Method of graphical analysis of 2 + 2 and 3 + 3 biological assays with graded responses

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Three examples of 2 + 2 and 3 + 3 biological assays with graded responses are analysed graphically. The method described has the advantage that only two "general purpose" nomograms are necessary for the analysis of assays with a wide range of ratios of successive doses and numbers of observations; assays with block restrictions may also be analysed. The accuracy of the method is discussed. An appendix gives the theory of the method and of the construction of the nomograms.

THE biological assay of solutions of known composition but unknown concentration can be considered complete only when the relative potency and its fiducial (or confidence) limits have been determined. Preferably, in my opinion, the experimenters themselves should make these determinations; however, biologists often find the standard statistical computations tedious and liable to error. In this paper some alternative graphical methods are described; they are offered for use as a check on the standard methods and, in many cases, as a substitute for them.

A similar procedure was first devised by the author for the analysis of routine tests of synthetic compounds in an industrial pharmacological research laboratory. However, the statistical bases of many of the tests and of parallel line dilution assays (Finney, 1952) are equivalent. Hence two "general purpose" nomograms have been prepared for 2 + 2 and 3 + 3 assays with graded responses.

The method described here differs from previous graphical approaches in three main ways. First, a log-dose response diagram is plotted, and hence less arithmetic is required. Secondly, the present method differs from others (Healy, 1949; Gridgeman, 1951; Leech & Grundy, 1953) by using an approximation to the fiducial limits of the relative potency instead of the exact limits, although it resembles these and most other graphical procedures in estimating variability of the observations by the range. Thirdly, these nomograms may be used for a wide range of ratios of successive doses and numbers of observations, and for assays with block restrictions. However, as with other methods, the ratio of successive doses of both test and standard preparations must be the same within any one assay, and there must be equal numbers of observations on each dose of both preparations.

The nomograms and their use

The nomograms are illustrated in Figs 1 and 2. For use they are printed on transparent photographic film. Each nomogram is about 12 × 10 inches in size, the response sum scales on the left hand sides being in inches. The theoretical basis of constructing the nomograms is described in a statistical appendix.

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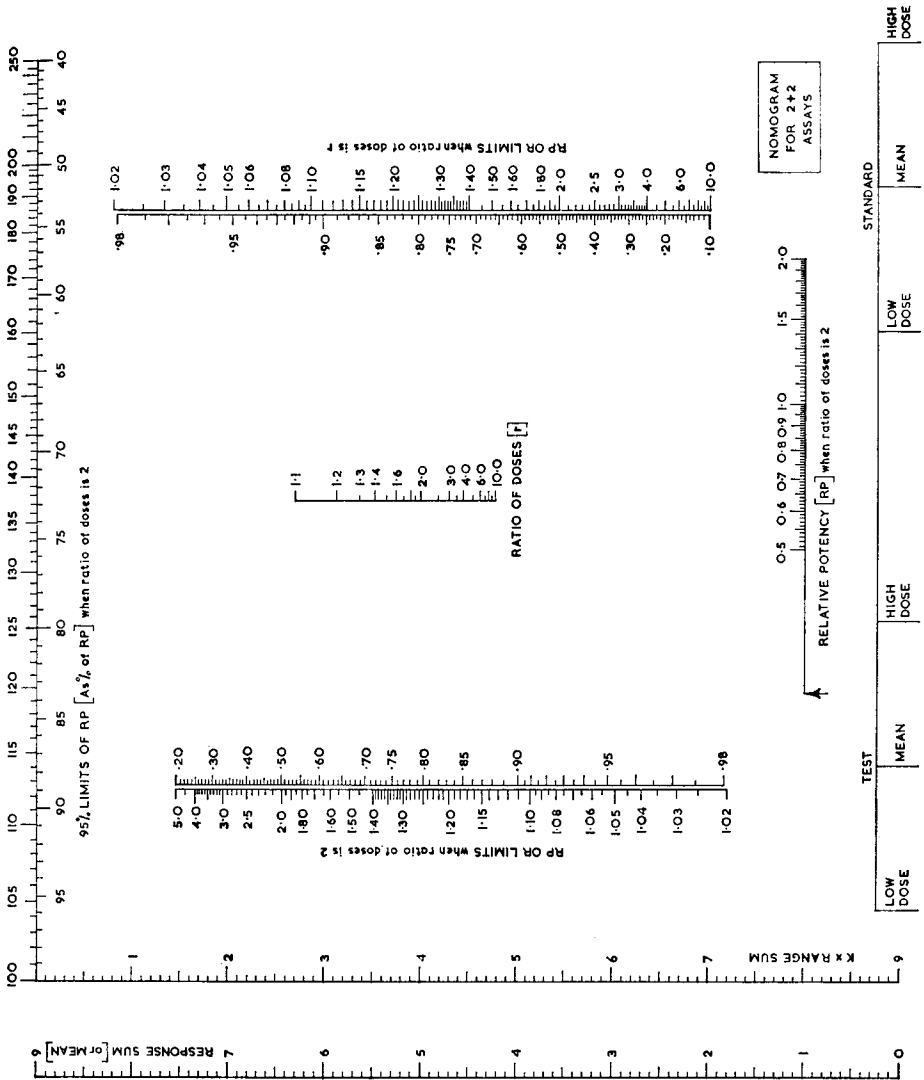
FIG. 1. Nomogram for 2 + 2 assays. Diagram is exactly half size.

