NEW LINEAR PLOTS FOR THE SEPARATE ESTIMATION OF MICHAELIS–MENTEN PARAMETERS

Cs. FAJSZI and L. ENDRENYI

Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, 6701 Szeged, Hungary

and

Department of Pharmacology, Department of Epidemiology and Biometrics, University of Toronto, Toronto, Canada M5S 1A8

Received 2 July 1974

1. Introduction

The relationship between observed reaction velocities (v) and substrate concentrations (c) of the reaction

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

is often described by the hyperbolic Michaelis–Menten equation:

$$v = Vc/(K_m + c) \quad (1)$$

Eq. (1) is used also in binding studies, with c_b and c_f (concentration of bound and free ligand) instead of v and c, respectively.

The two parameters, the maximal, asymptotic velocity (V) and the Michaelis constant (K_m), have been traditionally evaluated from the various linearisations of the hyperbola, such as the double reciprocal Lineweaver–Burk relationship. However, these transformations tend to yield incorrect (biassed) parameter estimates with comparatively large errors. The disadvantageous properties of most linear plots were predicted by intuitive and statistical arguments [1–4] and substantiated by computer simulated experiments [5–7], and consequently, parameter estimation by nonlinear regression has been recommended [3–13].

Indeed, with asymptotically large samples, nonlinear least-squares estimates of nonlinear parameters are known to have some favourable, optimal properties: they are, under certain conditions, consistent, normally distributed and, in the case of normal observation errors, efficient [14–16]. However, at limited sample sizes the least-squares method is expected to yield good, robust, but not optimal nonlinear parameter estimates. Thus, it is quite possible that alternative procedures can be developed which lead to improved estimated values of the constants. An approach aimed at conceivably reducing the errors of the calculated parameters could be based on their separate evaluation since, by paying attention to these constants one at a time, improved precision may be attained.

Two methods of separate parameter estimation will be considered. Both of these evaluate the constants from sets of suitably paired data (usually observations). The first may be applied, in any experimental design, to models which can be arranged to a linear form having a slope related to only one of the constants. The second approach is suited to a more general class of models (those permitting the elimination of one or more of their constants) but is restricted in the permissible experimental designs.

The application of these techniques to the estimation of Michaelis–Menten parameters will be explored in this communication.

 North-Holland Publishing Company – Amsterdam

2. Method of slope identification

2.1. *Development of the method*

Quite often the slope (B) of a straight line,

$$y = A + Bx$$

is related to a single parameter of an investigated model and this parameter can be evaluated from two observations y_1 and y_2 which are obtained at the corresponding values of the independent variables x_1 and x_2, i.e.

$$B = (y_1 - y_2) / (x_1 - x_2) = \Delta y / \Delta x. \quad (2)$$

For example, in the Eadie–Hofstee representation of the Michaelis–Menten equation,

$$v = V - K_m (v/c),$$

the slope is $-K_m$, and the Michaelis constant can be evaluated from

$$\Delta v / \Delta (v/c) = -K_m, \quad (3)$$

where $\Delta v = v_2 - v_1$ and $\Delta (v/c) = v_2/c_2 - v_1/c_1$, v_1 and v_2 are the reaction rates using c_1 and c_2 substrate concentration, respectively.

Similarly, based on the Hanes–Woolf transformation of the Michaelis–Menten equation,

$$c/v = K_m/V + (1/V)c,$$

the asymptotic velocity can be obtained from

$$\Delta (c/v) / \Delta c = 1/V, \quad (4)$$

where:

$$\Delta (c/v) = c_2/v_2 - c_1/v_1 \text{ and } \Delta c = c_2 - c_1.$$

If more than two, say N observations are made then, in expression (2), N-1 independent pairs of x's and y's can be found and, therefore, the parameter B can be evaluated from the average of the corresponding N-1 ratios of $\Delta y/\Delta x$. At the same time, $1/B$ can be similarly estimated by averaging N-1 independent ratios of $\Delta x/\Delta y$. The two methods can be expected to yield close but not identical values of B.

