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Improved Non-Parametric Statistical Methods for the Estimation of Michaelis–Menten Kinetic Parameters by the Direct Linear Plot

By WILLIAM R. PORTER and WILLIAM F. TRAGER
Department of Pharmaceutical Sciences, School of Pharmacy, University of Washington, Seattle, WA 98195, *U.S.A.*

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The theoretical basis for the direct linear plot [Eisenthal & Cornish-Bowden (1974) *Biochem. J.* **139**, 715–720], a non-parametric statistical method for the analysis of data-fitting the Michaelis–Menten equation, was reinvestigated in order to accommodate additional experimental designs and to provide estimates of precision more directly comparable with those obtained by parametric statistical methods. Methods are given for calculating upper and lower confidence limits for the estimated parameters, for accommodating replicate measurements and for comparing the results of two separate experiments. Factors that influence the proper design of experiments are discussed.

Many fundamental relations in chemistry, biology and medicine can be described by the general equation:

$$y = \frac{k_2 x}{k_1 + x} \qquad (1)$$

where k_1 = value of x required to produce a half-maximal value of y, and k_2 = maximum value attained by y. Among the many relations that are described by eqn. (1) are the adsorption of molecules to surfaces (and the binding of small molecules to proteins) as a function of concentration, the velocity of enzyme-catalysed reactions as a function of substrate concentration, and the pharmacological response as a function of dose. The quantities x, y, k_1 and k_2 are represented in the literature by certain commonly used symbols, some of which are listed in Table 1.

Thus in the field of enzyme kinetics, eqn. (1) would be rewritten:

$$v = \frac{V_{max.}[S]}{K_m + [S]} \qquad (1a)$$

Eqn. (1a) is, of course, the familiar Michaelis–Menten equation. For the sake of clarity, the remainder of this discussion will be restricted to the terminology of enzyme kinetics (unless otherwise noted) even though the methods developed herein are more generally applicable.

Since the experimental measurement of [S] or v is not error-free, objective methods for estimating the parameters K_m and $V_{max.}$ and the reliability with which they have been determined are required.

In addition, when experiments are performed to test the effect of some perturbation on the system (e.g. the addition of an inhibitor to an enzyme/substrate reaction mixture), it is necessary to determine the new values of the parameters K_m and $V_{max.}$ and to estimate both the direction (increase or decrease) and magnitude of the change. An estimate of the reliability with which the change has been measured is also required. These problems of interpretation of the experimental data can be resolved by an adequate statistical technique.

The problems inherent in the parametric statistical analysis of data to be fitted to eqn. (1a) have been amply discussed by Cornish-Bowden & Eisenthal (1974). A non-parametric method has been introduced by these authors (Eisenthal & Cornish-Bowden, 1974) that is less sensitive to outliers (observations which have a much higher error than is expected from the distribution of errors for the remaining observations) and that requires fewer assumptions about the nature of the experimental error. This method, termed the direct linear plot, requires that the data be plotted as lines in K_m–$V_{max.}$ parameter space. The intersections of the lines provide estimates of K_m and $V_{max.}$ (Fig. 1). The published method is simple and direct, but it has certain disadvantages: no provision has been made for the inclusion of replicate measurements of v at each value of [S] (as is frequently done in actual practice), the effects of systematic deviations (lack of fit) have not been thoroughly investigated, and only an awkwardly defined joint estimate of the precision of the estimated K_m and $V_{max.}$ values can be obtained. Therefore we have undertaken a study of the assumptions involved in the formation of the non-parametric estimates of K_m and $V_{max.}$.

Table 1. *Common symbols used in eqn.* (1)

Enzyme kinetics
- $y = v$ = rate of formation of product
- x = [S] = concentration of substrate
- $k_1 = K_m$ = Michaelis constant
- $k_2 = V_{max.}$ = maximum rate of formation of product

Langmuir adsorption
- $y = r$ = mol bound per unit of adsorbant
- x = [A] or C = concentration of adsorbed substance
- $k_1 = K_D$ = dissociation constant = $1/K_A$ = 1/association constant
- $k_2 = n$ = number of binding sites per unit of adsorbant

Pharmacology
- y = pharmacological response
- x = [D] = concentration of drug dosage
- $k_1 = K_D$ = drug–receptor dissociation constant
- k_2 = maximal response

Method

Point estimates and rectangular confidence regions for $(K_m, V_{max.})$

The direct linear plot is used to find the $\frac{1}{2}n(n-1)$ intersections of the n lines obtained from the sets of observations $([S]_i, v_i)$, $i = 1, \ldots, n$. This corresponds to solving the $\frac{1}{2}n(n-1)$ sets of simultaneous equations

$$V_{max.} = v_i + \frac{v_i}{[S]_i} K_m \qquad 1 \leqslant i < j \leqslant n$$

$$V_{max.} = v_j + \frac{v_i}{[S]_j} K_m$$

The solutions yield $\frac{1}{2}n(n-1)$ estimates of K_m and $V_{max.}$:

$$(K_m)_{ij} = \frac{v_j - v_i}{\frac{v_i}{[S]_i} - \frac{v_j}{[S]_j}} \tag{2}$$

$$(V_{max.})_{ij} = \frac{[S]_i - [S]_j}{\frac{[S]_i}{v_i} - \frac{[S]_j}{v_j}} \tag{3}$$