Full-size copies of both the nomograms, Figs. 1 & 2, printed on acetate sheet, will be made available until December 1965 at the inclusive price of 12s. 6d. Cash with order to The Pharmaceutical Press, 17, Bloomsbury Square, London, W.C.1.
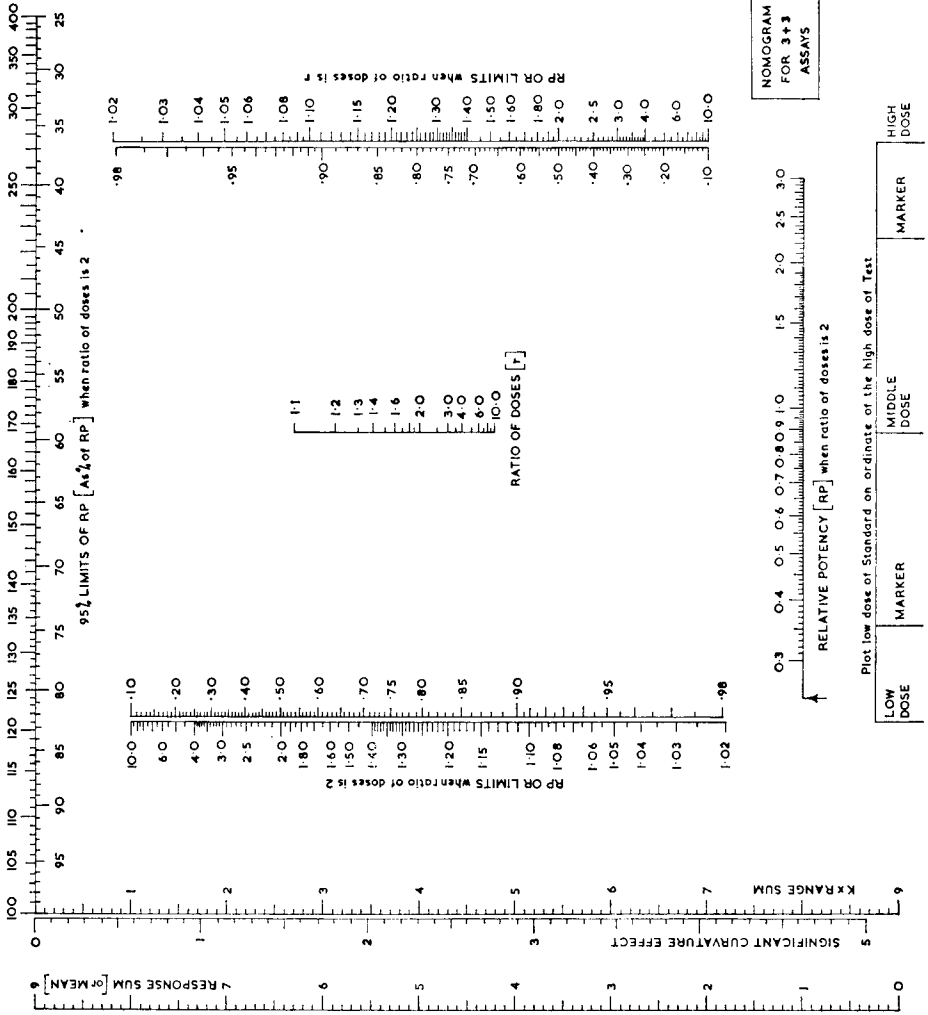
FIG. 2.   Nomogram for 3 + 3 assays.   Diagram is exactly half size.

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The results given initially by the nomograms are those that would be obtained if the ratio of successive doses was 2. Each nomogram includes an alignment diagram to convert the results if the ratio is not 2.

The use of the nomograms is illustrated by 3 examples.

EXAMPLE 1 : A 2 + 2 ASSAY WITHOUT BLOCK RESTRICTIONS

Table 1 shows the data and calculations and Fig. 3 the graphical construction for a 2 + 2 assay without block restrictions. For each dose the response sum and range (i.e. highest − lowest response) are determined. Paper graduated in inches and tenths (or twentieths) is used to plot the data. A suitable scale for plotting the response sums is chosen: in this example, 50 units per inch. If it is more convenient, the response means may be plotted.