Further procedures estimating the parameter B can be derived from rearrangements of relation (2), such as

$$\Delta y = B \cdot \Delta x. \quad (5)$$

Here B is the slope of a straight line passing through the origin when Δy is plotted against Δx and, therefore, it can be obtained from the corresponding linear regression calculations. Analogously, $1/B$ can be evaluated as the slope of a straight line passing through the origin in the plot of Δx against Δy,

$$\Delta x = (1/B)\, \Delta y. \quad (6)$$

These procedures can be extended substantially by multiplying both sides of expression (5) by some function of the x's and y's. One example of this kind of analysis involves the function

$$x_1 x_2\, \Delta y = B x_1 x_2 \Delta x. \quad (7)$$

The evaluation of the inverse relation (6) can be similarly extended, and B is calculated again by linear regression.

The relationships (2)–(7) can be written in the form of

$$Y = B'X.$$

B' can be calculated by averaging the Y values when X is defined as unity ($X \equiv 1$) and by linear regression when $X \not\equiv 1$.

Applications of the various estimation methods to the Michaelis–Menten parameters are listed in table 1. All methods evaluate the same constants, V or K_m; In error-free experiments they would yield identical values. However, in the presence of random observational errors the calculated parameters and their errors can be substantially different.

2.2. *Choice of data pairs*

Observations can be paired in various ways in order to form the data points Δy and Δx. For instance, from an ordered sequence of concentrations the neighbouring values may be paired (together with the corresponding velocities). However, this procedure

Table 1
Methods for the evaluation of Michaelis–Menten parameters from paired observations

	Estimation of K_m [a]			Estimation of V [a]		
No.	Y	B'	X	Y	B'	X
1 [b]	$\Delta v/\Delta(v/c)$	$-K_m$	1	$\Delta c/\Delta(c/v)$	V	1
2 [b]	$\Delta(v/c)/v$	$-1/K_m$	1	$\Delta(c/v)/\Delta c$	$1/V$	1
3 [c]	Δv	$-K_m$	$\Delta(v/c)$	Δc	V	$\Delta(c/v)$
4 [c]	$\Delta(v/c)$	$-1/K_m$	Δv	$\Delta(c/v)$	$1/V$	Δc
5 [c]	$c_1c_2\cdot\Delta v$	$-K_m$	$c_1c_2\cdot\Delta(v/c)$	$v_1v_2\cdot\Delta c$	V	$v_1v_2\cdot\Delta(c/v)$
6 [c]	$c_1c_2\Delta(v/c)$	$-1/K_m$	$c_1c_2\cdot\Delta v$	$v_1v_2\cdot\Delta(c/v)$	$1/V$	$v_1v_2\cdot\Delta c$

[a] Evaluation of the parameter is based on the expression $Y = B'X$.
[b] B' is estimated by averaging Y.
[c] B' is estimated from the linear regression of Y on X forced through the origin.

combines readings with only small differences in the velocities which could be very strongly distorted by experimental errors.

Consequently, an alternative approach could pair concentrations and velocities which are as far from each other as possible. With N=10 observations, these two point-pair selections would be (by indicating the order members of the concentrations):

No. 1: 1–2; 2–3; 3–4; 4–5; 5–6; 6–7; 7–8; 8–9; 9–10
No. 2: 1–6; 1–7; 1–8; 1–9; 1–10; 2–10; 3–10; 4–10; 5–10.

The latter method is quite asymmetrical since the largest and smallest observations appear in it with much greater frequency than the others. Therefore, compromise pair selections may be devised which maintain reasonable balances among the various observations while keeping quite large the distance within each pair. Such a selection could be;

No. 3: 1–6; 1–7; 2–7; 2–8; 3–8; 3–9; 4–9; 4–10; 5–10.

The effect of point-pair selection and method of evaluation on the precision and accuracy of the estimated parameters was investigated. The results of computer simulated experiments are described in the next section.

2.3. *Evaluation of the method*

Various factors affecting the results of parameter estimation [7] were considered for the analysis of the proposed methodology: the error structure, the experimental design and the method of evaluation.