Cornish-Bowden & Eisenthal (1974) proposed that the vector of parameters $(K_m, V_{max.})$ be estimated by

$$\hat{K}_m = \text{median of the } \{(K_m)_{ij}\}$$
$$\hat{V}_{max.} = \text{median of the } \{(V_{max.})_{ij}\}$$

Eqns. (2) and (3) are formally equivalent to finding the slopes of the regression lines

$$-v = K_m\left(\frac{v}{[S]}\right) - V_{max.} \tag{4}$$

$$[S] = V_{max.}\left(\frac{[S]}{v}\right) - K_m \tag{5}$$

Eqn. (4) is formally equivalent to an Eadie–Hofstee plot, and eqn. (5) may be easily rearranged to an [S]/v versus [S] plot. Theil (1950) has proposed a simple point estimate for the slope of the regression line $Y = \beta X + \alpha$, namely $\hat{\beta}$ = median of the $\{B_{ij}\}$ where:

$$B_{ij} = \frac{Y_j - Y_i}{X_j - X_i}, \quad 1 \leqslant i < j \leqslant n \tag{6}$$

The B_{ij} values proposed by Theil (1950) to be used in the estimation of β are in fact identical with the $(K_m)_{ij}$ and $(V_{max.})_{ij}$ for regression lines (4) and (5) respectively. The estimates $\hat{K}_m$ and $\hat{V}_{max.}$ of Cornish-Bowden & Eisenthal (1974) are then mathematically identical in all respects with Theil's (1950) $\hat{\beta}$.

The procedure of Theil (1950) has been extended by Sen (1968) and, as we shall show, can be used to obtain confidence intervals for the individual parameters K_m and $V_{max.}$ by using rank-correlation methods and Kendall's S distribution (Kendall, 1970). These are distribution-free statistical equivalents of the confidence intervals that may be obtained from the standard error of the parameters and Student's t distribution in conventional parametric statistical analysis (which presupposes a Gaussian error distribution). The utility of rank-correlation methods for the estimation of enzyme kinetic parameters can be demonstrated by a comparison with conventional parametric statistical analysis and with the runs-of-signs method of Cornish-Bowden & Eisenthal (1974) for determining confidence limits.

Example 1: *non-parametric estimation of* $(K_m, V_{max.})$ *with confidence limits obtained by rank-correlation methods*

The procedure for obtaining non-parametric estimates of $(K_m, V_{max.})$ from experimental data is simple: all necessary calculated values are given in Table 2 and all graphical procedures are illustrated by Fig. 1. The first step in the analysis is to tabulate the experimental [S] and v values (Table 2). Data of Michaelis and Menten as cited by Johansen & Lumry (1961) were used as an example. The data are then used to construct a direct linear plot (Fig. 1), as outlined by Eisenthal & Cornish-Bowden (1974). By inspection of Fig. 1, it is apparent that all of the plotted lines intersect, as expected, in a confined region of K_m–$V_{max.}$ parameter space.

Since five observations ([S], v) were obtained without replication, there are $N = (5/2)(5-1) = 10$ points of intersection. The probability distribution for Kendall's (1970) S for $n = 5$ with no replication is obtained from tables [e.g. Hollander & Wolfe (1973) pp. 384–393]. Note that the probability values given in Table 2 do not depend on the numerical values of

Table 2. *Calculations required for the non-parametric estimation of* (K_m, $V_{max.}$) *with confidence limits obtained by rank-correlation methods*

Experimental data (Johansen & Lumry, 1961)	
[S](mM)	v(μmol/min)
5.21	152.0
10.42	242.3
20.83	370.2
41.67	484.8
83.33	589.3

Rank	Calculated co-ordinates from direct linear plot (Fig. 1) $(K_m)_{ij}$ (mM)	$(V_{max.})_{ij}$ (μmol/min)	Cumulative probability*
1	15.25	596.91	0.008403
2	18.67	702.01	0.042017
3	18.97	705.54	0.117647
4	19.14	710.31	0.243697
5	19.78	727.62	0.411765
6	20.48	729.22	0.588235
7	20.87	734.10	0.756303
8	21.44	740.95	0.882353
9	22.90	751.28	0.957983
10	23.34	784.93	0.991597
Median	20.13	728.42	0.500000

* Kendall's (1970) S statistic, $n = 5$ no replicates.

the experimental data; rather, the probabilities are obtained from the number of observations and the number of replications included, if any.

The ranking of the points of intersection from left to right and from bottom to top can be obtained directly from Fig. 1. For purposes of illustration, the co-ordinates of all the points of intersection were calculated and are listed in Table 2. In practice, only the fifth and sixth points (used to calculate the median) and the two points required for the desired confidence limits (obtained by inspection of the probability distribution) need actually be calculated. For example, the probability of finding the true value of K_m between the tabulated values of $(K_m)_{ij}$ having ranks 1 and 10 is 98.3%, obtained by subtracting the cumulative probability associated with rank 1 from that for rank 10. Similarly, the probability of finding the true value of $V_{max.}$ between the $(V_{max.})_{ij}$ having ranks 1 and 10 is also 98.3%. The rectangle in K_m–$V_{max.}$ parameter space bounded by these values of $(K_m)_{ij}$ and $(V_{max.})_{ij}$ encloses a region in which the probability of finding the true values (K_m, $V_{max.}$) jointly is, from the Bonferroni inequality (below), greater than 96.7%. The individual confidence limits obtained from the $(K_m)_{ij}$ or $(V_{max.})_{ij}$ having ranks 2 and 9 enclose the true values of K_m or $V_{max.}$ with 91.6% probability. The corresponding joint-confidence rectangle will contain (K_m, $V_{max.}$) 83.2% of the