TABLE 1. A 2 + 2 ASSAY OF PENICILLIN
(data from British Pharmacopoeia, 1958, p. 904)

	Test		Standard	
	0·6 ml (T_1)	1·2 ml (T_2)	0·6 ml (S_1)	1·2 ml (S_2)
Responses	156 155 163 158	190 194 190 190	172 171 157 177	202 199 200 200
Sum	632	764	677	801
Range	8	4	20	3

The response sums are plotted on a scale of 50 units per inch :
Relative potency, RP = 0·80
Range sum = 35, K = 2·08, K × range sum = 72·8
95% limits of RP = 90%, 111% of 0·80
　　　　　　 = 0·72, 0·888
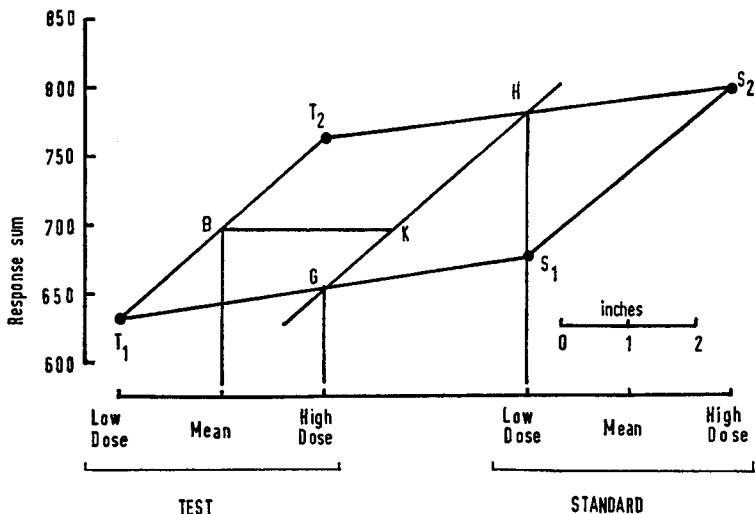Parallelism effect, L'_1 = 764 − 632 − (801 − 677) = 8, which is not significant at the 5% level, since $2|L'_1| < K ×$ range sum.

FIG. 3. Graph for 2 + 2 assay (data from Table 1).

The dosage scale is shown at the bottom of the nomogram (Fig. 1); the low and high doses of the test preparation and the low and high doses of the standard preparation are placed successively at intervals of 3 inches. Denoting the plotted points by T_1, T_2, S_1, S_2 respectively, the quadrilateral $T_1T_2S_2S_1$ is drawn (Fig. 3). The line GH joining the points where T_1S_1 cuts the ordinate for the high dose of the test preparation and T_2S_2 cuts the ordinate for the low dose of the standard preparation has a slope equal to the mean slope of the log-dose response lines of the two preparations. The horizontal distance, BK, from the point where T_1T_2 cuts the "test mean" ordinate to the line GH is measured against the scale on the nomogram to determine the relative potency.

To find the 95% fiducial limits (abbreviated to 95% limits in the remainder of this paper) of the relative potency, the sum of the ranges is multiplied by the factor K found from Table 2. The nomogram is placed over the

TABLE 2.   VALUES OF K IN 2 + 2 ASSAYS

Number of responses on each dose	Response sums plotted		Response means plotted	
	No block restrictions	With block restrictions	No block restrictions	With block restrictions
2	3·36	5·83	1·68	2·92
3	2·31	3·27	0·770	1·09
4	2·08	2·50	0·520	0·624
5	2·01	2·10	0·403	0·419
6	2·00	1·84	0·334	0·306
7	2·01	1·66	0·287	0·237
8	2·02	1·52	0·253	0·190
9	2·05	1·41	0·228	0·157
10	2·08	1·32	0·208	0·132

graph so that the line GH cuts the scale for "K × range sum" at the point corresponding to its calculated value measured on the same scale as the response sums (or means) have been plotted; it is essential that the "K × range sum" scale is vertical, i.e. parallel to the ordinates on the graph. In this example, the value of 72·8 at 50 units per inch is represented by a length of 1·456 inches on the scale. The point where the line GH cuts the scale for the 95% limits gives these limits as percentages of the relative potency.

Two validity tests are usually made in 2 + 2 assays: the slope should be significant, but the parallelism effect should not. If, when determining the 95% limits, the line GH cuts the scale for these limits, the slope is significant, at least at the 5% level. The parallelism effect, L_1', is found by substituting the response sums or means, whichever have been plotted, in the formula

$$L_1' = T_2 - T_1 - (S_2 - S_1)$$

In 2 + 2 assays, this effect is significant at the 5% level if

$$2|L_1'| > K \times \text{range sum}$$

In this example, the assay is valid and, since the ratio of high to low doses is 2, the relative potency and its limits do not have to be converted.

EXAMPLE 2: A 2 + 2 ASSAY WITH BLOCK RESTRICTIONS

Table 3 shows the data and calculations for a 2 + 2 assay in which the doses were given in random order, in blocks of 4; the standard analysis is a randomised block analysis. The ratio of the high to the low dose of each preparation is 1·5, and the relative potency and its limits have to be converted at the final stage of the graphical analysis.