A computer simulation study was performed to test accuracy (lack of bias) and precision (reproducibility) of the estimates, obtained by using expressions of table 1.

For the purpose of simulation we used the dimensionless form of the Michaelis–Menten equation [6]:

$$v' = c'/(1 + c') \qquad (8)$$

where $v' = v/V$ is the relative velocity and $c' = c/K_m$ is the relative concentration. This equation shows that all hyperbolic functions are similar in form, so that one can simulate the parameter estimation procedure as if the true values of K_m and V were unity. Noting this, we can omit the primes and use eq. (8) as the starting equation in the following.

In the simulated experiments normally distributed errors were assumed which in some cases were constant in the range of experimentation (constant absolute error), in others proportional to the velocity (constant relative error).

In designing the experiments various numbers of observations (5 or 10), experimental ranges (0.05 or 0.20 for the lowest 'true' relative velocity, and 0.60, 0.80 or 0.95 for the highest values) and concentra-

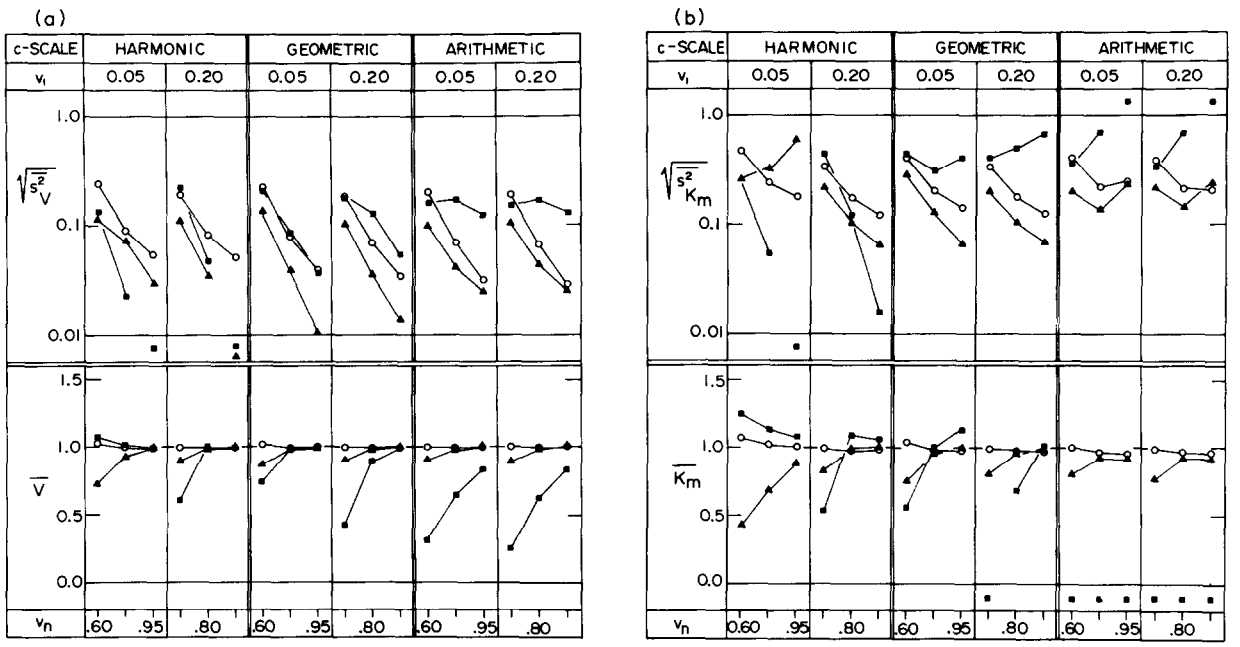

Fig. 1. Estimation of Michaelis–Menten parameters by separate evaluation and by nonlinear regression when the absolute observational error is constant, $\sigma = 0.05$ $V = 0.05$. (a) Evaluation of V; (b) Evaluation of K_m. Each simulated experiment containing 10 observations was repeated 100 times. Several experimental designs were considered as indicated in the diagram. The parameters were evaluated by the fifth method listed in table 1 (linear expressions (9) and (10) in the text) with 9 data points formed either from the neighbouring observations (selection No. 1, indicated by ■) or by a compromise point-pair selection (No. 3, designated by ▲). Nonlinear least-squares parameter estimates are designated by o. In the lower half of the diagram average estimated parameter values are shown: Deviations from the true value of 1.0 indicate bias. The upper half of the figure shows the square-root of the average estimated variance of the parameters.

