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PARALLEL LINE ASSAY WITH SUCCESSIVE ADJUSTMENT OF DOSES

BY

D. J. FINNEY* AND H. O. SCHILD†

From the Department of Statistics, University of Edinburgh, and the Department of Pharmacology, University College London†*

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Parallel line assays in sequences of randomized blocks on single subjects (Schild, 1942) have become a standard procedure, because of their biological convenience and their statistical efficiency. In practice, however, difficulty often arises in choosing the doses of standard and unknown preparations so that corresponding levels are almost equipotent. An unsuccessful choice of doses means a relatively inefficient assay, unless the experimenter restarts the assay with a new set of doses; in either case, he obtains less than the maximum information.

The paper that follows explains how advantage may be taken of the inherently sequential conduct of the assay, in such a way as to change the doses from block to block with the aim of approaching equipotency sequentially. The procedure is described and illustrated for a 4-point (or 2+2) assay, but it can easily be modified to suit other systems of doses.

1. MODEL

Suppose that a biological assay is to be conducted on a single subject, exposed successively to a series of doses of the standard test preparations. Provided that no residual effects of previous doses need be feared, an excellent simple design is to order the doses by means of a series of randomized blocks; for a 4-point design, each block of four successive trials on the subject includes two doses of each preparation in random order (Finney, 1964, §10.7).

Recognizing his inability to make an ideal choice of doses for an assay, an experimenter may seek to change the doses of one or both preparations. In particular, he may aim at finding doses in the ideal range of the response curve and, for an assay design symmetric in the two preparations, such that each dose of the test preparation, T, is matched by an approximately equipotent dose of the standard prepartion, S. He may thus hope to improve the precision of the assay relative to what he would obtain if the doses used for the first block were retained for all others.

There is no theoretical objection to this procedure; the use of results for completed blocks to guide the choice of doses for the next block does not introduce any bias. However, some special problems of statistical analysis arise. The analysis is inevitably more complicated than for the conventional 4-point assay, and in the most general form

quite considerably so, but certain important special cases are relatively easily described and calculated.

For a satisfactory assay in which each block contains only two doses of each preparation, good prior evidence of linearity of response curves is important: the statistical analysis cannot discriminate between non-linear response curves and non-parallelism. Suppose that x, y represent log dose and response respectively. The most general response lines, relating the expectation of y to x, may then be written for block i as:

$$\text{for S, } E(y) \equiv Y_S = \gamma_i - \tfrac{1}{2}\alpha + (\beta - \emptyset)x,$$
$$\text{and for T, } E(y) \equiv Y_T = \gamma_i + \tfrac{1}{2}\alpha + (\beta + \emptyset)x,$$

with variance σ^2 about the expectations. Here γ_i is a block parameter for block i and $\emptyset$ is a measure of departure for parallelism. The assay would not be undertaken unless there were reason to believe that $\emptyset = O$, but a test of this condition is desirable as a test of assay validity. If and only if $\emptyset = O$, the two regression lines are parallel and μ, the logarithm of relative potency, is defined by

$$\alpha = \beta\mu. \tag{2.2}$$

Note that α, β are assumed constant from block to block, and the object of the assay is to use estimates of them in the estimation of μ.

2. DOSES

The most general 4-point assay in r blocks of four can have its log doses, S_1, S_2, T_1, T_2, expressed as

$$x_{1i} - \tfrac{1}{2}d_{1i},\ x_{1i} + \tfrac{1}{2}d_{1i},$$
$$x_{2i} - \tfrac{1}{2}d_{2i},\ x_{2i} + \tfrac{1}{2}d_{2i},\ \text{for } i = 1, 2, \ldots, r.$$

In practice, however, the dose interval in any block is commonly taken as the same for both preparations:

$$d_{1i} = d_{2i} = d_i. \tag{3.1}$$

Often restriction may go further so that

$$d_i = d \text{ for all } i, \tag{3.2}$$

a common dose interval in all blocks.

3. GENERAL ANALYSIS

The assay provides a total of 4r responses, and the model in equations (2.1) implies (r + 3) parameters. These may be estimated by a general least-squares procedure. Indeed, since r of the parameters are the γ_i, the remaining three can be obtained from least squares applied to intra-block variation. If this analysis shows no reason to fear that $\emptyset \neq O$, the analysis can be recomputed with the constraint $\emptyset = O$ imposed, and new estimates of α, β can be used to estimate μ from (2.2).