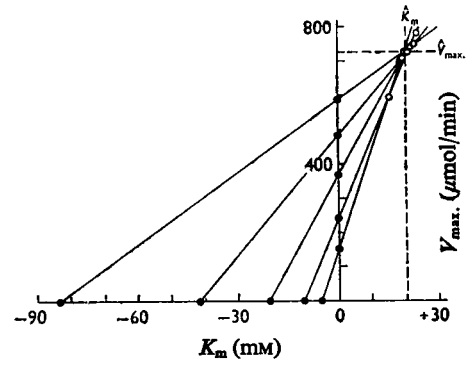

Fig. 1. *Direct linear plot of the data in Table* 2 Experimental data: ○, slope estimator points [the K_m and $V_{max.}$ co-ordinates of these points are the $(K_m)_{ij}$ and $(V_{max.})_{ij}$ values listed in Table 2].

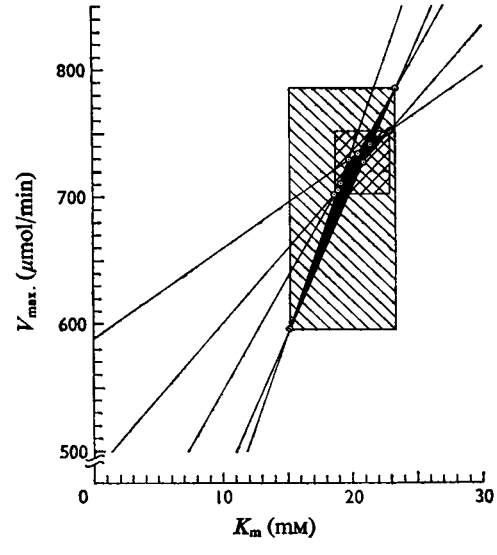

Fig. 2. *Comparison of rectangular non-parametric joint-confidence rectangle for the data plotted in Fig.* 1 *with the irregular confidence region of Cornish-Bowden & Eisenthal* (1974)

The outer rectangle with diagonal lines represents the 96.7% confidence region, the inner rectangle with cross-hatching the 83.2% confidence region and the irregular shape the 68.8% confidence region.

time. These regions are plotted in Fig. 2. For purposes of comparison, the irregularly shaped joint-confidence region ($P \geqslant 0.688$) of Cornish-Bowden & Eisenthal (1974) is also plotted in Fig. 2.

Note that the rectangular confidence regions provide conservative estimates for the joint probability of (K_m, $V_{max.}$) in the sense that the rectangles

Table 3. *Comparison of parametric and non-parametric estimates of K_m and $V_{max.}$ from the data in Table 2*

K_m (mM)	$V_{max.}$ (μmol/min)	Method
20.59	731.8	Non-linear least-squares‡
20.58±0.51* (±2.32)†	731.5±8.9* (±40.4)†	Weighted least-squares fit to eqn. (7)‡
20.60±0.51* (±2.32)†	731.8±8.9* (±40.4)†	Weighted least-squares fit to eqn. (8)‡
19.5	709	Lineweaver–Burk plot [graph of eqn. (7)]‡
20.1	724	$\frac{[S]}{v}$ versus [S] plot [graph of eqn. (8)]‡
20.13(+3.21, −4.88)†	728.42(+56.51, −131.51)†	Non-parametric median estimate

* Standard error.

† 98% confidence limits [obtained from Student's t distribution with 3 degrees of freedom for the least-squares estimates and from Kendall's (1970) S distribution for the non-parametric estimates].

‡ Johansen & Lumry (1961).

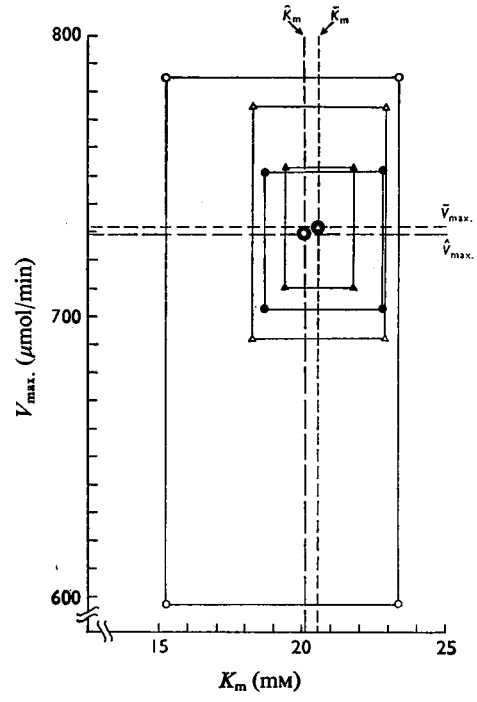

Fig. 3. *Comparison of non-parametric and parametric joint-confidence rectangles for the data plotted in Fig.* 1 The areas defined by circles indicate non-parametric joint-confidence rectangles and those defined by triangles indicate parametric joint-confidence rectangles. ○, 96.7%; △, 95% ●, 83.2%; ▲, 80% confidence intervals.

contain large areas of relatively low probability density. The irregular region of Cornish-Bowden & Eisenthal (1974) contains over two-thirds of the total probability density, but encompasses only a small fraction of the total area of either rectangle. Thus in cases requiring fine distinctions, the irregular region may prove to be more useful. However, the rectangular joint-confidence region is easily obtained from tabulated values of Kendall's (1970) S statistic and can be uniquely defined for a pre-selected level of significance. An additional advantage is that separate confidence intervals for each parameter may be obtained and compared directly with the results of simple parametric statistical procedures. Since K_m and $V_{max.}$ are highly correlated, certain pitfalls may be encountered if such comparisons are not made with care; this problem has been discussed in some detail by Cleland (1967).