TABLE 3. A 2 + 2 ASSAY OF VASOPRESSIN ON RAT BLOOD PRESSURE
(from Walsh, J., personal communication)

Block	Responses to				Deviations from response mean				Range
	T_1	T_2	S_1	S_2	T_1	T_2	S_1	S_2	
1	31	40	29	38	− 8·75	− 11·25	− 8·25	− 9	3·00
2	40	56	40	46	0·25	4·75	2·75	− 1	5·75
3	46	55	40	53	6·25	3·75	2·75	6	3·50
4	42	54	40	51	2·25	2·75	2·75	4	1·75
Sum	159	205	149	188	0	0	0		14·00
Mean	39·75	51·25	37·25	47					

Ratio of doses, r = 1·5, K = 2·50, K × range sum = 2·50 × 14·00 = 35·0
Parallelism effect, L'_1, = 205 − 159 − (188 − 149) = 7, which is not significant at the 5% level, since $2|L'_1| < K$ × range sum.
Assuming r = 2, relative potency, RP, = 1·25
and 95% limits of RP = 86%, 116% of 1·25
= 1·075, 1·45
Since r = 1·5, converted relative potency = 1·14
with 95% limits of 1·045, 1·24

Assuming the ratio of doses is 2, the relative potency is determined by the graphical procedure described for Example 1.

As shown in Table 3, the deviation of each observation from the mean of the responses to its particular dose is found; these deviations should add to zero, and this provides a check on their determination. The range of the deviations of the 4 observations in each block is determined. The sum of these ranges is used in the same way as the range sum in an assay without block restrictions to determine the 95% limits of the relative potency and to test the parallelism effect.

The three scales labelled "RP or limits when ratio of doses is 2", "RP or limits when ratio of doses is r", and "Ratio of doses" form the alignment diagram which is used to convert the relative potency and its 95% limits when the ratio of doses differs from 2. The procedure is the same for the relative potency and each of its limits. A straight-edge is placed to cut the appropriate scales at the value found assuming the ratio of doses is 2 and at the value of the actual ratio of doses. The straight-edge will then cut the third scale at the correct value with the actual ratio of doses.

Each "RP or limits" scale has separate calibrations for values greater and less than 1. The relative potency (and its limits) will remain greater or less than 1 when converted to any other value of the ratio of doses. For relative potencies or limits between 0·98 and 1·02, the following approximate formula can be used.

RP (or limit) when ratio of doses is r =

$$1 + \frac{\log_{10} r}{\log_{10} 2} \{[\text{RP (or limit) when ratio of doses is 2}] - 1\}$$

EXAMPLE 3.   A 3 + 3 ASSAY WITH BLOCK RESTRICTIONS

Table 4 shows the data and calculations, and Fig. 4 the graphical construction for a 3 + 3 assay with block restrictions.

TABLE 4.   A 3 + 3 ASSAY OF THE POTENCY OF KALLIDIN RELATIVE TO BRADYKININ ON GUINEA-PIG ILEUM

(from Shorley, P. G., personal communication)

	Responses to						Deviations from response mean						
	Kallidin			Bradykinin			Kallidin			Bradykinin			
Block	T_1	T_2	T_3	S_1	S_2	S_3	T_1	T_2	T_3	S_1	S_2	S_3	Range
1	16·0	36·5	53·5	27·5	46·5	54·5	4·3	−1·7	1·3	4·0	4·2	1·7	6·0
2	10·5	33·5	52·0	23·5	34·0	51·0	−1·2	−4·7	−0·2	0	−8·3	−1·8	8·3
3	8·5	44·5	51·0	19·5	46·5	53·0	−3·2	6·3	−1·2	−4·0	4·2	0·2	10·3
Sum	35·0	114·5	156·5	70·5	127·0	158·5	−0·1	−0·1	−0·1	0	0·1	0·1	24·6
Mean	11·7	38·2	52·2	23·5	42·3	52·8							