The wide availability of high-speed computers makes such an analysis no great task. Existing general programmes could be readily adapted, or a special programme written for assay use. Moreover, extension to larger blocks with more doses of each preparation would be easy. However, an alternative approach is perhaps illuminating and certainly

aids discussion of efficiency. The main presentation assumes condition (3.1), but Section 11 relaxes this.

4. CONTRASTS

Write y_{1i}, y_{2i}, y_{3i}, y_{4i} as the observed responses in block i to the doses S_1, S_2, T_1, T_2, respectively. Define *intra-block contrasts* as

$$\left.\begin{aligned} L_{1i} &= -y_{1i} - y_{2i} + y_{3i} + y_{4i}, \\ L_{2i} &= -y_{1i} + y_{2i} - y_{3i} + y_{4i}, \\ L_{3i} &= y_{1i} - y_{2i} - y_{3i} + y_{4i}. \end{aligned}\right\} \tag{5.1}$$

These are mutually orthogonal. From (2.1), their expectations are

$$E(L_{1i}) = 2\alpha + 2\beta(x_{2i} - x_{1i}) + 2\emptyset(x_{1i} + x_{2i}), \tag{5.2}$$

$$E(L_{2i}) = 2\beta d_i, \tag{5.3}$$

$$E(L_{3i}) = 2\emptyset d_i. \tag{5.4}$$

The variance of each contrast is $4\sigma^2$.

Evidently the L_{1i} and L_{2i} provide information on β, and the L_{1i} and L_{3i} provide information on $\emptyset$. A logical procedure would be to estimate $\emptyset$ from the L_{3i} and also to estimate $\emptyset$ simultaneously with other parameters from the L_{1i}. The orthogonality of L_{1i}, L_{3i} then makes possible a combination of estimates in a weighted mean, which can be tested against an estimated standard error. With strong prior belief in parallelism, a test based on the L_{3i} alone may seem adequate. The optimally weighted mean of the r values $L_{3i}/2d_i$ is

$$p = \Sigma L_{3i} d_i / 2\,\Sigma d_i^2, \tag{5.5}$$

where Σ denotes summation over $i = 1,2, \ldots, r$. The variance of p is

$$V(p) = \sigma^2 / \Sigma d_i^2 \tag{5.6}$$

The test for parallelism may be taken as a comparison of p with a theoretical value O, using an estimate of σ^2 in (5.6); the estimation of σ^2 is discussed in Section 6.

5. ESTIMATION OF POTENCY

If $\emptyset$ can be taken as O, estimation of the remaining parameters follows easily from the rewritten forms of (5.2)–(5.4), which may conveniently be discussed in reverse order.

By consideration of the variation of the individual values of $L_{3i}/2d_i$ about their weighted mean p, a sum of squares

$$\left[\Sigma L_{3i}^2 - \frac{(\Sigma L_{3i} d_i)^2}{\Sigma d_i^2}\right] / 4 \tag{6.1}$$

with (r–1) d.f. is obtained; its mean square estimates σ^2. In an exactly similar manner, the values of L_{2i} yield an estimate of β as a weighted mean,

$$b_2 = \Sigma L_{2i} d_i / 2\,\Sigma d_i^2, \tag{6.2}$$

with variance

$$V(b_2) = \sigma^2 / \Sigma d_i^2. \tag{6.3}$$

The sum of squares

$$\left[\Sigma L_{2i}^2 - \frac{(\Sigma L_{2i} d_i)^2}{\Sigma d_i^2}\right] / 4 \tag{6.4}$$

with (r–1) d.f. also estimates σ^2.

Equation (5.2), when $\emptyset = O$, can be regarded as the specification of a linear regression relation between L_{1i} and

$$u_i = 2(x_{2i} - x_{1i}), \tag{6.5}$$

where 2α and β are the parameters of the regression. First calculate

$$\bar{u} = \Sigma u_i / r. \tag{6.6}$$

$$\bar{L}_1 = \Sigma_{1i} / r. \tag{6.7}$$

Then, using the notation (applicable to all subsequent expressions in square brackets)

$$[f_i, g_i] = \Sigma f_i g_i - \frac{(\Sigma f_i)(\Sigma g_i)}{r}, \tag{6.8}$$

estimates of β, α are:

$$b_1 = [L_{1i}, u_i]/[u_i, u_i], \tag{6.9}$$

$$a = (\bar{L}_1 - b_1\bar{u})/2. \tag{6.10}$$

The relevant variances are

$$V(\bar{L}_1 (= 4\sigma^2/r, \tag{6.11}$$

$$V(b_1) = 4\sigma^2 / u_1, u_1]. \tag{6.12}$$

Also, the residual sum of squares about the regression leads to

$$\left\{ [L_{1i}, L_{1i}] - \frac{[L_{1i}, u_i]^2}{[u_i, u_i]} \right\} / 4 \tag{6.13}$$

as a sum of squares with (r-2) degrees of freedom estimating σ^2.