The results obtained by Johansen & Lumry (1961) using graphical techniques and parametric statistical-estimation methods are reported in Table 3. Weighted least-squares estimates were obtained by fitting the data to either a Lineweaver–Burk plot:

$$\frac{1}{v}=\left(\frac{K_m}{V_{max.}}\right)\frac{1}{[S]}+\frac{1}{V_{max.}} \quad (7)$$

or to the single reciprocal form:

$$\frac{[S]}{v}=\left(\frac{1}{V_{max.}}\right)[S]+\frac{K_m}{V_{max.}} \quad (8)$$

Individual confidence limits for K_m and $V_{max.}$ were found from the standard error and Student's t distribution and were used to construct parametric joint-confidence regions with greater than 80% and 95% probabilities for the inclusion of the true value of (K_m, $V_{max.}$). These regions are plotted in Fig. 3 along with the non-parametric rectangular confidence regions encompassing nearly the same probabilities. Rectangular parametric joint-confidence regions suffer the same drawback as their non-parametric counterparts with respect to including some areas of very low probability density. The rectangles represent outer bounds for the elliptical joint-confidence regions described by Cleland (1967).

Example 2: non-parametric estimation of (K_m, $V_{max.}$) with replicate observations

The procedure outlined for the case where single observations were made is followed with only one modification: the intersections of those lines of the direct linear plot representing replicate observations

Table 4. *Calculations required for the non-parametric estimation of K_m, $V_{max.}$ when some observations are replicates*

Data (from Table 2 plus *fictitious replicates)	
[S] (mM)	v (μmol/min)
5.21	152.0
10.42	240.0*
10.42	242.3
10.42	245.0*
20.83	370.2
41.67	484.8
83.33	589.3

Calculated co-ordinates from direct linear plot (not shown)			
Rank	$(K_m)_{ij}$ (mM)	$(V_{max.})_{ij}$ (μmol/min)	Cumulative probability†
1	14.33	570.00	0.001190
2	15.25	596.91	0.005952
3	16.43	631.19	0.017857
4	18.67	702.01	0.041667
5	18.97	705.54	0.082143
6	19.14	710.31	0.142857
7	19.79	719.68	0.225000
8	20.19	727.62	0.326190
9	20.48	729.22	0.440476
10	20.87	734.10	0.559524
11	20.94	734.67	0.673810
12	21.44	734.40	0.775000
13	21.48	740.95	0.857143
14	21.81	744.07	0.917857
15	21.89	751.28	0.958333
16	22.91	757.85	0.982143
17	23.34	784.93	0.994048
18	24.75	810.10	0.998810
Median	20.67	731.66	0.500000

† Kendall's (1970) S statistic, $n = 7$, one triplicate.

(i.e. the intersections occurring on the horizontal axis of the plot) are ignored when finding the median or when calculating the probability distribution. To illustrate this, two fictitious observations were added to the data of Table 2: (10.42, 240.0) and (10.42, 245.0). There are now three observed values for v (240.0, 242.3 and 245.0) for one value of [S] (10.42), simulating a triplicate determination. The number of intersections (excluding those along the K_m axis) is now $N = \frac{1}{2}n(n-1) - p, r\frac{1}{2}r(r-1) = (7/2)(7-1) - (1) \times (3/2)(3-1) = 18$. The probability distribution is obtained for Kendall's (1970) S statistic for $n = 7$ and one tie of size 3 (Sillitto, 1947). The calculated results are shown in Table 4.

Since Kendall's (1970) S statistic may be computed for any possible combination of replicated observations, no problems are encountered in defining probabilities associated with the ranked co-ordinates of the points of intersection in the direct linear plot. Because the runs-of-signs method of Cornish-Bowden & Eisenthal (1974) has no simple extension to experiments containing replicate measurements, rank-correlation methods have an obvious advantage in permitting more flexible experimental designs.