The response means are plotted on a scale of 10 units per inch
Ratio of doses, r = 1·4, K = 0·409, K × range sum = 0·409 × 24·6 = 10·06
Parallelism effect, L'_1, = 52·2−11·7−(52·8−23·5) = 11·2, which is significant at the 5% level, since $|L'_1| >$ K × range sum
Combined curvature effect, L_2, = 0·40+0·25 = 0·65, which is significant at the 5% level, as judged by the "significant curvature effect" scale.
Difference of curvature effects, L'_2, = 0·40−0·25 = 0·15, which is not significant at the 5% level, as judged by the "significant curvature effect" scale.
Assuming r = 2, relative potency, RP = 0·80
and 95% limits of RP = 85%, 118% of 0·80
= 0·68, 0·94
Since r = 1·4, converted relative potency = 0·90
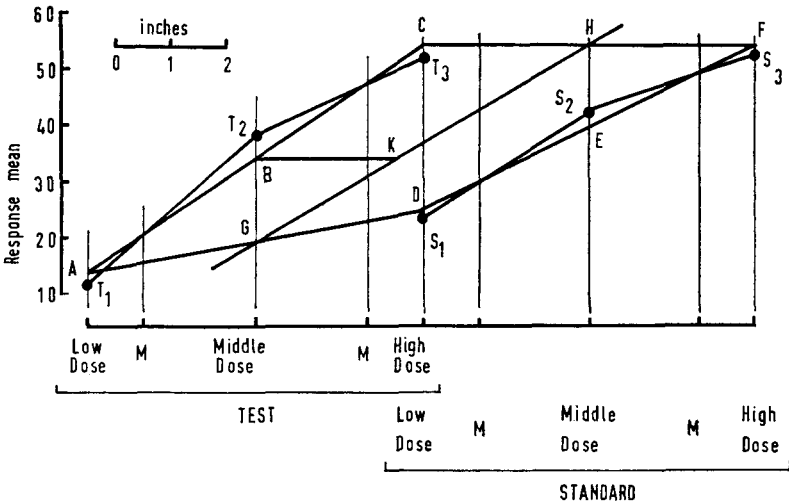with 95% limits of 0·83, 0·97

FIG. 4.   Graph for a 3 + 3 assay (data from Table 4).

The dosage scale is shown at the bottom of the nomogram (Fig. 2); the low, middle and high doses of each preparation are plotted at intervals of 3 inches, with the high dose of the test preparation and the low dose of the standard preparation at the same point on the scale.   Let the plotted

points, either response sums or response means, be T_1, T_2, T_3, S_1, S_2, S_3. For each preparation two marker ordinates, M, between each pair of doses and 1 inch from the low dose or high dose ordinate are used.

The regression line for the test preparation is constructed by drawing a line through the points where T_1T_2 and T_2T_3 cut the marker ordinates; suppose it cuts the ordinates of the three doses of the test preparation at A, B, C respectively. The regression line for the standard preparation is similarly constructed to cut the ordinates of the doses of the standard preparation at D, E, F respectively.

The line GH joining the points where AD and CF cut the ordinates for the middle doses of the test and standard preparations respectively has a slope equal to the mean slope of the two regression lines. The horizontal distance BK from B to the line GH is measured against the scale on the nomogram to determine the relative potency.

The range sum is determined as in 2 + 2 assays with block restrictions. In this example, the deviations for the doses do not add exactly to zero because the response means are corrected to one decimal place. The values of K for 3 + 3 assays are given in Table 5.

TABLE 5.   VALUES OF K IN 3 + 3 ASSAYS

Number of responses on each dose	Response sums plotted		Response means plotted	
	No block restrictions	With block restrictions	No block restrictions	With block restrictions
2	1·00	1·96	0·501	0·981
3	0·73	1·23	0·245	0·409
4	0·67	0·96	0·169	0·241
5	0·66	0·82	0·132	0·163
6	0·65	0·72	0·109	0·120
7	0·66	0·65	0·094	0·093
8	0·66	0·60	0·083	0·075
9	0·68	0·59	0·075	0·062
10	0·68	0·53	0·068	0·053

Four validity tests may be made in 3 + 3 assays. If when determining the 95% limits, the line GH cuts the scale for the limits, the slope is significant, at least at the 5% level.

The parallelism effect, L_1', is found by substituting the response sums or means, whichever have been plotted, in the formula

$$L_1' = T_3 - T_1 - (S_3 - S_1)$$

In 3 + 3 assays, this effect is significant at the 5% level if

$$|L_1'| > K \times \text{range sum}$$

Note that this criterion for significance differs from that for 2 + 2 assays.

The combined curvature effect, L_2, and the difference of curvature effect, L_2', are found from the lengths of T_2B and S_2E. The length T_2B is given a positive or negative sign according to whether T_2 is above or below B; similarly, S_2E is positive or negative according to whether S_2 is above or below E. The effects are given by

$$L_2 = T_2B + S_2E$$
$$L_2' = T_2B - S_2E$$

Either of these effects is significant at the 5% level if the distance indicated by $|L_2|$ or $|L_2'|$ on the scale for the "significant curvature effect" is greater than the distance on the "K × range sum" scale used in determining the 95% limits. Thus, in this example, $|L_2| = 0.65$, and the distance corresponding to 0·65 on the "significant curvature effect" scale is greater than 1·006 (= 10·06/10) on the "K × range sum" scale; hence L_2 is significant.

In this example, both L_1' and L_2 are significant and it would be conventional to regard the assay as invalid. However, to illustrate the method, the relative potency and its 95% limits have been determined and converted to allow for the ratio of successive doses being 1·4.

Discussion

The accuracy of the graphical method described may be assessed by comparing the results of the three worked examples with those obtained by standard computational methods using the root mean square estimate of the residual standard deviation and determining the exact fiducial limits using Fieller's theorem.