The orthogonality of the L_{1i} and the L_{2i} ensures that the b_1 and b_2 are independent estimators of b ; they can be combined by weighting inversely as their variances, to give

$$b = \frac{b_1[u_i, u_i] + 4b_2 \Sigma d_i^2}{[u_i, u_i] + 4 \Sigma d_i^2}, \tag{6.14}$$

with

$$V(b) = \frac{4\sigma^2}{[u_i, u_i] + 4 \Sigma d_i^2}. \tag{6.15}$$

Moreover, comparison between b_1, b_2 leads to one additional estimate of α^2, which may be most compactly written

$$\frac{[u_i, u_i] \Sigma d_i^2}{[u_i, u_i] + 4 \Sigma d_i^2} (b_1 - b_2)^2. \tag{6.16}$$

The sums of squares (6.1) and (6.4) can be combined to give an estimate of σ^2 with 2(r-1) d.f., which is independent of the value of $\emptyset$ and so is properly used in a test of significance of p. However, if $\emptyset$ is zero, the components (6.13 and (6.16) can also be included to give a mean square, s^2, with 3(r-1) d.f. This can be inserted in (6.11), (6.15), and then employed in assessment of the precision of the estimated relative potency in the usual manner. In fact, now with

$$a = (L, - b\bar{u})/2$$

$$M = \frac{a}{b} = \frac{L_1}{2b} - \frac{\bar{u}}{2} \tag{6.17}$$

is an estimator of the logarithm of relative potency. Since $\bar{u}$ has no sampling variation, $V(\bar{L}_1)$ and V(b) can be used in Fieller's theorem (Finney, 1964, § 2.5) to obtain fiducial limits for M ; if b is large relative to its standard error, an asymptotic standard error for M can be stated in the usual manner.

6. NUMERICAL EXAMPLE

Table 1 contains records of a histamine assay on guinea-pig ileum. The doses were chosen sequentially by an experimenter who was unaware of the true activity ratio. He maintained a constant d_i throughout, so satisfying condition (3.2) and giving

$$\Sigma_i d_i^2 = rd^2 \tag{7.1}$$

where, for this assay, $r=5$ and $d=\log 2$. The doses of T were held constant throughout. but from the statistical viewpoint this is immaterial.

TABLE 1
RECORDS OF A HISTAMINE ASSAY

Block	Dose symbol	Dose*	Response
I	T_1	1	43
	T_2	2	68
	S_2	16	40
	S_1	8	22
II	S_1	20	56
	T_1	1	44
	T_2	2	70
	S_2	40	92
III	S_1	12	39
	S_2	24	64
	T_2	2	71
	T_1	1	42
IV	T_2	2	68
	T_1	1	46
	S_2	28	72
	S_1	14	42
V	T_1	1	41
	T_2	2	67
	S_1	17	58
	S_2	34	80

* Units of μg/l histamine for S, arbitrary units for T.

7. ANALYSIS OF THE EXAMPLE

In the example in Section 6, $r=5$ and the x_{2i} are constant for all blocks. A first step is the tabulation of the contrasts for each block, after which the formulae obtained earlier can be applied. Table 2 shows these contrasts, which are easily formed from equations (5.1). Values of d_i and u_i are included; by taking logarithms to base 2, d_i could have been made 1.0, but this represents little saving of labour.

