Design Workshop Lecture Notes
ISI, Kolkata, November, 25-29, 2002, pp. 61-76

Design and Analysis for Bioassays

G. M. Saha
Theoretical Statistics and Mathematics Unit, Indian Statistical Institute
Kolkata, India

---

1 Introduction

1. Purpose of biological assay

Biological assays are methods for the estimation of nature, constitution, or potency of a material (or of a process) by means of the reaction that follows its application to living matter.

(I) Qualitative Assays	(II) Quantitative Assays
These do not present any statistical problems. We shall not consider them here.	These provide numerical assessment of some property of the material to be assayed, and pose statistical problems.

Definition. An assay is a form of biological experiment; but the interest lies in comparing the potencies of treatments on an agreed scale, instead of in comparing the magnitude of effects of different treatments.

This makes assay different from varietal trials with plants and feeding trials with animals, or clinical trials with human beings. The experimental technique may be the same, but the difference in purpose will affect the optimal design and the statistical analysis. Thus, an investigation into the effects of different samples of insulin on the blood sugar of rabbits is not necessarily a biological assay; it becomes one if the experimenter's interest lies not simply in the changes in blood sugar, but in their use for the estimation of the potencies of the samples on a scale of standard units of insulin. Again, a field trial of the responses of potatoes to various phospatic fertilizers would not generally be regarded as an assay; nevertheless, if the yields of potatoes are to be used in assessing the potency of a natural rock phosphate relative to a standard superphosphate, and perhaps even in estimating the availability of phosphorus in the rock phosphate, the experiment is an assay within the terms of the description given herein.

2. History of biological assay.

In the Bible, in the description of Noah's experiment from his ark by sending a dove repeatedly until it returns with an olive leaf, by which Noah knows or estimates the level of receding waters from the Earth's grounds, we find that it has all the three essential constituents of an assay – namely "stimulus" (depth of water), "subject" (the done) and "response" (plucking of an olive leaf).

Serious scientific history of biological assay began at the close of 19th century with Ehrlich's investigations into the standardization of diphtheria antitoxin. Since then, the standardization of materials by means of the reactions of living matter has become a common practice, not only in pharmacology, but in other branches of science also, such as plant pathology. However the assays were put on sound bases only since 1930's when some statisticians contributed with their refined methods to this area.

3. **Structure of a Biological Assay**

The typical bioassay involves a stimulus (for example, a vitamin, a drug, a fungicide), applied to a subject (for example, an animal, a piece of animal tissue, a plant, a bacterial culture). The intesity of the stimulus is varied by using the various "doses" by the experimenter. Application of stimulus is followed by a change in some measurable characteristic of the subject, the magnitude of the change being dependent upon the dose. A measurement of this characteristic, for example, a weight of the whole subject, or of some particular organ, an analytical value such as blood sugar content or bone ash percentage, or even a simple record of occurrence or non-occurrence of a certain muscular contraction, recovery from symptones of a dietary deficiency, or death — is the response of the subject.

4. **Types of Bioassays**

Three main types (other than qualitative assays)are :

(i) DIRECT ASSAYS;

(ii) INDIRECT ASSAYS based upon quantitative responses;

(iii) INDIRECT ASSAYS based on quantal responses ("all-or-nothing").

5. **Direct Assays**

We shall first take up DIRECT ASSAYS. In such assays doses of the standard and test preparations are sufficient to produce a specified response, and can be directly measured. The ratio between these doses estimates the potency of test preparation relative to the standard. If z_S & z_T are doses of standard & test preparations producing the same effect, then the relative potency ρ is given by

$$\rho = \frac{z_S}{z_T}.$$

Thus, in such assays, the response must be clear-cut & easily recognized, and exact dose can be measured without time lag or any other difficulty.

A typical example of a direct assay is the "cat" method for the assay of digitalis. Preparation is infused until its heart stops (causing death). The dose is immediately measured.

Preparations	Tolerences	mean
Strophanthus A (Test Prep.) (in .01 cc/kg.)	1.55, 1.58, 1.71, 1.44, 1.24, 1.89 2.34	1.68
Strophanthus B (Stan. Prep.) : (in .01 cc/kg.)	2.42, 1.85, 2.00, 2.27, 1.70, 1.48, 2.20	1.99

$$R = \hat{\rho} = \frac{\overline{x}_S}{\overline{x}_T} = \frac{0.0199}{0.0168} = 1.18 \text{ (Relative Potency Estimates)}$$

Thus 1 cc of tincture A is estimated to be equivalent to 1.18 cc of tincture B.

Precision of the estimate ?

We shall work out the precision of our estimate. But before that, please note that in the example considered, we have assumed that the two preparations contain the same effective ingredient which proceduces the response. Such assays are called ANALYTICAL DILUTION ASSAYS. We shall confine ourselves only to ADA's in this course. [An assay with two preparations which have a common effect but do not contain the same effective ingredient is called a COMPARATIVE DILUTION ASSAY.]

Let $F = \overline{x}_B/\overline{x}_A$
Then

$$V(R) = \frac{1}{\overline{x}_A^2}[V(\overline{x}_B) + R^2 V(\overline{x}_A)]$$

For tincture $A, \sum(x-\overline{x})^2 = 0.7587$ & tincture $B, = 0.6815$. Both S.S. are based on 6 d.f.