In all cases the tests of validity gave the same results by both methods. The relative potencies and their limits are compared in Table 6. It will be seen that the comparison is good in all cases.

TABLE 6. COMPARISON OF RELATIVE POTENCIES AND 95% LIMITS IN 3 EXAMPLES

	Standard method		Present graphical method	
	Relative potency (RP)	95% limits of RP	Relative potency (RP)	95% limits of RP
Example 1 ..	0·801	0·705–0·898	0·80	0·72 –0·888
Example 2 ..	1·137	1·040–1·245	1·14	1·045–1·24
Example 3 ..	0·898	0·824–0·975	0·90	0·83 –0·97

It should be emphasised that limits determined from the nomograms are "exact" only when the relative potency of corresponding doses of the two preparations is 1. Table 7 shows that as the relative potency deviates more from 1, the approximate limits differ more from the exact fiducial limits, and the fiducial probability of the approximate limits becomes less than 0·95.

In the author's experience, the approximation is accurate enough for most research purposes when the fiducial probability is 0·90 or more, i.e. (with ratios of doses of 2) when the relative potency lies between 0·6 and 1·67 in a 2 + 2 assay, or between 0·5 and 2·0 in a 3 + 3 assay. When accuracy is of critical importance it may be necessary to use the approximation only for relative potencies closer to 1, e.g., between 0·8 and 1·25. In a particular assay, the difference between the approximate and exact limits may be roughly estimated from Table 7 by interpolation; this would be a guide to whether the exact limits should be computed.

The nomograms described here have a general applicability to a wide range of assays. They may be used as the basis of simpler nomograms for

TABLE 7. EXACT PERCENTAGE FIDUCIAL LIMITS CORRESPONDING TO APPROXIMATE LIMITS DETERMINED FROM NOMOGRAMS, AND FIDUCIAL PROBABILITIES OF APPROXIMATE LIMITS

The relative potencies and limits given are for ratios of doses, r, equal to 2. The fiducial probabilities of the approximate limits are given in parenthesis; they were determined for a residual mean square with 24 degrees of freedom. The derivation of this table is described in the Appendix.

2 + 2 Assays

Approximate % limits from nomograms	Relative Potency								
	0·4	0·6	0·8	0·9	1·0	1·11	1·25	1·67	2·5
90–111	82–117 (0·78)	86–113 (0·89)	89–111 (0·94)	89–111 (0·95)	90–111 (0·95)	90–112 (0·95)	90–113 (0·94)	89–116 (0·89)	85–122 (0·78)
80–125	62–133 (0·79)	72–126 (0·90)	77–124 (0·94)	79–124 (0·95)	80–125 (0·95)	81–127 (0·95)	81–130 (0·94)	80–140 (0·90)	75–161 (0·79)
60–167	23–162 (0·84)	38–151 (0·91)	51–153 (0·94)	56–159 (0·95)	60–167 (0·95)	63–178 (0·95)	65–196 (0·94)	66–264 (0·91)	62–439 (0·84)
40–250	3–150 (0·88)	10–174 (0·92)	24–191 (0·94)	32–213 (0·95)	40–250 (0·95)	47–309 (0·95)	52–420 (0·94)	57–1030 (0·92)	67–3700 (0·88)

3 + 3 Assays

Approximate % limits from nomograms	Relative Potency								
	0·4	0·6	0·8	0·9	1·0	1·11	1·25	1·67	2·5
90–111	86–114 (0·88)	88–112 (0·93)	89–111 (0·95)	90–111 (0·95)	90–111 (0·95)	90–112 (0·95)	90–112 (0·95)	89–113 (0·93)	88–116 (0·88)
80–125	72–129 (0·88)	77–125 (0·93)	79–124 (0·95)	80–125 (0·95)	80–125 (0·95)	80–126 (0·95)	80–127 (0·95)	80–130 (0·93)	78–138 (0·88)
60–167	42–164 (0·89)	51–160 (0·93)	57–161 (0·95)	59–163 (0·95)	60–167 (0·95)	61–171 (0·95)	62–176 (0·95)	63–196 (0·93)	61–239 (0·89)
40–250	15–206 (0·90)	25–206 (0·93)	34–223 (0·95)	37–235 (0·95)	40–250 (0·95)	43–270 (0·95)	45–298 (0·95)	48–403 (0·93)	49–684 (0·90)

specialised use in routine assays or tests with a fixed number of observations and a fixed ratio of successive doses.

In this Department, a specialised nomogram has been prepared for the rat tail-pinch test for analgesics. The antagonism by nalorphine of the compound under test is measured by the dose ratio, which is mathematically equivalent to a relative potency. A test of significance of the antagonism is made by seeing whether the value of unity, corresponding to no antagonism, lies between the lower and upper limits of the dose ratio. Our experience with this nomogram has been favourable; it is reasonably accurate and has been welcomed by the experimenters.