tion spacings (harmonic: when $1/c_{i+1} = 1/c_i + d$, i.e. the reciprocals of the concentrations are uniformly spaced; geometric; logarithmic: when $c_{i+1} = c_i \cdot q$, i.e. the ratios of successive concentrations are identical; arithmetic: when $c_i + d$, i.e. the concentrations are uniformly spaced; or c^2–arithmetic) were considered.

In addition to the parameter estimation methods listed in table 1, the two constants were evaluated also by nonlinear regression [3–13]. In studies assuming constant relative errors weighted nonlinear regression was applied with weights inversely proportional to the squares of predicted velocities [4,7].

In a series of preliminary investigations several combinations of evaluation procedures and observation-pair selections were found to yield strongly biased and imprecise parameter estimates and, therefore, were eliminated from further considerations. Results of the remaining methods are shown in fig. 1 for experiments with constant absolute error, and in fig. 2 for cases in which the relative error is constant.

Among the methods listed in table 1 for the evaluation of the two constants, the slopes of the fifth relationship (cf. eq. (7)),

$$c_1 c_2 (v_2 - v_1) = K_m (v_2 c_1 - v_1 c_2) \qquad (9)$$

and

$$v_1 v_2 (c_2 - c_1) = V (v_1 c_2 - v_2 c_1), \qquad (10)$$

yield the best results in studies assuming constant absolute error (fig. 1). With the third (the compromise) point-pair selection, the estimated parameter errors tend to be somewhat lower than those obtained by nonlinear regression. In contrast, the first pairing scheme (from neighbouring observations) provides