Estimate of common variance $s^2 = \frac{0.7587+0.6815}{12} = 0.1200$.
$V(\overline{x}_A) = V(\overline{x}_B) = \frac{s^2}{7}$. Hence $R = 1.18$
& $V(R) = \frac{s^2}{x_A^2}[\frac{1}{7} + \frac{R^2}{7}] = 0.0145$.
So we have $R = 1.18 \pm 0.120[\sqrt{0.0145} = 0.120]$.

[Here we have used the result:

$$V[f(x,y)] = V(x)(\frac{\partial f}{\partial x})^2_{(E(x),E(y))} + V(y)(\frac{\partial f}{\partial y})^2_{(E(x),E(y))}.$$

Thus

$$\begin{aligned} V(\tfrac{a}{b}) &= V(a).\tfrac{1}{\beta^2} + V(b)\tfrac{\alpha^2}{\beta^4}, \quad \alpha = E(a),\ \beta = E(b) \\ &= \tfrac{1}{\beta^2}[V(a) + \mu^2 V(b)] \quad \text{where} \quad \mu = \alpha/\beta.] \end{aligned}$$

We shall now try to give Fiducial limits for the estimate of relative potency using a theorem, called *FIELLER'S THEOREM*. This is due to Fieller (1940).

Let α & β be two parameters & $\mu = \alpha/\beta$ respectively s.t. $E(a) = \alpha$ & $E(b) = \beta$. Also let $m = a/b$ be an estimate of μ. We shall assume that a & b are normally distributed and that

$$V(a) = v_{11}s^2,\ V(b) = v_{22}s^2,\ \mathrm{cov}(a,b) = v_{12}s^2$$

where s^2 is an unbiased estimator for common variance σ^2 based on f d.f..

FIELLER'S THEOREM. Upper & lower fiducial limits of μ are :

$$m_L, m_U = [m - \frac{gv_{12}}{v_{22}} + \frac{t_0 s}{b}\{v_{11} - 2mv_{12} + m^2 v_{22} - g(v_{11} - \frac{v_{12}^2}{v_{22}})\}^{1/2}]/(1-g)$$

where $g = t_0^2 s^2 v_{22}/b^2$, where t_0 is the two-sided α percentage point of Student's t distribution with f d.f.

Proof. Consider the linear comb. $(a - \mu b)$.

$$E(a - \mu b) = 0\ \&\ V(a - \mu b) = s^2(v_{11} - 2\mu v_{12} + \mu^2 v_{22}).$$

$$\text{So, } P[(a-\mu b)^2 \le t_0^2 s^2 (v_{11} - 2\mu v_{12} + \mu^2 v_{22})] = 1 - \alpha$$

The expression inside [ ] is expanded as an inequality for a quadratic expression in μ. Thus, the event inside [ ] is equivalent to the following :

$$a^2 - 2\mu ab + \mu^2 b^2 - t_0^2 s^2 (v_{11} - 2\mu v_{12} + \mu^2 v_{22}) \le 0$$

i.e., $\mu^2(b^2 - t^2 s^2 v_{22}) + 2\mu(t_0^2 s^2 v_{12} - ab) + (a^2 - t_0^2 s^2 v_{11}) \le 0$.

[Now recall that $px^2 + qx + r \le 0 \Leftrightarrow r_1 \le x \le r_2$ where $r_1 \le r_2$ are the two roofs of the quadratic equation $px^2 + qx + r = 0$ and $p > 0$, since $px^2 + qx + r = p(x - r_2)(x - r_2) = 0$ becomes the equation. If $p < 0$, then it is equivalent to $x \le r_1 \cup x \ge r_2$.]
So, we find the two roots of the above Q.E. in μ :

$$\frac{-(t_0^2 s^2 v_{12} - ab) \pm \sqrt{(t_0^2 s^2 v_{12} - ab)^2 - (b^2 - t_0^2 s^2 v_{22})(a^2 - t_0^2 s^2 v_{11})}}{(b^2 - t_0^2 s^2 v_{22})}$$

Dividing both num^r & denom^r by b^2, we get :

$$\frac{m - \frac{gv_{12}}{v_{22}} \pm \sqrt{(\frac{t_0^2 s^2 v_{12}}{b^2} - m)^2 - (1-g)(m^2 - \frac{t_0^2 s^2 v_{11}}{b^2})}}{1-g}$$

The term under square root ($\sqrt{\ \ }$) is :

$$\frac{g^2 v_{12}^2}{v_{22}^2} - \frac{2mgv_{12}}{v_{22}} + g\frac{v_{11}}{v_{22}} + gm^2 - g^2\frac{v_{11}}{v_{22}}$$

$$= (\frac{g}{v_{22}}[v_{11} - 2mv_{12} + v_{22}m^2 - gv_{11} + g\frac{v_{12}^2}{v_{22}}]$$

& $\frac{g}{v_{22}} = \frac{t_0^2 s^2}{b^2}$. Hence the result.

6. **Fiducial limits for the strophanthus assay**.

If the tolerances of individual cats are assumed to be normally distributed with constant variance, Fieller's theorem is directly applicable to the calculation of fiducial limits for the potency of tincture A relative to tincture B. We have :

$$s^2 v_{11} = s^2 v_{22} = \frac{0.1200}{7}, \quad v_{12} = 0. \quad \text{Hence}$$

$g = \frac{(2.18)^2 \times 0.1200}{(1.68)^2 \times 7} = 0.0289$, a value small enough to be practically negligible. So, the limits are :

$$R_L, R_U = [1.18 \pm \frac{2.18}{1.68}\{\frac{0.1200}{7} \times (0.9711 + 1.18^2)^{1/2}]/0.9711] = 0.95, 1.48.$$

So, we can say that 1 cc of A may be asserted to have a potency lying between 0.95 cc and 1.48 cc of B.

