(Eq. 39)}$$

$$x_T = \frac{1}{\log 4}\{\log z_T - \log 0.0625\} \qquad \text{(Eq. 40)}$$

give $x_S = x_T = 0$ at the two lower doses and $x_S = x_T = 1$ at the two upper doses.

The usual assay analysis of variance then gives:

	d.f.	Mean Squares
Between preparations	1	0.45
Common regression	1	2486.45
Divergence	1	281.25
Residual	16	16.15

Since the $\alpha = 0.05$ critical F-value for 1 and 16 degrees of freedom is 4.49 it can be seen (*a*) from the between preparations term that closely similar response levels were achieved and (*b*) from the divergence term that the slopes of the regression lines for the standard and test preparations were significantly different.

We are therefore in a situation for which the preceding procedures are appropriate with $n_S = n_T = 2$ doses for each preparation and $r = 5$ responses at each preparation-dose combination. Conventional regression calculations, as indicated by Eqs. 8, 9, and 11 and similar equations for the test preparation, then give

$$\bar{x}_S = \bar{x}_T = {}^1/_2$$

and

$$\bar{y}_S = (396 + 247)/10 = 64.3$$

$$\bar{y}_T = (357 + 283)/10 = 64.0$$

Again, from Eq. 27

$$\Sigma_S' = \Sigma_T' = 5\left(\frac{1}{4} + \frac{1}{4}\right) = \frac{5}{2}$$

and it follows from Eqs. 11 and 12, or because the interval between the two values of x_S is unity, that

$$b_S = -(79.2 - 49.4) = -29.8$$

and similarly,

$$b_T = -(71.4 - 56.6) = -14.8$$

The equation for the prediction of an X_S value corresponding to a specified X_T value can now be written down from Eq. 14 as

$$\hat{X}_S - \frac{1}{2} = \frac{1}{29.8}\left\{64.3 - 64.0 + 14.8\left(X_T - \frac{1}{2}\right)\right\}$$

$$= \frac{1}{29.8}(-7.1 + 14.8\,X_T)$$

that is,

$$\hat{X}_S = 0.26 + 0.50\,X_T \qquad \text{(Eq. 41)}$$

With $F_c = 4.49$, for $\alpha = 0.05$, and $s^2 = 16.15$

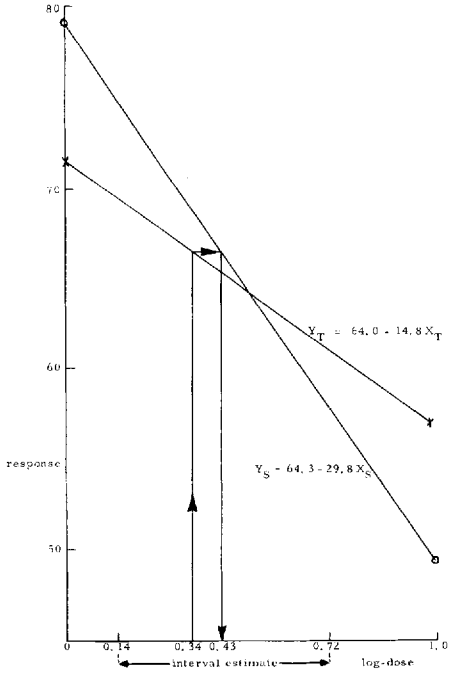

Fig. 2.—Assay of LH in swine pituitary tissue (18).

(16 d.f.) the fiducial interval can now be calculated. For this, from Eq. 31,

$$A = 29.8^2 - \frac{2}{5}(4.49)(16.15)$$
$$= 888.04 - 29.01$$
$$= 859.03$$

Hence, from Eq. 37

$$X_{SL}', X_{SH}' = \frac{1}{859.03}\left[29.8^2\hat{X}_S' \mp \sqrt{(4.49)(16.15)\left\{859.03\left(\frac{1}{2}+\frac{1}{2}+\frac{2X_T'^2}{5}\right)+\frac{2(29.8^2)}{5}\hat{X}_S'^2\right\}}\right] \quad \text{(Eq. 42)}$$

$$= 1.03\hat{X}_S' + \sqrt{0.0844\,(1 + 0.40\,X_T'^2 + 0.42\,\hat{X}_S'^2)} \quad \text{(Eq. 43)}$$