TABLE 2
CONTRASTS FOR THE NUMERICAL EXAMPLE

i	Block total	d_i	u_i	L_{1i}	L_{2i}	L_{3i}
1	173	0·3010	−1·8062	49	43	7
2	262	0·3010	−2·6021	−34	62	−10
3	216	0·3010	−2·1584	10	54	4
4	228	0·3010	−2·2923	0	52	−8
5	246	0·3010	−2·4609	−30	48	4
Total	1125		−11·3199	−5	259	−3

Now

$$\Sigma d^2_i = 5 \times (0.3010)^2 = 0.4530. \tag{8.1}$$

Therefore by (5.5)

$$p = \frac{-3 \times 0.3010}{0.9060} = -0.997. \tag{8.2}$$

The constancy of d_i means that (6.1) simplifies to

$$\left[\Sigma L^2_{3i} - \frac{(\Sigma L_{3i})^2}{r}\right] / 4 \tag{8.3}$$

and similarly for (6.4). These two sums of squares, each with 4 d.f. in the example, are found to be 60.80 and 50.20 respectively; an estimate of σ^2 for use in (5.6) is

$$\frac{60.80 + 50.20}{4+4} = 13.88 \text{ with } 8 \text{ d.f.} \tag{8.4}$$

Hence the estimated standard error of p is

$$\sqrt{\frac{13.88}{0.4530}} = 5.54. \tag{8.5}$$

Clearly p is so small relative to its standard error that it makes no suggestion of non-parallelism.

From (6.2),

$$b_2 = \frac{\Sigma L_{2i}}{10d} = \frac{259}{3.010} = 86.05. \tag{8.6}$$

Then from (6.6) to (6.13),

$$\bar{u} = -2.2640, \tag{8.7}$$

$$\bar{L}_1 = -1.00, \tag{8.8}$$

$$b_1 = \frac{40.8907}{0.374614} = 109.15, \tag{8.9}$$

and the residual sum of squares (3 d.f.) is

$$\frac{1}{4}\left[4552.00 - \frac{40.8907^2}{0.374614}\right] = 22.15. \tag{8.10}$$

Combination of b_1, b_2 according to (6.14) gives

$$b = \frac{40.8907 + 155.9226}{0.374614 + 1.8120} = \frac{196.81}{2.1866} = 90.01. \tag{8.11}$$

The contribution to the error sum of squares from the difference between regression coefficients, expression (6.16), is then

$$\frac{0.3746 \times 0.4530}{2.1866}(109.15 - 86.05)^2 = 41.41. \tag{8.12}$$

Table 3, the analysis of variance, can now be completed. In this analysis, the components for preparations, regression, and parallelism are readily obtainable from formulae in Section 5 as

$$(\Sigma L_{1i})^2/4r, \tag{8.13}$$

$$\{b_1[u_i, u_i]+4b_2\Sigma d_i^2\}^2/4\{[u_i, u_i]+4\Sigma d_i^2\}, \tag{8.14}$$

and

$$(\Sigma L_{3i}d_i)^2/4\Sigma d_i^2 \tag{8.15}$$

TABLE 3

ANALYSIS OF VARIANCE FOR TABLE 1

Source	Degrees of freedom	Sums of squares	Mean squares
Blocks	4	1151·00	
Preparations (L_1)	1	1·25	1·25
Regression (b)	1	4428·49	
Parallelism (L_3)	1	0·45	0·45
Errors			
From L_{2i}	4	50·20	14·55
From L_{3i}	4	60·80	
Residuals from b	3	22·15	
Difference (b_2-b_1)	1	41·41	
Total	19	5755·75	

The mean squares for the separate contributions to error seem satisfactorily homogeneous, despite the moderately large single degree of freedom for (b_2-b_1), and a pooled mean square is used henceforth as s^2 with 12 d.f. There is no suggestion of lack of parallelism, and the mean square for preparations is also satisfactorily small.

8. POTENCY ESTIMATION

From (6.17), the logarithm of relative potency is estimated as

$$M=\frac{-1.00}{180.02}+1.1320$$
$$=1.1264 \tag{9.1}$$

The detailed arithmetic for finding limits need not be displayed here; it leads to 1.0847, 1.1680 as 95% fiducial limits. Evaluation of antilogarithms then gives the estimated potency of T as 13.38 μg histamine per unit dose, with 95% limits at 12.15, 14.72 (the true potency was 13 μg per unit dose).

9. EFFICIENCY

In the example just discussed, b is so much greater than its standard error (and "g" therefore so small) that the asymptotic variance formula could adequately have described the precision of M and led to the fiducial limits. This is*

$$V(M)=\frac{s^2}{4b^2}\left[\frac{4}{5}+\frac{0.0056^2\times 16}{2.1866}\right]$$
$$=\frac{s^2}{4b^2}\times 0.8002. \tag{10.1}$$

* In the notation of this paper, equation (2.10) of Finney (1964) gives the estimate of asymptotic variance as

$$V(M)=\frac{V(\bar{L}_1)}{4b^2}+\left(M+\frac{\bar{u}}{2}\right)^2\frac{V(b)}{b^2},$$

whence (6.11), (6.15) lead to (10.1).