The purpose of a bioassay is to discover equally effective doses of the standard and test preparations, that is to say, doses whose inverse ratio will estimate the potency of the test preparation relative to the standard. One objection to the direct procedure discussed earlier, is bias produced by time-lag. Even when this danger is absent, technical difficulties may prevent the experimenter from ensuring that subjects receive just the right dose to produce the characteristic response : to determine individual toleences of cats for digitalis may require no more than reasonable skill and care, but to determine individual tolerances of aphids for an insecticide is impossible, and a different method of assay must be sought. This leads us to INDIRECT ASSAYS. We shall first discuss such assays with quantitative responses (and not quantal).

2 Indirect Bio-Assays

In indirect bio-assays the relationship between the dose and response of each preparation is first ascertained. Then the dose corresponding to a given response is obtained from the relation for each preparation separately. Consider two drugs, A and B, each administered at $k(\geq 2)$ prefixed levels (doses) $d_1, \ldots, d_k$. Let Y_{Si} and Y_{Ti} be the response variables for the standard and test preparations, respectively. It is not necessary to have the same doses for both preparations, but the modifications are rather straightforward, and hence we assume this congruence. We assume first that both Y_{Si} and Y_{Ti} are continuous (and possibly nonnegative) random variables. Suppose, further, that there exist some dosage $x_i = \xi(d_i), i = 1, \ldots, k$, and response-metameter $Y^* = g(X)$, for some strictly monotone $g(\cdot)$, such that the two dosage-response regressions may be taken as linear, namely that

$$\begin{aligned} Y^*_{Ti} &= \alpha_T + \beta_T x_{ti} + e_{Ti}, \\ Y^*_{Si} &= \alpha_S + \beta_S x_{si} + e_{Si}, \quad i = 1, \ldots, k, \end{aligned} \tag{2.1}$$

where, for statistical inferential purposes, certain distributional assumptions are needed for the error components e_{Ti} and $e_{Si}, i = 1, \ldots, k$. Generally, in the context of log dose transformations we have a parallel -line assay, while slope-ratio assays arise typically for *power transformations*.

When $x_i = \log(\text{dose})$ is the linearizing transformation, let for $i = 1, \ldots, k$

$$E(Y^*_{Si}) = \alpha_S + \beta x_{si} \tag{2.2}$$

denote the relation between the expected response and x_s where $x_s = \log(d_s)$ and d_s denotes the dose of the standard preparation. Denoting by d_t a dose equipotent to d_s, we have $\rho = d_s/d_t$ that is

$$\log \rho = \log d_s - \log d_t = x_s - x_t.$$

That is, $x_s = \log \rho + x_t$. Substituting for x_s in the relation of the standard preparation (2.2) , we get the relation for the test preparation as

$$\begin{aligned} & E(Y_{Si}^*) &=& \alpha_S + \beta(\log \rho + x_{ti}) \\ \text{that is} \qquad & E(Y_{Si}^*) &=& \alpha_T + \beta x_{ti} = E(Y_{Ti}^*) \end{aligned} \tag{2.3}$$

where $\alpha_T = \alpha_S + \beta \log \rho$. Hence, the relationship for the test preparation is also linear like that of the standard preparation for the same transformation. An examination of the two equations for the two preparations shows that the lines have the same slope and are, therefore, parallel.

Thus, in a parallel-line assay, the two dose-response regression lines (2.1) are taken to be parallel and, further, that the errors e_{Ti} and e_{Si} have the same distribution (often taken as normal). In this setup we then have $\beta_S = \beta_T = \beta$ (unknown), while $\alpha_T = \alpha_S + \beta \log \rho$, where ρ is the relative potency of the test preparation with respect to the standard one. This leads to the basic estimating function

$$\log \rho = \{\alpha_T - \alpha_S\}/\beta, \tag{2.4}$$

so that if the natural parameters β, α_S, and α_T are estimated from the acquired bioassay dataset, statistical inferences on $\log \rho$ (and hence ρ) can be drawn in a standard fashion. If in an assay k doses are taken for each of the two preparations and $\bar{x}_s$ and $\bar{x}_t$ denote the averages of the dose metameters and $\bar{y}_s$ and $\bar{y}_t$ are the average responses for the preparations, then it is known that

$$\begin{aligned} & \alpha_S = \bar{y}_s - \beta \bar{x}_s \\ \text{and} \qquad & \alpha_T = \bar{y}_t - \beta \bar{x}_t. \end{aligned} \tag{2.5}$$

Substituting these values in $\log \rho = \{\alpha_T - \alpha_S\}/\beta$, we get an estimate R of ρ from

$$\log R = \bar{x}_s - \bar{x}_t - \{\bar{y}_s - \bar{y}_t\}/\beta. \tag{2.6}$$

¿From equations (2.2) and (2.3) it is seen that the two lines for the two preparations should be parallel when the dose metameter is log(dose). The assays corresponding to this transformation are, therefore, called parallel line assays.