Now, in particular, suppose it is desired to estimate the dose of the standard preparation equivalent to 0.1 mg. of the test preparation. First, from Eq. 40, the specified value of X_T is

$$X_T = \frac{1}{\log 4}\{\log 0.1/0.0625\} = 0.3390$$

Hence, from Eq. 41,

$$\hat{X}_S = 0.26 + (0.50)(0.3390) = 0.4295$$

and, from Eq. 39,

$$\log(z_S/0.4) = 0.4295 \log 4$$

from which the estimated equipotent dose is

$$\hat{Z}_S = 0.73 \text{ mg.}$$

Noting that $X_T' = 0.3390 - 0.50 = -0.1610$ and $X_S' = -0.0705$, the corresponding 95% fiducial interval is obtained from Eq. 43 as,

$$X_{SL}', X_{SH}' = -(1.03)(0.0705) \mp \sqrt{0.0844\,\{1 + (0.40)(0.1610^2) + 0.42(0.0705)^2\}}$$
$$= -0.365, +0.220$$

From Eqs. 26 and 39 the interval for the equipotent dose can then be calculated as

$$Z_{SL}, Z_{SH} = 0.48, 1.09 \text{ mg.}$$

Alternatively, since for these data $A/b_S^2 = 0.97$, the approximate formula in Eq. 38 may be used to give,

$$X_{SL}, X_{SH} = 0.4295 \mp \sqrt{\frac{(4.49)(16.15)}{29.8^2}(1 + 0.4X_T'^2)}$$
$$= 0.14, 0.72$$

and on subtraction of $\bar{x}_S = 0.5$, values are obtained which closely agree with those obtained above for X_{SL}' and X_{SH}'.

The results are illustrated in Fig. 2. In this the width of the interval estimate serves to emphasize the fact that an experiment designed to give sufficient precision for a parallel line assay will give poor precision for an estimate of an equipotent dose if divergence has to be admitted as the more realistic situation.

For simplicity of exposition, the above development and the example have been carried through for a constant number r of response observations at each preparation-dose level combination. The extension to the unequal numbers case readily follows, *mutatis mutandis*, using the standard statistical procedures for dealing with unequal numbers in single classification experiments. Additionally, assays based on designs other than the completely randomized design can be treated by the basic techniques described above.

QUADRATIC RESPONSE CURVES

The principles described above are also applicable when one or both of the log-dose response relationships can be described by quadratic curves about which the responses of individual experimental units are normally distributed. As expected, however, more computation is required.

If the two quadratic relationships are

$$\bar{y}_S = a_S + b_S X_S + c_S X_S^2 \quad \text{(Eq. 44)}$$

and

$$\bar{y}_T = a_T + b_T X_T + c_T X_T^2 \quad \text{(Eq. 45)}$$

the log-dose of the standard preparation equivalent to a specified log-dose X_T of the test preparation can be estimated as one solution of the quadratic equation,

$$c_S\hat{X}_S^2 + b_S\hat{X}_S + a_S - a_T - b_T X_T - c_T X_T^2 = 0 \quad \text{(Eq. 46)}$$

Identification of the appropriate root can be made without difficulty because the two dose-response curves must be monotonic (though not necessarily in the same sense) in the region of interest and because the specified X_T and its correspondent $\hat{X}_S$ should be within the dose ranges over which the curves themselves were estimated.

In many practical cases extreme accuracy will not be required of such estimation procedures and, particularly if numerous equipotent doses are required, it may be more convenient to read them from graphs of the two curves.

Fiducial intervals for the estimate defined in Eq. 46 can also be obtained on the above principles as the appropriate solutions of Eq. 23 with u equal to the expression on the left of Eq. 46. Solution of a fourth degree equation is required in this case. The interpretation of solutions of quartic equations in a similar inverse estimation problem has been discussed by Williams (19).

RELATIVE POTENCY AS A FUNCTION OF DOSE

Divergent line assays may occur in some contexts where it may be of interest to estimate the relative potency itself, although this quantity is now of more restricted use than in the simple case when it is constant. For example, if we now find that 1 mg. of the test preparation is equipotent to ρ(1 mg.) of the standard preparation it is no longer true that 1 Gm. of the test and 1000ρ(1 mg.) of the standard preparations are equipotent.