Example 3: *comparing two experimental results: inhibitor studies*

Data obtained from a study by Black *et al.* (1975), in which changes in dog blood pressure were measured in response to histamine in the presence or absence of certain antagonists, are tabulated in Table 5 and plotted as direct linear plot in Fig. 4. The non-parametric and parametric estimates of the parameters for both control and treatment experiments are given in Table 5. Examination of Fig. 3 shows that a very large increase in the estimated value of K_D (Table 1) occurs after addition of the inhibitors, but a small increase in response (p) also appears to have occurred. A non-parametric test of the hypothesis $\Delta\hat{p} = 0$ can be done by means of Hollander's (1970) test, an extension of the Wilcoxon (1945) signed-rank test to linear-regression problems. Hollander's (1970) test is a distribution-free test of the hypothesis that the slopes of two lines are identical. A detailed computational procedure is available [Hollander & Wolfe (1973) pp. 27–38, 209–217, 269–271] and will not be repeated here. For the convenience of the reader, the symbolism and computational procedures cited will be adhered to as much as possible. Hollander's (1970) test requires the co-ordinates of the points of intersection of the first and fourth, second and fifth, and third and sixth lines (ranked from lowest dose to highest dose) from the direct linear plot of both the control and treatment experiments. These quantities are identified in Table 5. These estimates were then paired at random, pairing one estimate from the control experiment with one estimate from the treatment experiment. The results of these pairings are listed in Table 6. This is not the only way in which the estimates could be paired, but as has been emphasized [Hollander & Wolfe (1973) pp. 209–217] in the absence of any pre-determined plan, random pairing is essential. The pair differences Z_i [Hollander & Wolfe's (1973) notation, pp. 27–38, 209–217] are then calculated; these differences are used to compute the Wilcoxon signed-rank statistic T^+. In this particular example there is less than a 12.5% chance that no increase in either K_D or p has occurred in the treatment experiment. For the number of observations analysed in this example, no greater level of significance may be obtained from this test; it is necessary to increase the number of experimental observations tested. The Wilcoxon shift parameters may be calculated from the $W_k = (\frac{1}{2})(Z_i + Z_j)$, $1 \leqslant i \leqslant j \leqslant n$; a 50% joint confidence rectangle for the

Table 5. *Effect of the co-administration of mepyramine and metiamide on the depressor response to histamine of the mean blood pressure of five anaesthetized dogs*
The results are from Black *et al.* (1975).

[D] = intravenous dose (nmol/kg)	p = change in blood pressure (mmHg)	
	p_1 (control)	p_2 (treatment)
0.31622	7 (A)*	
1.0	12 (B)	
3.1622	32 (C)	
0.0	54 (A)	3 (A)
31.622	74 (B)	9 (B)
100.0	93 (C)	26 (C)
316.22		52 (A)
1000.0		70 (B)
3162.2		98 (C)

Calculated co-ordinates from direct linear plot				
Control		Treatment		
$(K_D)_{ij}$	p_{ij}	$(K_D)_{ij}$	p_{ij}	Cumulative probability†
0.493	17.919	190.592	83.341	0.001389
2.080	53.053	231.579	86.211	0.008333
2.808 (A)	69.164 (A)	272.087	89.896 (B)	0.027778
3.384	79.172	284.234 (B)	90.391	0.068056
4.055	81.920	291.304	96.743	0.136111
4.661	86.634	314.398 (C)	107.744 (C)	0.234722
5.399	88.364	344.695	108.682	0.359722
6.364‡	89.020‡ (B)	350.917‡	108.875‡	0.500000
6.418 (B)	89.296	353.148	108.944	0.640278
6.536	96.771	357.828	110.842	0.765278
6.638 (C)	99.173 (C)	361.470 (A)	111.441 (A)	0.863889
7.317	99.805	389.914	119.974	0.931944
8.725	101.114	575.000	120.245	0.972222
10.636	105.531	690.721	175.555	0.991667
13.474	139.628	717.785	205.587	0.998611

* Identifies the data used to find the co-ordinates in the direct linear plot (Fig. 4) (identified below by the same letter) required for Hollander's (1970) test.
† Kendall's (1970) S statistic, $n = 6$, no replicates.
‡ Median.

shift parameter vector ($\Delta\hat{K}_D$, $\Delta\hat{p}$) can then be formed by finding the individual 75% confidence limits (in this example given by W_1 and W_6). This region is plotted in Fig. 5. The imprecision with which (ΔK_D, Δp) is determined suggests that further experimentation would be desirable.

Theory

The regression model of Sen (1968) is:

$$Y_j = \alpha + \beta(W_i - v_i) + e_j$$

where v_i is the random error associated with $W_{i,j}$ e is the random error associated with Y_j, and α and β are parameters to be estimated.

The experimental ($[S]_i$, v_i) are used to compute the $(K_m)_{ij}$ and $(V_{max.})_{ij}$ according to eqns. (2) and (3), excluding all pairs i,k which have $[S]_i = [S]_k$. Since the same procedure is used to compute the median value and obtain $(1-\alpha)$ confidence limits for each parameter, it is convenient to use a single nomenclature: B_{ij} is used to represent either $(K_m)_{ij}$ or $(V_{max.})_{ij}$ in the following discussion.

The total number of elements B_{ij} is given by the following formula (Sillitto, 1947):

$$N = \frac{n(n-1)}{2} - p_2 - 3p_3 - \cdots \frac{-r(r-1)}{2} p_r$$

where N is the total number of calculated slope estimates, p_2 is the number of duplicate observations, p_3 is the number of triplicate observations, ..., p_r is the number of r-replicate observations. Having

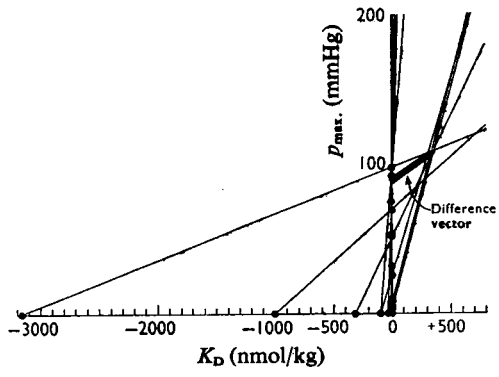

Fig. 4. *Direct linear plot of a dose–response experiment, illustrating the effect of added inhibitors*
Data are taken from Table 5; the K_D and p-co-ordinates of the points of intersection of the plotted lines are listed in rank order in Table 5.