For normally distributed errors, the whole set of observations pertains to a conventional linear model with a constraint on the two slopes, β_S and β_T , so that the classical maximum likelihood estimators and allied *likelihood ratio tests* can be incorporated for drawing statistical conclusion on the relative potency or the fundamental assumption of parallelism of the two regression lines. However, the estimator of $\log \rho$ involves the ratio of two normally distributed statistics, and hence it may not be unbiased; moreover, generally the classical *Fieller's theorem* (see Finney, 1964) is incorporated for constructing a confidence interval for $\log \rho$ (and hence, ρ). Because of this difference in setups (with that of the classical linear model), design aspects for such parallel-line assays need a more careful appraisal. For equispaced (log), doses, a symmetric $2k$-point design has optimal information contents, and is more popularly used in practice. We refer to Finney(1964) for a detailed study of such bioassay designs in a conventional normally distributed errors model. Two main sources of nonrobustness of such conventional inference procedures are the following:

1. Possible nonlinearity of the two regression lines (they may be parallel but yet curvilinear).

2. Possible nonnormality of the error distributions.

On either count the classical normal theory procedures may perform quite nonrobustly, and their (asymptotic) optimality properties may not hold even for minor departures from either postulation. However, if the two dose-response repressions (linear or not) are not parallel, then the fundamental assumption of parallel-line assays is vitiated, and hence statistical conclusions based on the assumed model may not be very precise.

In a slope-ratio assay, the intercepts α_S and α_T are taken to be the same, while the slopes β_S and β_T need not be the same and their ratio provides the specification of the relative potency ρ. In such slope-ratio assays, generally a power transformation: dosage $=$ (dose)$^\lambda$, for some $\lambda > 0$, is used, and we have

$$\rho = \{\beta_T/\beta_S\}^{1/\lambda}, \tag{2.7}$$

which is typically a nonlinear function of the two slopes β_T and β_S, and presumes knowledge of λ. In such a case the two error components may not have the same distribution even if they are normal. This results in a heteroscedastic linear model (unless $\rho = 1$), where the conventional linear estimators or allied tests may no longer possess validity and efficiency properties. Moreover, because ρ^λ is a ratio of two slopes, its conventional estimator based on the usual estimators of the two slopes is of the ratio type. For such ratio-type estimators, again the well-known Fieller theorem is usually adopted to attach a confidence set to ρ or to test a suitable null hypothesis. Such statistical procedures may not have the exact properties for small to moderate sample sizes. Even for large sample sizes, they are usually highly nonrobust for departures from the model-based assumptions (i.e. linearily of regressions, the fundamental assumption, and normality of the errors). Again the design aspects for such slope-ratio assays need careful study, and Finney(1964) contains a detailed account of this study. Because of the common intercept, usually a $2k + 1$ point design, for some nonnegative integer k, is advocated here.

3 Parallel Line Assays

A parallel line assay in which each of the preparations has an equal number of doses and an equal number of subjects is allotted to each of the doses, is called a symmetrical parallel line assay. We shall discuss here only symmetrical parallel line assays.

Let the number of doses of each of the preparations be k. As there are in all $2k$ doses in this assay, it is called a $2k$-point symmetrical parallel line assay or simply $2k$-point assay.

Let n subjects be allotted to each of the doses and a suitable response be measured from each subject. Suppose further that $s_1, s_2, \ldots, s_k$ denote the doses of the standard preparation and $t_1, t_2, \ldots, t_k$ the same for the test preparation. Denoting the response of the rth subject allotted to the pth dose of the standard preparation by y_{spr} and the rth response from the qth subject of the test preparation by y_{tqr}, the response data are first arranged as in Table 1.

TABLE 1
Response Data from $2k$-Point Assay

	Standard preparation				Test preparation			
Response	Doses				Doses			
	s_1	s_2	$\cdots$	s_k	t_1	t_2	$\cdots$	t_k
	y_{s11}	y_{s21}	$\cdots$	y_{sk1}	y_{t11}	y_{t21}	$\cdots$	y_{tk1}
	y_{s12}	y_{s22}	$\cdots$	y_{sk2}	y_{t12}	y_{t22}	$\cdots$	y_{tk2}
	$\cdots$	$\cdots$	$\cdots$	$\cdots$	$\cdots$	$\cdots$	$\cdots$	$\cdots$
	$\cdots$	$\cdots$	$\cdots$	$\cdots$	$\cdots$	$\cdots$	$\cdots$	$\cdots$
	y_{s1n}	y_{s2n}	$\cdots$	y_{skn}	y_{t1n}	y_{t2n}	$\cdots$	y_{tkn}
Total	S_1	S_2	$\cdots$	S_k	T_1	T_2	$\cdots$	T_k

The analysis of the assay for conducting validity tests and for estimating relative potency becomes very much simplified when the doses of each of the preparations are taken in geometric progression as shown below:

$$s, cs, c^2s, \ldots, c^{k-1}s \quad \text{and} \quad t, ct, c^2t, \ldots, c^{k-1}t$$

where s and t are suitable starting doses of the standard and test preparation respectively and c is a constant which is the same for both the preparations. A further precaution necessary while choosing the doses is that the doses should be evenly distributed in the range of response in which the dose response relationship was investigated for obtaining the linearizing transformation.