Appendix: notes on the construction of the nomograms and on Table 7

Following Giet (1956), the term "modulus" is used to indicate the scale for plotting a variable: if a variable x has modulus μ, it is plotted at a distance μx along the scale. For illustration we shall consider the use of the nomogram for a $3 + 3$ assay with n observations on each dose, a ratio of doses of 2 and no block restrictions in the assay design.

Graphical construction. The method of construction of the individual regression lines for 3 + 3 assays is a special case of the method of advancing centroids given by Askovitz (1957). The relative potency scales on the nomograms are logarithmic with lengths of $1\frac{1}{2}$, 3 and $4\frac{1}{2}$ inches corresponding to relative potencies of 0·5, 1·0 and 2·0 respectively. Suppose the response sums are plotted with modulus λ. The modulus of the logarithm to base 2 of the dose is the distance between successive doses on the dose scale; let this distance be d. Then the modulus of the slope of the regression line will be $n\lambda/d$.

Estimation of the residual standard deviation. The range sum is used to estimate the residual standard deviation following the methods described in the preface of Pearson & Hartley (1958). The mean range, $\bar{w}$, is distributed approximately as

$$\frac{c\chi\sigma}{\sqrt{\nu}}$$

where σ is the true residual standard deviation, χ is a chi-variate on ν degrees of freedom, and ν and the scale factor c are found from Table 30 of Pearson & Hartley (1958). In the illustrative 3 + 3 assay without block restrictions, σ is estimated by s, given by $s = \bar{w}/c = W/6c$, where W is the sum of the ranges.

Determination of fiducial limits. The geometry of the method of determining the approximate fiducial limits has previously been used, for example by De Beer (1945). In Fig. 5, GH is a line with slope b and OV
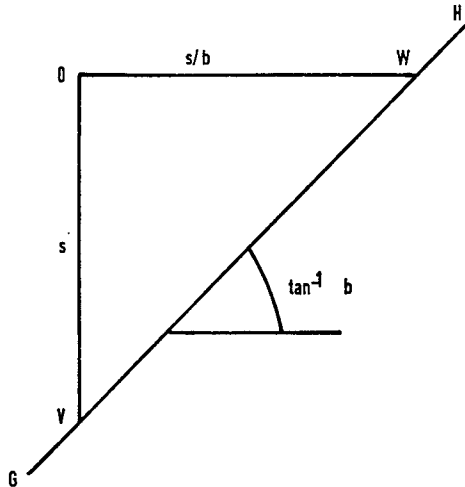

FIG. 5. Diagram to illustrate principle of determination of fiducial limits.

has the length s, so that OW has the length s/b. The fiducial limits (as a percentage of the relative potency) are approximated by a single-valued function of s/b, so that OW may be calibrated with these limits.

The logarithm of the relative potency is the ratio, m, given by

$$m = a/b$$

where, in a 2 + 2 assay,

$$a = \tfrac{1}{2}(T_1+T_2)-\tfrac{1}{2}(S_1+S_2)$$
$$b = \tfrac{1}{2}(T_2-T_1+S_2-S_1)$$

and, in a 3 + 3 assay,

$$a = \tfrac{1}{3}(T_1+T_2+T_3)-\tfrac{1}{3}(S_1+S_2+S_3)$$
$$b = \tfrac{1}{4}(T_3-T_1+S_3-S_1)$$

In either case, the exact fiducial limits, m_U and m_L, for m are

$$m_U,\ m_L = \left[m \pm \frac{ts}{b}\left\{v_{11}(1-g)+m^2v_{22}\right\}^{\frac{1}{2}} \right] \div (1-g)$$

where s^2 is the residual mean square estimate of σ^2 and has f degrees of freedom; v_{11} and v_{22} are constants such that the variances of a and b are $\sigma^2 v_{11}$ and $\sigma^2 v_{22}$ respectively; t is the t-deviate with f degrees of freedom corresponding to the fiducial probability being used; and $g = t^2s^2v_{22}/b^2$.

The logarithms of the exact limits, expressed as proportions of m, are p_U and p_L, given by

$$p_U,\ p_L = \frac{mg}{1-g} \pm \frac{ts}{b}\left\{\frac{v_{11}}{1-g} + \frac{m^2v_{22}}{(1-g)^2}\right\}^{\frac{1}{2}}$$

The approximate limits, p_U', p_L' used in the nomograms are obtained by putting m = 0, i.e.

$$p_U',\ p_L' = \pm \frac{ts}{b}\sqrt{\frac{v_{11}}{1-g}} = \pm t\sqrt{\frac{v_{11}}{(b/s)^2 - t^2v_{22}}}$$