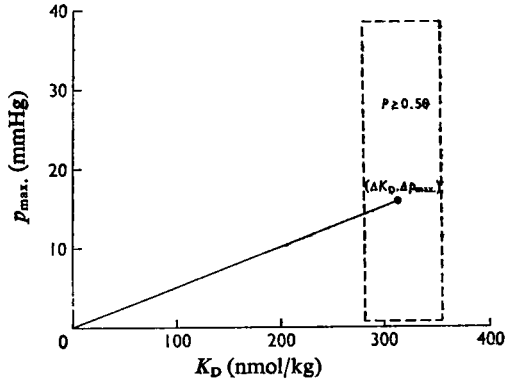

Fig. 5. *Plot in K_D–p parameter space of the vector of shift parameters (ΔK_D, Δp) and 50% joint-confidence rectangle for the difference in K_D and p for the data in Table 5*

Table 6. *Calculation of difference vector (ΔK_D, Δp) from data in Table 5*

Random pairings and calculated differences

Comparison of K_D and K_D'	Comparison of p and p'
$Z_1 = K_D' - K_D = 281.426$	$Z_1 = p' - p_A = 38.580$
$Z_2 = K_D' - K_D = 307.980$	$Z_2 = p' - p_B = 0.876$
$Z_3 = K_D' - K_D = 354.832$	$Z_3 = p' - p_C = 12.268$
$T^+ = 6$*	$T^+ = 6$*

* Wilcoxon signed-rank statistic: $P\{T^+ \geq 6\} = 0.125$.

Rank	Calculated Wilcoxon estimators W_k: ΔK_D	Δp	Cumulative probability†
1	281.426	0.876	0.125
2	294.703	6.572	0.250
3	307.980	12.268	0.375
4	318.129	19.728	0.625
5	333.406	25.424	0.750
6	354.832	38.580	0.875
Median	313.155	15.998	0.500

† Wilcoxon signed-rank statistic, T^+, $n = 3$.

obtained the N values of B_{ij} satisfying eqn. (6), they are ranked in order from smallest to largest.

The following derivation summarizes the results of Sen (1968) and retains his symbolism. The function $c(u)$ can be defined as follows:

$$c(u) = 1, \quad \text{if } u > 0$$
$$c(u) = -1, \quad \text{if } u < 0$$
$$c(u) = 0, \quad \text{if } [S]_i = [S]_k \qquad (9)$$

Then it can be shown that:

$$N = \sum_{i=1}^{i=n-1} \sum_{j=i+1}^{j=n} c(W_j - W_i)$$

Define $Z_i(b) = Y_i - bW_i$, and define

$$U_n(b) = \frac{\sum_{i=1}^{i=n-1} \sum_{j=i+1}^{j=n} c(W_j - W_i)c[Z_j(b) - Z_i(b)]}{[N(\frac{1}{2}n)(n-1)]^{\frac{1}{2}}} \qquad (10)$$

The function $U_n(b)$ is Kendall's (1970) rank coefficient of correlation between the $Z_i(b)$ and the W_i, and the quantity $[N(\frac{1}{2}n)(n-1)^{\frac{1}{2}} U_n(b)]$ is a discrete random variable having the same distribution as Kendall's (1970) S statistic corrected for ties (Sillitto, 1947). The problem of finding the best estimate, $\hat{\beta}$, of β and upper bound β_U and lower bound β_L for a $(1-\alpha)$ confidence interval for β reduces to selecting values of b such that:

$$\hat{\beta} = b, \quad \text{if } U_n(b) = 0$$
$$\beta_U = b, \quad \text{if } U_n(b) > S(n, \alpha/2)$$
$$\beta_L = b, \quad \text{if } U_n(b) < -S(n, \alpha/2)$$

where $S(n, \alpha/2)$ is the value of Kendall's (1970) S, corrected for ties, for which

$$P\{|S| \geq x\} = \alpha$$

The function Z_i, used to define $U_n(b)$ in eqn. (10), provides a way of estimating β, since the difference $(Z_j - Z_i)$ reduces to a term containing the random errors v_i, v_j, e_i and e_j when $b = \beta$:

$$Z_j(\beta) - Z_i(\beta) = \beta v_j - \beta v_i + e_i - e_j \qquad (11)$$

There should not be any correlation between the W_i and the error term on the right side of eqn. (11), since

the errors are assumed to be independent random variables.

Kendall's (1970) rank-correlation methods thus provide a way of estimating β. It is only necessary to select a real number b such that $Z_i(b)$ is uncorrelated with W_i; then b is an estimate of β. The value of b chosen in this manner is precisely the arithmetic median of B_{ij} ranked in numerical order.