Let

$$x_{s_i} = \log c^i s = \log s + i \log c \ \ (i = 0, 1, 2, \ldots, k-1)$$

and

$$x_{t_i} = \log c^i t = \log t + i \log c \ \ (i = 0, 1, 2, \ldots, k-1).$$

Denoting by $\bar{x}_s$ and $\bar{x}_t$ the averages of the doses of the two preparations, we get

$$\bar{x}_s = \log s + \frac{k-1}{2} \log c \tag{3.1}$$

and

$$\bar{x}_t = \log t + \frac{k-1}{2} \log c \tag{3.2}$$

So,

$$x_{s_i} - \bar{x}_s = \left(i - \frac{k-1}{2}\right) \log c$$

and

$$x_{t_i} - \bar{x}_t = \left(i - \frac{k-1}{2}\right) \log c.$$

Case 1 When k is odd we choose the base of the logarithm as c so that $\log c$ is 1. The log dose as deviates from their mean can now be written as below:

Standard preparation

$$-\frac{k-1}{2}, -\frac{k-3}{2}, \ldots, -1, 0, 1, \ldots, \frac{k-1}{2}$$

Test preparation

$$-\frac{k-1}{2}, -\frac{k-3}{2}, \ldots, -1, 0, 1, \ldots, \frac{k-1}{2}$$

By choosing the base of the logarithm as above, these deviate values could be made integers.

Case 2 When k is even, the base of the logarithm is taken as $\sqrt{c}$ so that $\log c$ becomes 2 and hence all the dose deviates $(i-(k-1)/2)\log c$ become odd integers as shown below.

Standard preparation

$$-(k-1), -(k-3), \ldots, -1, 1, 3, \ldots, (k-1)$$

Test preparation

$$-(k-1), -(k-3), \ldots, -1, 1, 3, \ldots, (k-1)$$

The regression contrast for each preparation can now be obtained by multiplying these deviate values by the coresponding dose totals and adding them.

Analysis

As stated earlier the purpose of analysis of indirect bio-assays is two fold. First, it is tested through the analysis of variance technique if, (i) the dose metameter and response relationship is linear and (ii) the two lines for the two preparations are parallel. If the tests reveal that the relationship is linear and the lines are parallel, then the relative potency of the test preparation is estimated from the relation

$$\log R = \bar{x}_s - \bar{x}_t - \frac{\bar{y}_s - \bar{y}_t}{b}$$

We have already obtained $\bar{x}_s$ and $\bar{x}_t$ at (3.1) and (3.2), $\bar{y}_s - \bar{y}_t$ is given by

$$\bar{y}_s - \bar{y}_t = \frac{\sum_i S_i - \sum_i T_i}{kn}$$

The combined regression coefficient of the two preparations as obtained at (3.3) below gives the value of b.

For the first part of the analysis the following contrasts among the dose totals are obtained.

Preparation contrast:

$$(L_p) = -\sum_i S_i + \sum_i T_i \tag{3.3}$$

Combined regression contrast:

$$(L_1) = -\frac{k-1}{2}(S_1+T_1) - \frac{k-3}{2}(S_2+T_2) - \cdots + \frac{k-1}{2}(S_k+T_k)$$

when k is odd.

Combined regression contrast

$$(L_1) = -(k-1)(S_1+T_1) - (k-3)(S_2+T_2) - \cdots + (k-1)(S_k+T_k)$$

when k is even.

The difference between the two regression contrasts of the two preparations is the parallelism contrast.

Parallelism contrast

$$(L_1') = -\frac{k-1}{2}(S_1-T_1) - \frac{k-3}{2}(S_2-T_2) - \cdots + \frac{k-1}{2}(S_k-T_k)$$

when k is odd.

$$= -(k-1)(S_1 - T_1) - (k-3)(S_2 - T_2) - \cdots + (k-1)(S_k - T_k)$$

when k is even.

It is seen that $\bar{y}_s - \bar{y}_t = -\frac{L_p}{kn}$.

Again, when k is odd

$$\begin{aligned} b &= \frac{L_1}{4\left\{\left(\frac{k-1}{2}\right)^2 + \left(\frac{k-3}{2}\right)^2 + \cdots + 1^2\right\}} \\ &= \frac{6L_1}{kn(k^2-1)}. \end{aligned}$$

When k is even

$$\begin{aligned} b &= \frac{L_1}{4\{(k-1)^2 + (k-3)^2 + \cdots + 1^2\}} \\ &= \frac{3L_1}{2kn(k^2-1)}. \end{aligned}$$

The following analysis of variance table is then written for the validity tests and estimation of error variance.

TABLE 2
Analysis of Variance in $2k$-point Assays for Validity Tests

Sources of variation	d.f.	s.s.	m.s.	F
Preparation (L_p)	1	$L_p^2/2kn$		
Regression (combined) (L_1)	1	L_1^2/D		
Prallelism (L_1')	1	$L_1'^2/D$	s_b^2	s_b^2/s^2
Deviation from regression	$2k-4$	By subtraction	s_d^2	s_d^2/s^2
Doses	$2k-1$	$\frac{\sum_i S_i^2 + \sum_i T_i^2}{n} - \frac{\{\sum(S_i+T_i)\}^2}{2kn}$		
Within doses (error)	$2k(n-1)$	By subtraction	s^2	
Total	$2kn-1$	$\sum_{pr} y_{spr}^2 + \sum_{qr} y_{tqr}^2 - \frac{\{\sum(S_i+T_i)\}^2}{2kn}$		

The value of D, the divisor for the regression and the parallelism sums of squares in the above table is $(kn(k^2-1))/6$ when k is odd and $(2kn(k^2-1))/3$ when k is even.

For testing the linearity of regression, the mean squares for the deviations from regression is tested by the F-test using the within mean squares as error. For testing parallelism, the "parallelism" component is tested.