In a 3 + 3 assay where a and b are given in terms of the mean responses for each dose,

$$v_{11} = 2/3n,\ v_{22} = 1/4n$$

Substituting these values in the formula for p_U', p_L' and writing $p'^2 = p_U'^2 = p_L'^2$, we find

$$\frac{s}{b} = \frac{\sqrt{4n}}{t}(1 + 8/3p'^2)^{-\frac{1}{2}}$$

If the percentage limits corresponding to p' are plotted at a distance of

$$R(1+8/3p'^2)^{-\frac{1}{2}}$$

along the limits scale, the implied modulus of s/b is $Rt/\sqrt{4n}$. Then the modulus for s on a vertical scale should be $Rt/\sqrt{4n} \times n\lambda/d = Rt\sqrt{n}\lambda/2d$, i.e. the modulus for W should be $Rt\sqrt{n}\lambda/2d \times 1/6c = Rt\sqrt{n}\lambda/12cd$. The 3 + 3 nomogram is constructed with R = 12 inches, d = 3 inches; in Table 3, $K = t\sqrt{n}/3c$ when response sums from an assay with no block restrictions are plotted. Hence the modulus for $K \times W$ must be λ, i.e. $K \times W$ must be measured on the same scale as the response sum was plotted.

Tests of validity. The slope b is significant if $b/s\sqrt{v_{11}} > t$ i.e. if $g < 1$. As $g \to 1$, $p'^2 \to \infty$, so that if finite limits are found, the slope will be significant at the 5% level, and possibly at a much smaller level.

The parallelism effect L_1' determined from the response sums has variance $4n\sigma^2$. It is therefore significant at the 5% level if

$$|L_1'| > t.2s \sqrt{n} = 2t\sqrt{n}W/6c = K \times W.$$

The curvature effects may be found to have lengths given by

$$L_2 = \frac{\lambda}{3} (-T_1 + 2T_2 - T_3 - S_1 + 2S_2 - S_3)$$

$$L_2' = \frac{\lambda}{3} (-T_1 + 2T_2 - T_3 + S_1 - 2S_2 + S_3)$$

where the letters represent response sums or means, whichever have been plotted. For dose sums, the variance of L_2 or L_2' is $4\lambda^2 n\sigma^2/3$. Hence L_2 is significant at the 5% level if $|L_2| > 2t\lambda\sqrt{ns}/\sqrt{3}$ i.e. if $|L_2| > \lambda KW/\sqrt{3}$. Hence, if the distance corresponding to $|L_2|$ on a scale with modulus $\sqrt{3}$ is greater than $K \times W$ plotted on the same scale as the response sums, then L_2 is significant at the 5% level. The same result holds for L_2'.

Alignment diagram. If R_2 and R_r are corresponding relative potencies assuming the ratios of successive doses are 2 and r respectively, then

$$\log_2 R_2 = \log_r R_r$$

i.e. $\log_{10}\log_{10}R_2 - \log_{10}\log_{10}R_r - \log_{10}\log_{10}2 + \log_{10}\log_{10}r = 0$

This equation may be expressed in determinantal form as

$$\begin{vmatrix} -1 & \log_{10}\log_{10}R_2 & 1 \\ 0 & \frac{1}{2}(\log_{10}\log_{10}2 - \log_{10}\log_{10}r) & 1 \\ 1 & \log_{10}\log_{10}R_r & 1 \end{vmatrix} = 0$$

and this is the basic determinant of the alignment diagram (see Allcock & Jones, 1950).

Derivation of Table 7. The exact limits of p_U, p may be expressed in terms of $(p')^2 = (p_U')^2 = (p_L')^2$ by noting that

$$(p')^2 = gv_{11}/(1-g)v_{22}$$

and p_U, $p_L = mg/(1-g) \pm [g(1-g)v_{11}/v_{22} + m^2 g]^{\frac{1}{2}} /(1-g)$

Eliminating g from these formulae:

$$p_U, p_L = mv_{22}(p')^2/v_{11} \pm p'\sqrt{1 + m^2 v_{22}/v_{11} + m^2 v_{22}^2(p')^2/v_{11}^2}$$

This is the expression used to determine the exact fiducial limits in Table 7.

The fiducial probability corresponding to the approximate limits was determined by finding the values of g in the formula for the exact limits such that

$$p_U, p_L = \pm p'$$

After some algebraic manipulation, it is found that

$$g = \frac{(p')^2}{(m \pm p')^2 + v_{11}/v_{22}}$$

Then if we write $g^2 = t_E^2 s^2 v_{22}/b^2$ where t_E is the value of the t-deviate for the exact limit equal to one of the pair of approximate limits we find

$$\frac{t_E^2}{t^2} = \frac{v_{22}(p')^2 + v_{11}}{v_{22}(m \pm p')^2 + v_{11}}$$

where t is the t-deviate corresponding to the fiducial probability being used. With this formula, a value of t_E can be determined for each approximate limit, and hence the exact fiducial probability of the pair of approximate limits.

In Table 7, the exact limits are correct whatever the value of f. The exact fiducial probability varies however with f, the tabulated values being correct for $f = 24$. When $f < 24$, the exact fiducial probability differs less from the nominal value of 0·95; for $f > 24$, the difference is greater, but even when $f \to \infty$, the fiducial probability is never more than 0·01 less than the value in Table 7.

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