Confidence limits are easily obtained from the frequency distribution of S. From the results of Sen (1968):

$\hat{\beta}$ is the median of the B_{ij}

β_U is the element of B_{ij} having the rank $\frac{1}{2}[N+S(n, \alpha/2)]+1$

β_L is the element of B_{ij} having the rank $\frac{1}{2}[N+S(n, \alpha/2)]$

and

$$P\{\beta_L \leqslant \beta \leqslant \beta_U\} = 1-\alpha$$

The values of $\hat{\beta}$, β_U and β_L obtained in this way are translation-invariant (i.e. the ranks are not affected by changes in the co-ordinate system which involve only linear transformations of the co-ordinates, such as changes in the units in which the variables [S] and v are measured). The statistic $U_n(b)$ satisfies the conditions of Puri & Sen (1971) for the components of a p-dimensional random vector, and therefore the estimates $(\hat{K}_m, \hat{V}_{max.})$ are estimates of the true vector of parameters $(K_m, V_{max.})$ in K_m–$V_{max.}$ parameter space. In addition, rectangular (Bonferroni) confidence regions for $(K_m, V_{max.})$ can be constructed by the method of Puri & Sen (1968) by finding $(K_m)_L$, $(K_m)_U$, $(V_{max.})_L$ and $(V_{max.})_U$ such that:

$$P\{(K_m)_L \leqslant K_m \leqslant (K_m)_U\} \geqslant 1-(\alpha/2)$$

$$P\{(V_{max.})_L \leqslant V_{max.} \leqslant (V_{max.})_U\} \geqslant 1-(\alpha/2)$$

from which it can be shown that the joint probability for the vector $(K_m, V_{max.})$ is given by

$$[P\{(K_m)_L \leqslant K_m \leqslant (K_m)_U\}] \cap [P\{(V_{max.})_L \leqslant V_{max.} \leqslant (V_{max.})_U\}] \geqslant 1-\alpha \quad (12)$$

Eqn. (12) is the Bonferroni inequality; it provides a conservative estimate of the joint probability. What this means in practice is that if we select $\alpha = 0.05$ (i.e. we wish to find a 95% joint-confidence region), then the rectangular confidence region found by eqn. (12) will give at least a 95% confidence region, although the exact level of confidence may be somewhat larger.

The point estimate $(\hat{K}_m, \hat{V}_{max.})$ of the true vector of parameters K_m, $V_{max.}$ obtained in this way is identical with the arithmetic median estimate proposed by Cornish-Bowden & Eisenthal (1974). Replicate measurements of v for a single value of [S] can be easily accommodated. Uniformly shaped confidence regions, derived from the distribution of Kendall's (1970) S statistic, corrected for ties, can be obtained for all suitable values of n (n = number of observations) at any required level of significance.

Distribution of Kendall's (1970) S statistic

Given two sets of observations on n objects, (X_i, Y_i), $i = 1, \ldots, n$, Kendall's S (Kendall, 1970) is defined as:

$$S = \sum_{i=1}^{i=n-1} \sum_{j=i+1}^{j=n} c(X_j - X_i)\, c(Y_j - Y_i)$$

where, for the present purpose, $c(u)$ is defined by eqn. (9). S is a discrete random variable and is defined for $S = -\frac{1}{2}n(n-1)$, $-\frac{1}{2}n(n-1)+2$, ..., $+\frac{1}{2}n(n-1)-2$, $+\frac{1}{2}n(n-1)$, when there are no replications. S can therefore have any of $\frac{1}{2}n(n-1)$ integer values, evenly spaced in units of 2. The value of S is the total score that would be obtained if the X_i were ranked in numerical order from smallest to largest and the corresponding Y_i were then compared in the following way: for each Y_i, if the value of $Y_j, j > i$, is numerically larger, score +1, but if the value of Y_j is numerically smaller, score −1 [for replications, $c(u) = 0$, and the contribution to S is defined as zero]. There are $n!$ possible permutations of the ranks of the Y_i (when there are no replications) and therefore there are $n!$ ways of forming the total score S. If the X_i and Y_i are independent, there ought to be a total lack of correlation between the ranks of the X_i and the ranks of the Y_i, and the value of S should be close to zero. If the X_i and the Y_i are highly correlated, however, very large positive or very large negative values of S will be obtained.

Problems occur if replicate observations are made for a single value of X; in general, an r-replicate determination gives rise to $r!$ pairings, each of which contribute 0 to the total score S. The effect of replication is not only to decrease the maximum value that S can attain by a factor of $\frac{1}{2}r(r-1)$ for each replicate, but also to decrease the number of permutations possible by a factor of $1/r!$ for each replicate (Sillitto, 1947). Therefore it is necessary to recalculate the cumulative probability of S for each possible combination of replications for each value of n. This has been done for all duplicates, triplicates and combinations thereof for $n = 1, \ldots, 10$ (Sillitto, 1947). The cumulative probability for S without ties has been extensively tabulated (Kendall, 1970; Hollander & Wolfe, 1973, pp. 384–393; Kaarsemaker & van Wijngaarden, 1953).

Fortunately, for sufficiently large values of n, the distribution of S can be approximated by a standard unit normal probability curve if the number and size of the replications are not excessive. The normal

approximation holds for $n \geqslant 10$ with no replications at $\alpha = 0.025$ (two-tailed); it also holds for $n \geqslant 10$ with any number of duplicates at $\alpha = 0.025$ and for $n \geqslant 12$ with any number of triplicates at $\alpha = 0.025$. It may be necessary to verify by direct computation of the discrete probability that the normal probability approximation is valid in other cases.

The normal approximation of S is given by the following formula (Kendall, 1970):

$$S^*(n, \alpha/2) = z_{\alpha/2} \sqrt{\frac{n(n-1)(2n+5) - \sum_{i=2}^{r} p_i i(i-1)(2i+5)}{18}}$$

where $z_{\alpha/2}$ is the deviation from the mean of the standard unit normal distribution for which $P\{x \leqslant z_{\alpha/2}\} = \alpha/2$ (e.g. for $\alpha = 0.025$, $z_{\alpha/2} = 2.24$), and n is the number of observations of y; p_i is the number of replicates of size i, and r is the size of the largest replicate.