If both these tests are not significant, then the relative potency can be estimated as below.

$$\begin{aligned} \log R &= \bar{x}_s - \bar{x}_t - \frac{\bar{y}_s - \bar{y}_t}{b} \\ &= \log s - \log t + \frac{L_p}{kn} \cdot \frac{kn(k^2-1)}{6L_1} \end{aligned}$$

when k is odd

$$= \log \frac{s}{t} + \frac{(k^2-1)}{6} \cdot \frac{L_p}{L_1}$$

or

$$R = \frac{s}{t} \ \text{antilog} \ \left\{\frac{d(k^2-1)}{6}\frac{L_p}{L_1}\right\}$$

where $d = \log_{10} c$.

When k is even

$$\log R = \log \frac{s}{t} + \frac{L_p}{kn} \cdot \frac{2kn(k^2-1)}{3L_1}.$$

That is

$$R = \frac{s}{t} \text{ antilog } \left\{ \frac{d(k^2-1)}{3} \frac{L_p}{L_1} \right\}.$$

Precision of R can be estimated through Fieller's theorem.

4 Block Designs for Parallel Line Assays

The use of incomplete block designs in bio-assays is limited mainly by the inflexibility of existing incomplete block designs, including the balanced incomplete block (BIB) designs. These designs aim at estimating the differences between all pairs of treatments with the same (or nearly the same) variance. But in bio-assays all contrasts are not of equal importance, especially in symmetrical parallel line and slope-ratio assays, in which two particular contrasts are of major importance. These contrasts for parallel line assays are:-

(i) the difference between the totals of the standard and test preparations (the 'preparation' contrast)

(ii) the pooled estimate of slope (the 'combined regression' contrast).

In symmetrical parallel line assays the numbers of doses of the two preparations (standard and unknown, or test) are the same, doses are equispaced logarithmically, and response depends linearly on log dose. Let $s_1, s_2, \ldots, s_k$ denote k doses (on the logarithmic scale) of the 'standard' preparation arranged in *ascending* order of magnitude, and $t_1, t_2, \ldots, t_k$ denote the k doses of the test preparation arranged in *descending* order of magnitude: the (constant) difference between successive log-doses is the same for both series. (The doses of the two preparations are ordered in opposite directions for notaional convenience.) Further, let S_i, T_i denote the totals of the observations (or responses) for the doses s_i, t_i respectively. For a design in complete blocks of $2k$ subjects, Finney (1964) defines contrasts of these total appropriate for estimating the relative potency of the test preparation and for testing validity (i.e. for verifying the assumptions underlying the method of estimation of relative potency). The first contrast, called the 'preparation' or 'material' contrast, is

$$L_p = \sum_{i=1}^{k} (T_i - S_i).$$

The second is the combined slope or regression contrast, denoted by L_1, and is the sum of the linear contrasts of the dose totals of the two preparations. The two contrasts L_p and L_1 are necessary for estimating the relative potency. The other contrasts provide validity tests: they are the 'parallelism' contrasts, denoted by L_1' (the difference between the two linear contrasts), L_2 and L_2' (the sum and difference of the quadratic contrasts of the dose totals of the two preparations) and further pairs of contrasts of the type L_m and L_m' (sums and differences of mth power contrasts among dose totals of the two preparations).

When an incomplete block design is used for such an assay, the blocks (litters, cages, etc.) are no longer orthogonal to the doses, and some at least of the defined contrasts of the dose total are not free from block effects. Dose effects must therefore first be estimated

by the methods appropriate to the design, and used in place of unadjusted dose totals to calculate the contrasts.

We describe first the method of construction of a series of incomplete block designs for symmetrical parallel line assays from which the 'preparation' contrast L_p and 'combined slope' contrast L_1 can be obtained from unadjusted dose totals, as in the case of ordinary complete block designs.

Any incomplete block design for the k doses of the standard preparation in blocks of size $k'(k' < k)$ is taken. The final design is then obtained by including k' doses of the test preparation in each block of the previous design, using the rule that if the dose s_i of the standard preparation occurs in the block, then the dose t_i should also be included. (We recall that the t_i are numbered in *descending* order of magnitude of dose.) We thus have an incomplete block bio-assay design, in $2k'$ units per block, from which 'preparation' and 'combined slope' contrasts can be obtained from unadjusted dose totals. This is evident from the following considerations. The preparation contrast is independent of block effects because equal numbers of doses of the two preparations occur in each block of the design. Also, since the dose totals S_i and T_i have the same coefficient, but with opposite signs, in the 'combined slope' contrast (as can be easily verified by referring to tables of orthogonal polynomials- (see Das and Giri, 1986), this contrast can be expressed as a weighted total of within-block contrasts, and so is free from block effects. For the same reason, all relevant contrasts of the types L_{2n+1} and L'_{2n+2} can be estimated from unadjusted dose totals. Such contrasts are estimated with full accuracy, and are, clearly, at least as precisely estimated by the incomplete block design as they would be by a complete block design; they will be more precise if the reduction in block size reduces variance. The other contrasts L'_{2n+1} and L_{2n+2} are affected by block differences, and must be estimated by the method appropriate to the design.

These designs are in fact equivalent to singular group divisible partially balanced incomplete block designs- (see Dey, 1986).

If the original design involving k doses of the standard preparation is a BIB design, all the estimated contrasts are uncorrelated. If the original design is any other incomplete block design, such as the cyclic design, estimates of those contrasts (L'_{2n+1} and L_{2n+2}) which are affected by block differences are correlated among themselves.

As an example, consider the construction of a 6-point parallel line assay design in blocks of 4.