When, however, the relative potency itself is required it can be estimated as a function of log-dose from Eq. 19. Since $\mu(X_T) = X_S - X_T$, where X_S is the equipotent log-dose of the standard preparation, an interval estimate for $\mu(X_T)$ can be obtained by subtracting X_T from the interval estimate previously determined for X_S. Alternatively, we may proceed directly by applying Fieller's procedure, *via* Eq. 23 with

$$u = b_S\{\mu(X_T) - \bar{x}_S + \bar{x}_T\} + (\bar{y}_S - \bar{y}_T) - (b_T - b_S)X_T' \quad \text{(Eq. 47)}$$

As a result if, for convenience, we write $\lambda = \mu(X_T) - \bar{x}_S + \bar{x}_T$ the interval can be determined by adding $(\bar{x}_S - \bar{x}_T)$ to each root of the quadratic equation

$$A\lambda^2 + 2B\lambda + C = 0 \quad \text{(Eq. 48)}$$

where

$$A = b_S^2 - \frac{F_c s^2}{\Sigma_S'} \quad \text{(Eq. 49)}$$

$$B = -b_S^2\{M(X_T) - \bar{x}_S + \bar{x}_T\} + \frac{F_c s^2}{\Sigma_S'} \quad \text{(Eq. 50)}$$

$$C = b_S^2\{M(X_T) - \bar{x}_S + \bar{x}_T\}^2 - F_c s^2\left\{\frac{1}{n_S} + \frac{1}{n_T} + X_T'^2\left(\frac{1}{\Sigma_S'} + \frac{1}{\Sigma_T'}\right)\right\} \quad \text{(Eq. 51)}$$

RELATIVE POTENCY AS A FUNCTION OF RESPONSE

The point estimate $M(Y)$ of $\mu(Y)$, the log relative potency at response Y can be calculated from Eq. 21, but exact interval estimation is not so straightforward as in the previous cases. In many practical cases, however, it will be sufficient to use approximate fiducial intervals which can be obtained as follows.

It is a well-known result that, if

$$r = \frac{u}{v}$$

is a ratio of two variates, u and v, which are statistically independent, and if the coefficient of variation of the denominator, v, is small, then

(coefficient of variation of r)^2 = (coefficient of variation of u)^2 + (coefficient of variation of v)^2 (Eq. 53)

That is, if $d_u s^2$ and $d_v s^2$ are the estimated variances of u and v, respectively, $V(r)$, the estimated variance of the ratio is given by,

$$V(r) = r^2 s^2\left(\frac{d_u}{u^2} + \frac{d_v}{v^2}\right) \quad \text{(Eq. 54)}$$

To apply this in the present context we have, from Eq. 21

$$M(Y) - \bar{x}_S + \bar{x}_T = \left\{\frac{(Y - \bar{y}_S)}{b_S} - \frac{(Y - \bar{y}_T)}{b_T}\right\} \quad \text{(Eq. 55)}$$

The difference between two independent ratios appears on the righthand side and hence

$$V\{M(Y) - \bar{x}_S + \bar{x}_T\} = V\left(\frac{Y - \bar{y}_S}{b_S}\right) + V\left(\frac{Y - \bar{y}_T}{b_T}\right) \quad \text{(Eq. 56)}$$

and now, applying Eq. 54

$$V\left(\frac{Y - \bar{y}_S}{b_S}\right) = \left(\frac{Y - \bar{y}_S}{b_S}\right)^2 s^2\left\{\frac{\frac{1}{n_S}}{(Y - \bar{y}_S)^2} + \frac{\frac{1}{\Sigma_S'}}{b_S^2}\right\} = \frac{s^2}{b_S^2}\left\{\frac{1}{n_S} + \frac{(Y - \bar{y}_S)^2}{b_S^2\Sigma_S'}\right\} \quad \text{(Eq. 57)}$$

The variance of $(Y - \bar{y}_T)/b_T$ can be similarly calculated and, on the assumption that the quantity $M(Y) - \bar{x}_S + \bar{x}_T$ in Eq. 55 is normally distributed, the approximate fiducial limits of $\mu(Y)$ are then,

$$\mu_L(Y),\ \mu_H(Y) = \bar{x}_S - \bar{x}_T \mp \sqrt{F_c s^2\left\{\frac{1}{n_S} + \frac{1}{n_T} + \frac{(Y - \bar{y}_S)^2}{b_S^2\Sigma_S'} + \frac{(Y - \bar{y}_T)^2}{b_T^2\Sigma_T'}\right\}} \quad \text{(Eq. 58)}$$