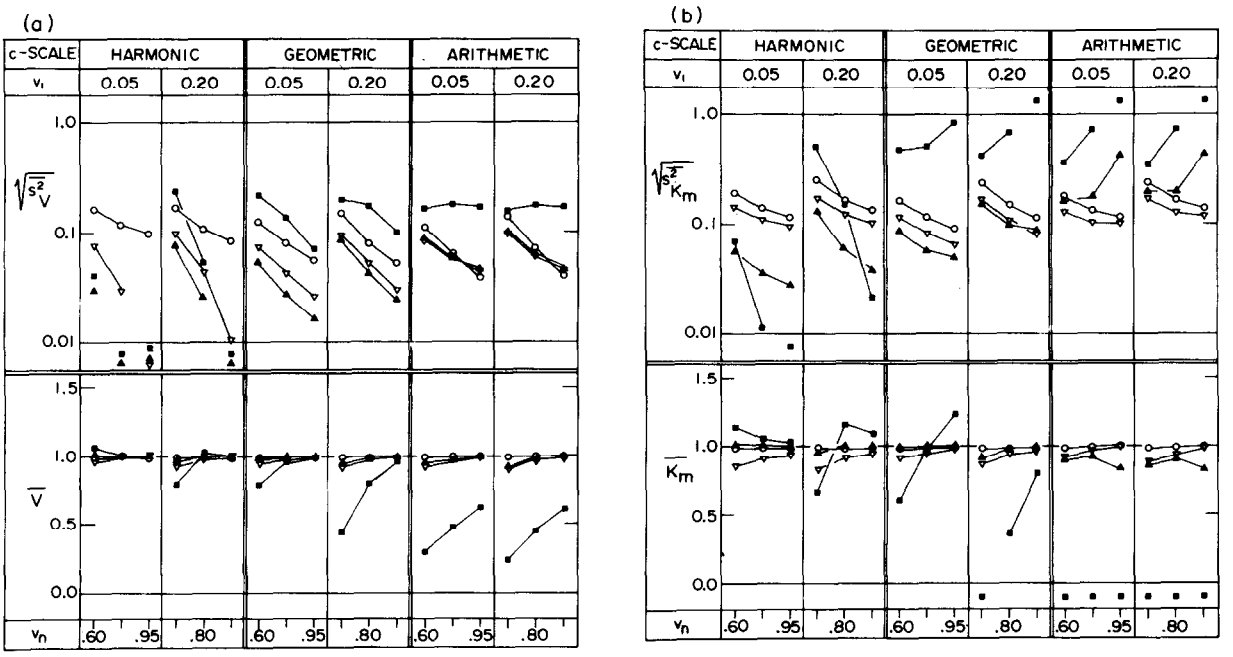

Fig. 2. Estimation of Michaelis–Menten parameters by separate estimation and by weighted nonlinear regression when the relative observational error is constant, $\sigma = 0.10\ v/V = 0.10\ v$. (a) Evaluation of V; (b) Evaluation of K_m. Interpretation of the diagram is identical with that of fig. 1 with the addition of results of estimation following expressions (11) and (12) after data pairing by scheme No. 3 (indicated by ∇).

quite low error estimates, especially if the range of observations can be extended to high relative velocities, but only if the concentrations are spaced in harmonic progression. The linearization yield higher biases than nonlinear regression estimations, even though the difference is not always substantial.

Similar conclusions can be drawn also in the case of constant relative errors (fig. 2), except that now the compromise point-pair selection (No. 3) does not yield favourable results with arithmetic concentration scaling. With this design, the third estimation method listed in table 1 (Eadie–Hofstee and reciprocal of Hanes–Woolf plots, cf. eqs. (3), (4)), involving the plots

$$v_2 - v_1 = -K_m \quad \frac{v_2}{c_2} - \frac{v_1}{c_1} \tag{11}$$

and

$$c_2 - c_1 = V \quad \frac{c_2}{v_2} - \frac{c_1}{v_1} \tag{12}$$

yield generally the smallest error estimates, provided that the compromise pairing scheme (No. 3) is used. This calculational procedure leads, at all experimental designs, to reasonably low estimated parameter error.

3. Method of parameter elimination

In a parallel work [17], evaluation of the parameters in the Hill equation,

$$v = Vc^n/(K^n + c^n), \tag{13}$$

is considered. (K is a Michaelis-type constant, n is the degree of the equation).

It is shown by eliminating the constant K from expressions for two observations that, if v_i and v_j are velocities observed at concentrations c_i and $c_j = \alpha c_i$,

respectively (where α is constant throughout an experiment), then they are related through a hyperbolic expression:

$$v_j = Lv_i/(M+v_i), \quad (14)$$

where $M=V/(\alpha^n-1)$ and $L=M\alpha^n$. Consequently, the parameters n and V can be evaluated by all methods applicable to the hyperbola, including linearizations. In the case of the Michaelis–Menten equation $n=1$ and, therefore, the same procedures can be used for the estimation of V.

V can be similarly eliminated from two hyperbolic expressions. As a result, by maintaining $v_j = \beta v_i$ with a constant β throughout the experiment a hyperbolic relation between c_j and c_i is obtained:

$$c_j = Pc_i/(Q+c_i), \quad (15)$$

with $Q=K_m/(1-\beta)$ and $P=\beta Q$. The relationship enables the evaluation of K_m.

Furthermore, it can be shown that, with $c_j = c_i+\gamma$, the linear relationship

$$c_j/v_j = c_i/v_i + \gamma/V \quad (16)$$

is obtained and that for $v_j = v_i + \delta$, another linear expression,

$$v_j/c_j = v_i/c_i + \delta/K_m, \quad (17)$$

is derived. From the intercepts of the corresponding plots V and K_m, respectively, can be calculated, provided that a constant c value of γ or δ is maintained in an experiment.

If v_i and v_j refer to actual observations then application of the four methods based on expressions (14)–(17) is restricted to the four corresponding experimental designs.