Signed-rank methods for comparing two experiments

A simple method for estimating the difference ($\Delta K_m, \Delta V_{max.}$) for the K_m and $V_{max.}$ values obtained in two experiments is to find the arithmetic difference (e.g. $K_{m1} - K_{m2}$) of the median estimates obtained from the direct linear plot. This method, although easily accomplished by simple graphical analysis, does not permit any estimate of the reliability of the measured differences. Only hypothesis-testing, which merely indicates the significance of the difference, can be performed by using the confidence regions for the separately estimated ($K_m, V_{max.}$). Since the K_m and $V_{max.}$ values found in each experiment are highly correlated, it is desirable to estimate not only the difference vector ($\Delta K_m, \Delta V_{max.}$) but also its joint-confidence region.

An alternative statistical procedure, which will provide such an estimate of reliability, can be devised by using Hollander's (1970) test for comparing the slopes of two regression lines. This distribution-free test is a purely computational method with no simple graphical equivalent, and is based on Wilcoxon signed-rank statistics (Wilcoxon, 1945). Estimates derived in this way satisfy the conditions of Puri & Sen (1971) for the elements of a p-dimensional random vector. Differences in K_m (ΔK_m) for two experiments can be estimated by comparing the slopes of the regression lines given by eqn. (4). Similarly, differences in $V_{max.}$ ($\Delta V_{max.}$) for two experiments can be estimated by comparing the slopes of the regression lines given by eqn. (5). The joint estimate ($\Delta K_m, \Delta V_{max.}$) obtained in this fashion has an associated joint-confidence interval obtained from the Bonferroni inequality, eqn. (12), and the individual confidence limits for ΔK_m and $\Delta V_{max.}$ found by Hollander's (1970) method.

Efficient design of experiments

Unfortunately, the errors of measurement encountered in the laboratory for the types of experiments that might be described by eqn. (1a) are frequently large (a relative error of 10% or more is not uncommon). Further, the cost in time, labour and material for obtaining additional measurements limits the feasibility of extended experimentation. Therefore it is desirable to design experiments in such a way that they may be completed with a minimum number of measurements yet give adequately precise results.

Essentially four cases may be considered in the proper design of experiments: the first has as its object the precise determination of K_m and $V_{max.}$ where the investigator is willing to assume, on the basis of prior experience, that eqn. (1a) describes the behavior of the system being studied; a second type of experiment has as its object the detection of departures from strict adherence to eqn. (1a); such experiments might be a crucial part of mechanistic studies, for example. A third type of experiment has as its object the determination of the effect of a perturbing agent (inhibitor, activator etc.) on the system; this type of experiment may either assume that eqn. (1a) described the data, or it may require a test of this assumption. The fourth type of experiment is exploratory: the object may be to determine acceptable ranges in [S] for further experiments, to test grossly for departures from eqn. (1a), to determine effects of variations in [S] on the precision with which v can be determined etc. The proper experimental design for this last case has been described in detail by Cleland (1967). The utility of the direct linear plot as a graphical method for obtaining estimates of K_m and $V_{max.}$ quickly during such exploratory experiments has been amply discussed by Eisenthal & Cornish-Bowden (1974).

The design of Cleland (1967) is very inefficient for the other three cases, however. The first case requires that data be collected at only two values of [S]: a very low value (limited by the precision of the analytical technique), and a high value (limited by saturation of the system). This case, discussed by Eisenthal & Cornish-Bowden (1974), yields a direct linear plot in which the lines intersect at the largest possible angle.

By making replicate observations at both values of [S], the experimental precision can be increased more readily than by making an equal number of observations at intermediate values of [S]. At least four observations at each value are needed in order to construct a 95% joint-confidence region by the methods described above. Sen (1968) has shown that under these conditions the experiment is optimally designed, in the sense that if the errors were in fact normally distributed, the non-parametric results would have an efficiency of 95% compared with the least-squares results.

The second case requires some prior knowledge about the alternatives that might reasonably be expected. Preliminary experiments may provide clues to the proper design of this type of experiment. In the absence of any specific alternative model, data should be collected over as wide a range in [S] as is feasible. If the choice must be made between replication at few values of [S] or single observations at many values of [S], the former course will give more information about lack of fit for a given number of observations. On the other hand, if a specific alternative model is being considered, it is only necessary to make observations at three values of [S]: a low and high value, as in the first type of experiment, and a value at which the alternative model is expected to produce a maximum deviation from eqn. (1a) (Cox, 1958*a*, *b*).

The third case is similar to the first case; presumably the necessary preliminary experiments that suggested that the perturbation was worth investigating have been completed and it is known that eqn. (1a) still adequately describes the system. It is again only necessary to obtain observations at two values of [S] in both the control and treatment experiments. Hollander's (1970) test requires seven pairs of slope estimates (28 observations) to construct a 95% joint-confidence region for the difference vector. If there is doubt about the adequacy of the model, some of these observations should be made at intermediate values of [S].

Whenever possible, the experiment should be performed as a paired-replicates design: every attempt should be made to duplicate exactly the experimental conditions under which one control observation and its corresponding treatment observation are obtained. For each pair of observations the order in which they are made should be randomized.

Whatever design is chosen for the final experiments, preliminary experiments must be carried out to probe for controllable sources of variation and to establish the accuracy and precision of the analytical method. Statistical evaluation of data is not a substitute for careful experimental technique or thoughtful planning.

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