SLOPE RATIO ASSAYS

Suppose that the two dose-response lines in a slope ratio assay are

$$Y_S = a_S + b_S z_S \quad \text{(Eq. 59)}$$

$$Y_T = a_T + b_T z_T$$

where z_S and z_T represent doses, and the intercepts a_S and a_T are estimates of the parameters α_S and α_T and, instead of α_S being equal to α_T as in the regular slope ratio assay case, we now have $\alpha_S \neq \alpha_T$. The dose Z_S which is equipotent with a specified dose Z_T of the test preparation is then estimated as

$$\hat{Z}_S = -\frac{1}{b_S}(a_S - a_T - b_T Z_T) \quad \text{(Eq. 61)}$$

It can now be seen that this is directly analogous to Eq. 13 for the previous case, except that we now have doses Z_S and Z_T instead of log-doses X_S and X_T, so that *mutatis mutandis*, the above procedures can readily be applied in slope-ratio assay situations.

DISCUSSION

Finney (1965) has recently given an interesting general discussion of the role of the concept of constant relative potency, or equivalently of the condition of similarity, in bioassay. It should be noted that the estimation procedures in the present paper are referred to situations when the condition of similarity does not obtain. Such situations are common in research situations for which the condition would often be an unrealistically ideal assumption.

Relatedly, although techniques have been presented for estimating relative potency as a function of dose (concentration) or response, it is considered that these are of less importance and value than those described for the estimation of equipotent doses. It is suggested that this latter is the more basic concept for bioassay in general because, even when relative potency is constant, applications of its estimation are often, in effect, made toward determinations of equipotent doses.

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Drug Standards

Standardization of Papain Activity

Report of a Collaborative Study

By EDGAR A. LAZO-WASEM

Methods of assay for the enzyme papain were evaluated, and those endorsed are presented. A procedure which measures the hydrolysis of casein under standardized conditions was found to be the method of choice.

PAPAIN, a crude or purified proteolytic enzyme derived from the tropical plant *Carica papaya*, has been used in the pharmaceutical and food industries for over half a century. Twenty years ago, a monograph for papain was included in the eighth edition of the "National Formulary" (1). The then official assay procedure consisted of a limit test based on digestion of beef muscle.

After deletion of papain from the "National Formulary," many procedures came into use for the standardization of commercial papain. For pharmaceutical and food grade papain, the most widely used procedures have been milk-clotting (2), casein digestion (3), and digestion of hemoglobin (4, 5). For crystalline papain, most laboratories have, at least recently, relied on the initial rate of hydrolysis of synthetic peptide substrates such as *N*-benzoyl-L-arginine ethyl ester hydrochloride.

In an attempt to bring about unification in methods of assay throughout United States laboratories, a committee was established within the Quality Control Section of the Pharmaceutical Manufacturers Association in the fall of 1962. This group was to study current prevailing methods and recommend a generally acceptable method for use throughout the industry. This report describes the findings and recommendations of that committee.

PLAN OF STUDY AND RESULTS

Member firms of the Pharmaceutical Manufacturers Association, representing manufacturing suppliers and pharmaceutical firms marketing papain in dosage forms, were invited to supply their procedures. The methods received involved either milk-clotting, casein digestion, or hemoglobin digestion. From the procedures received, three assays based on the above principles were prepared and forwarded to eight laboratories for collaborative study. "Standard" and "unknown" papain preparations were also forwarded, and thus an effort was initiated whereby

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A study by the Committee on Papain, Quality Control Section, Pharmaceutical Manufacturers Association. Committee Membership: J. E. Giesemann, Brayten Pharmaceutical Co.; N. Kartinos, Baxter Laboratories, Inc.; G. F. McCutcheon, S. B. Penick & Co.; C. F. Peterman, Kremers-Urban Co.; J. V. Saenger, Warner-Chilcott Laboratories; I. S. Shupe, Winthrop Laboratories; L. A. Underkoffler, Miles Chemical Co.; and E. A. Lazo-Wasem, Strong Cobb Arner, Inc. (Chairman).
After the study reported here was underway, it was learned that efforts toward uniformity of enzyme assays, including papain, were being made by the International Commission for the Standardization of Pharmaceutical Enzymes, Fédération Internationale Pharmaceutique. Since then this writer has been kept informed of the efforts of this predominantly European group, the initial studies of which have been excellently summarized in the commission's First Report (6). For papain, the commission has endorsed a method based on the initial rate of hydrolysis of a synthetic substrate, *N*-benzoyl-L-arginine ethyl ester hydrochloride, for both crystalline papain and less purified preparations. A comparison of the unit of activity reported here with that adopted by the commission will be the subject of a future report.