The three blocks of a BIB design with $3(= k)$ standard doses as treatments, with $2(= k')$ doses in each block are

$$s_1 \;\; s_2; \;\; s_1 \;\; s_3; \;\; s_2 \;\; s_3. \tag{4.1}$$

The assay design is constructed by adding to these blocks the corresponding doses of the test preparation:

$$\begin{array}{lllllll} \text{Block} & 1) & s_1 & s_2 & t_1 & t_2 \\ & 2) & s_1 & s_3 & t_1 & t_3 \\ & 3) & s_2 & s_3 & t_2 & t_3. \end{array}$$

The coefficients of the contrasts required in this assay are shown in Table 3 (again, we recall that $s_1 < s_2 < s_3$ and $t_1 > t_2 > t_3$).

TABLE 3
Contrasts for 6-point Parallel Line Assay

	log dose					
	s_1	s_2	s_3	t_1	t_2	t_3
L_p	-1	-1	-1	1	1	1
L_1	-1	0	1	1	0	-1
L_1'	-1	0	1	-1	0	1
L_2	1	-2	1	1	-2	1
L_2'	1	-2	1	-1	2	-1

It is easily verified that the preparation (L_p) the 'combined slope' (L_1) and the between-quadratic (L_2') contrasts so estimated from unadjusted dose totals are free from block effects.

A design of particular interest is that in which any block contains only two doses, s_i and t_i. There will thus be k blocks each with two doses: all the contrasts L_p, L_{2n+1} and L_{2n+2}' are estimable free from block effects, while the rest of the contrasts are completely confounded with blocks. This design must be replicated several times for estimation of error.

BIB designs can be used conveniently up to 6-point assays. For 8 or more points, BIB designs require more replicates than are usually convenient in assays. The number of replicates required can be considerably reduced by basing block contents on cyclic designs (See Das and Giri, 1986): a design for a $2k$-point assay in blocks of 4 units, obtained from a circular design of block size 2, is

$$s_1\ s_2\ t_1\ t_2;\quad s_2\ s_3\ t_2\ t_3; \cdots ; s_k\ s_1\ t_k\ t_1.$$

These blocks may be repeated several times to ensure enough degrees of freedom for error.

5 Analysis for Parallel Line Assays Based on BIB Designs

The analysis of parallel line assays consists essentially of two parts. The first part is the computation of the analysis of variance, including sums of squares due to the various contrasts defined earlier, and providing validity tests and the error mean square. The other part consists of estimating the relative potency and its variance, and limits.

The sum of squares for any contrast unaffected by block differences is calculated by

$$\left[\sum_{i=1}^{k} l_i S_i - \sum_{i=1}^{k} l_i T_i\right]^2 \Big/ r(2\sum_{i=1}^{k} l_i^2) \tag{5.1}$$

where $l_1, l_2, \ldots$ are coefficients of the contrast and r is the number of observations (or replicates) on which S_i or T_i is based. The *s.s.* due to the other contrasts are calculated from least squares estimates $s_1, s_2, \ldots, s_k$ and $t_1, t_2, \ldots, t_k$ of the effects of the various doses of the standard and test preparations (we use the same symbols for the doses and their estimated effects, since there is no danger of confusion), using the model

$$y_{mj} = \mu + \beta_j + \delta_m + e_{mj} \tag{5.2}$$

where y_{mj} is the response to dose m in the jth block, μ is the general mean and β_j the jth block effect, δ_m denotes s_m or t_m as may be appropriate, and the error e_{mj} is assumed

to be normally distributed with zero mean and variance, σ^2. The desired contrasts are then calculated from the estimated dose effects, and the corresponding *s.s.* obtained as indicated by earlier. The error *s.s.* is found in general as the difference between the total *s.s.* and (unadjusted block *s.s.* + adjusted dose *s.s.*). When estimates of all contrasts are uncorrelated, as for BIB designs, the adjusted dose *s.s.* can be obtained from the model of the *s.s.* due to all the contrasts. The details of the analysis for designs based on BIB designs follow.

Adjusted dose totals are

$$\begin{aligned} Q_i^S &= S_i - \sum_{j(i)} \bar{y}_j \\ Q_i^T &= T_i - \sum_{j(i)} \bar{y}_j \end{aligned} \tag{5.3}$$

where $\bar{y}_j (j = 1, 2, \ldots, b)$ is the jth block average, and summation is over allblocks containing dose i of the preparations. All the required contrasts are functions of these Q's. Those unaffected by block differences are functions of the differences $Q_i^s - Q_i^T (= S_i - T_i)$: sums of squares due to them are most conveniently obtained, as described earlier, directly from the dose totals. The remaining contrasts are functions of

$$Q_i = Q_i^S + Q_i^T : \tag{5.4}$$

the s.s. due to any affected contrasts, $\sum_{i=1}^{k} l_i Q_i$, is then

$$[\sum_{i=1}^{k} l_i Q_i]^2 / rE(2 \sum_{i=1}^{k} l_i^2) \tag{5.5}$$

where

$$E = (rk' - r + \lambda)/rk',$$

k' being the block size of the starting design and λ the number of blocks in each of which any two given doses of the standard preparation occur together.