Suppose that the procedure of changing doses had not been adopted. Some interest lies in inquiring how the precision of estimation might have been altered, and this can be done in terms of asymptotic variances with the assumption that s^2, b, and M would have been substantially the same. One benefit from the changes of doses is the information on β derivable from the values of L_{1i}. If all doses were kept the same throughout the assay, u_i would be constant, and the variance of the best estimator of β would be

$$\frac{\sigma^2}{\Sigma d_i^2}$$

instead of (6.15).

If the investigator had regarded his first block as containing the best choice of doses and had maintained these throughout, he would have had $u_i = 1.8062$ for five blocks, and in place of the quantity in [ ] in (10.1) the expression for the variance would have

$$\frac{20}{25} + \frac{(0.2233)^2 \times 16}{1.8120} = 1.2403$$

The design with change of doses therefore has an efficiency relative to this design of

$$\frac{1.2403}{0.8002} = 1.550.$$

Alternatively efficiency might be considered relative to a design in which the investigator had guessed the doses used in Block 5 as those to be used throughout. The efficiency of the assay with changes of dose on this basis is 1.119. These efficiencies will tend to be under-assessments of the real gain from the design actually used, because they neglect " g " in Fieller's theorem and this quantity will be slightly larger for the two alternative designs. Indeed, in this instance the assay achieved about the highest possible precision, and would not have been bettered if $\frac{1}{2}u_i = -M$ could have been maintained throughout.

10. CHOICE OF DOSES

The general principles of choosing doses for a biological assay will not be altered by the possibility of sequential change of doses. The aim will still be to spread the doses widely while evading the risk that any fall outside the linear sections of response curves. Somewhat greater caution in the avoidance of extreme doses for the first block is perhaps justified, because later blocks can compensate for this.

The additional consideration arising is that ΣL_{1i} should be close to zero. The doses chosen for each block should take advantage of the experience so far and, using preliminary estimates of β, should aim at compensating for any excess of values of L_{1i} on either side of zero. It is more important that ΣL_{1i} should be close to zero than that individual values of L_{1i} should be.

11. UNEQUAL DOSE INTERVALS

When equation (3.1) is not satisfied, the case for using a general least-squares analysis (Section 3) is stronger. Nevertheless, the method described above can be adapted. Two

of the three intra-block contrasts need to be modified; L_{1i} is unaltered, but the others are now best defined as

$$\left.\begin{aligned} L_{2i} &= -d_{1i}y_{1i}+d_{1i}y_{2i}-d_{2i}y_{3i}+d_{2i}y_{4i}, \\ L_{3i} &= \ d_{2i}y_{1i}-d_{2i}y_{2i}-d_{1i}y_{3i}+d_{1i}y_{4i}. \end{aligned}\right\} \tag{12.1}$$

The three remain orthogonal. Now $E(L_{1i})$ remains as in (5.2),

$$E(L_{2i})=\beta(d_{1i}^2+d_{2i}^2)+\emptyset(d_{2i}^2-d_{1i}^2), \tag{12.2}$$

$$(E(L_{3i})=\beta(d_{2i}^2-d_{1i}^2)+\emptyset^2(d_{1i}^2+d_{2i}^2). \tag{12.3}$$

with

$$V(L_{1i})=4\sigma^2, \tag{12.4}$$

$$(V(L_{2i})=V(L_{3i})=2\sigma^2(d_{1i}^2+d_{2i}^2) \tag{12.5}$$

Partial regression procedures can be used on (5.2), (12.2), (12.3) to lead to a significance test on a weighted mean estimate of $\emptyset$. If this mean is satisfactorily small, each of the three types of contrast can be used to contribute to estimation of β. The calculations are more laborious but not intrinsically more difficult than those described in Section 5.

SUMMARY

1. In a biological assay based upon a sequence of responses of a single subject to different doses of two preparations, it is sometimes convenient to change the doses during the course of the assay.

2. This paper describes the statistical analysis of a 4-point (2+2 dose) assay in which the doses are altered sequentially with the aim of approaching equipotency.

3. A numerical example is discussed in detail, with some account of its precision relative to assays with fixed doses.

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