4. Discussion

Parameters can be evaluated, at times, advantageously by separate estimation. This appears to be true in the case of Michaelis–Menten parameters, especially for regression calculations which utilize relations (9) and (10) and point-pairing from neighbouring observations and also by the compromise scheme (method No. 1 and 3). The estimated parameter errors obtained from these calculations are generally lower than those yielded by nonlinear regression while the corresponding biases are usually only somewhat less favourable. The expected values of the estimates remain to be evaluated.

At a first glance it is surprising that a linear regression yields as good estimates, or even better, than the nonlinear regression. There are two factors which make this possible. First, by estimating the parameters separately, improved precision may be attained. Second, the nonlinear least-square estimates are optimal only for asymptotically large samples [14–16]. At a limited sample size other procedures can yield better results. In the recommended estimation method (eqs. (9) and (10)), or in the case of arithmetic concentration arrangement with constant relative error (eqs. (11) and (12)) both of these factors are present.

The method of parameter elimination has been, at least in part, assessed elsewhere, applying to the Hill equation, the parameter n of which can be estimated by this method without the preliminary knowledge of the maximal velocity [17–19].

The various methods of separate parameter estimation involve data points formed from observation pairs. This kind of pairing imposes constraints on the experimental design when the method of parameter elimination is applied: depending on the calculation scheme, either the ratio of or the difference between paired concentrations or velocities must be maintained at a fixed, constant value. However, in practice, the data points need not be composed from actual observation. A curve my be fitted to the measurements in any of the usual plots and the paired data points obtained from these.

Finally, the importance of careful general experimental design [7] is re-emphasized. With constant absolute error (fig. 1), the observed velocities should extend to as high values as possible but they may (and preferably should) be constrained at the low end. With constant relative error (fig. 2), the range of measurements should be as wide as possible.

Acknowledgements

The expert and helpful programming assistance of

Dr F. H. F. Kwong and the useful suggestions of Dr T. Keleti are gratefully acknowledged. The work was supported by the Medical Research Council of Canada.

References

[1] Goldstein, A. (1949) Pharmacol. Rev. 1, 102–165.
[2] Riggs, D. S. (1963) The mathematical approach to physiological problems, pp. 276–280, Williams and Wilkins, Baltimore.
[3] Johansen, G. and Lumry, R. (1961) Compt. Rend. Trav. Lab. Carlsberg, 32, 185–214.
[4] Wilkinson, G. N. (1961) Biochem. J. 80, 324–332.
[5] Dowd, J. E. and Riggs, D. S. (1965) J. Biol. Chem. 240, 863–869.
[6] Colquhoun, D. (1969) Appl. Statist. 18, 130–140.
[7] Endrenyi, L. and Kwong, F. H. F. (1972) in: Analysis and simulation of biochemical systems (Hemker, H. C. and Hess, B., eds.), pp. 219–237, North-Holland, Amsterdam.
[8] Cleland, W. W. (1963) Nature 198, 463–465.
[9] Bliss, C. I. and James, A. T. (1966) Biometrics 22, 573–602.
[10] Cleland, W. W. (1967) Adv. Enzymol. 29, 1–32.
[11] Hanson, K. R., Havir, E. A. and Ling, R. (1967) Biochem. Biophys. Res. Commun. 29, 194–197.
[12] Lasch, J. (1969) Acta Biol. Med. Germ. 23, 747–757.
[13] Hoy, T. G. and Goldberg, D. M. (1971) Biomed. Comp. 2, 71–77.
[14] Hartley, H. O. and Booker, A. (1965) Ann. Math. Statist. 36, 638–650.
[15] Jennrich, R. I. (1969) Ann. Math. Statist. 40, 633–643.
[16] Malinvaud, E. (1970) Ann. Math. Stat. 41, 956–969.
[17] Endrenyi, L., Fajszi, Cs. and Kwong, F. H. F. (1974) Eur. J. Biochem., submitted for publication.
[18] Silonova, G. V., Livanova, N. B. and Kurganov, B. I. (1969) Molekul. Biol. 3, 768–784.
[19] Kurganov, B. I., Dorozhko, A. I., Sinel'nikova, E. M. and Kagan, Z. S. (1972) Dokl. Akad. Nauk SSR, 203, 1414–1417.