The variance of the estimate of an affected contrast is

$$(2 \sum_i l_i^2 / rE)\sigma_{2k'}^2,$$

where $\sigma_{2k'}^2$ is the error variance in incomplete blocks of size $2k'$. If a randomised (complete) block design is used, the corresponding variance is $(2 \sum_i l_i^2 / r)\sigma_{2k}^2$, where σ_{2k}^2 denotes the error variance in complete blocks of $2k$ units. If a BIB with $2k$ doses, blocks of size k_1, and r replications is used, the variance is $(2 \sum_i l_i^2 / rE')\sigma_{k_1}^2$, where E' is the efficiency factor of this BIB design and $\sigma_{k_1}^2$ its error variance. Thus, the efficiency of the present design in respect of affected contrasts is $E\sigma_{2k}^2 / \sigma_{2k'}^2$ when compared with the randomised block design and $(E/E')(\sigma_{k_1}^2 / \sigma_{2k'}^2)$ when compared with the basic BIB design. Hence if the reduction of variance is such that $\sigma_{2k'}^2 / \sigma_{2k}^2 < E$ the present series of bio-assay designs will be more efficient than randomised blocks even for the affected contrasts. Comparison with BIB designs with $2k$ doses is possible only for particular cases. But when the BIB designs are used some information is lost on each contrast, and particularly on L_p and L_1, the contrasts of major importance, which are estimated without loss by the designs mentioned here.

An Example

For illustrating the method of analysis of a 6-point symmetrical parallel line assay Bliss(1952, p. 498) presented a body of data collected on a vitamin D assay by Coward and Kassner(1941): the design used 12 litters of 6 rats as randomized blocks. Here we use these data with some modifications. To ensure comparability of the estimate of relative potency all observations were used, but were fitted into an incomplete block design of the present series by omitting two observations from each of the original blocks (litters), as shown by blanks in Table 4, and forming 6 additional blocks (13-18) from the 24 observations omitted, ignoring litter differences, but retaining the dose-observation relation. The design assumed is that for 6 treatments in blocks of 4 derived in Section 4 from a BIB design: the 3 blocks of the design were replicated 6 times, to accommodate the 72 observations of the original assay. The data and the assumed design are shown in Table 4.

TABLE 4
Data and BIB design based Parallel Line Assay (Doses in mg.)

	Standard			**Test**			
	s_1	s_2	s_3	t_3	t_2	t_1	Block
Block number	2.5	5	10	2.5	5	10	total
1	2	8	-	-	9	7	26
2	6	-	9	3	-	8	26
3	-	6	12	4	6	-	28
4	9	11	-	-	14	13	47
5	10	-	17	8	-	10	45
6	-	7	5	6	9	-	27
7	4	10	-	-	11	13	38
8	11	-	9	3	-	15	38
9	-	9	14	5	8	-	36
10	4	7	-	-	10	10	31
11	12	-	9	15	-	15	51
12	-	8	11	7	8	-	34
13	4	4	-	-	5	9	22
14	7	-	8	3	-	9	27
15	-	15	10	6	8	-	39
16	2	4	-	-	6	6	18
17	4	-	13	5	-	12	34
18	-	10	13	4	18	-	45
Totals	75	99	130	69	112	127	612
	S_1	S_2	S_3	T_3	T_2	T_1	
Adjusted	-25.75	1.25	22.50	-31.75	14.25	19.50	0
totals	Q_1^S	Q_2^S	Q_3^S	Q_3^T	Q_2^T	Q_1^T	

S.S. due to blocks = 358.00

TABLE 5
Contrasts, Divisors and Sums of Squares for Data of Table 4

	Contrast	Divisor	Sum of squares
L_p	4	$6 \times 12 = 72$	0.22
L_1	113	$4 \times 12 = 48$	266.02
L_2'	-35	$12 \times 12 = 144$	8.51
L_1'	-3.00	$4 \times 9 = 36$	0.25
L_2	-46.50	$12 \times 9 = 108$	20.02
Total			295.02

For this design, $r = 12, k' = 2$, and $\lambda = 6$, whence $rE = (rk' - r + \lambda)/k' = 9$. Using the contrast coefficients of Table 3, and formulae (5.1), (5.4) and (5.5), contrasts, divisors, and

sums of squares are obtained as shown in Table 5 for unaffected (L_p, L_1, L_2') and affected (L_1', L_2) contrasts: the analysis of variance is shown in Table 6.

As in the original analysis, all the validity tests are satisfied. Though the estimate of relative potency must remain identical with the original estimate, the mean squares due to error and the affected contrasts will be different. After regrouping the data there are 49 d.f. for error, of which 10 d.f. may be contaminated by differences between the original litters. The mean square for the contaminated component is 6.11, while that for the remaining 39 d.f. is 7.23: the error mean square in the original analysis was 7.22, with 55 d.f. Thus grouping of observations on animals from different litters into the same block has not increased the error mean square (as might have been expected from the significance of the between-block mean square).The estimate of the relative potency is $R = \frac{2.5}{2.5}\ antilog\ \{\frac{4d}{3}.\frac{L_p}{L_1}\} =\ antilog\ \{\frac{16d}{339}\}$ where $d = \log_{10} 2$.

TABLE 6
Analysis of Variance of Data of Table 4

Nature of variation	d.f.	s.s.	m.s.	F
Between Blocks	17	358.00	21.06	3.01**
Preparations	1	0.22	0.22	
Regression	1	266.02	266.02	38.00**
Prallelism	1	0.25	0.25	< 1
Comb. Quadratic	1	20.02	20.02	2.86NS
Diff. quadratic	1	8.51	8.51	1.22NS
All Contrasts	5	295.02		
Error (by substraction)	49	342.98	7.00	
Total	71	996.00		

References

Dey, A. (1986). Theory of Block Designs, Wiley, New Delhi.

Das, M.N. and Giri, N.C. (1986). Design and Analysis of Experiments, Wiley, New Delhi.

Finney, D.J. (1964). Statistical Methods in Biological Assay, 2nd ed. Griffine, London.