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A NEW METHOD FOR DETERMINING ENZYME KINETIC CONSTANTS

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INTRODUCCION

There is nowadays a growing interest on the statistical reliability of kinetic parameters estimates. Although some authors state that the accuracy of data obtained by conventional methods suffices for general biochemical purposes, it is obvious that to study such items as the kinetics of products formation via a long chain of reactions, theoretical predictions can largely deviate from true results unless robust data of V and Km for individual steps are available. Since kinetic parameters reliability partly depends on the method used for their estimation, several procedures for obtaining them have recently appeared to add to the traditional ones. ATKINS and NIMNO[1] have compared seven of the best known of these methods (2-8) and reported that the procedure of WILKINSON[5] was the most reliable when initial rate data contained errors of constant absolute magnitude, while that of EISENTHAL & CORNISH-BOWDEN[7] gave the best estimates when data contained outliers or errors were of constant relative magnitude; on view that some of the new methods gave clearly worst results than the traditional ones, ATKINS and NIMNO[1] strongly recommended that each newly proposed procedure be rigorously tested before published.

In this paper a method for estimating Km and V is described and compared with those of WILKINSON[5], EISENTHAL AND CORNISH-BOWDEN[7] and the three lineal transformations of the Michaelis-Menten equation currently used (2-4).

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THEORY

General considerations

The three most important properties of any system for fitting the Michaelis-Menten equation are versatility, accuracy (lack of bias) and precision (grouping of data around the mean). To increase the versatility of the method here described, no assumption about the type of errors in initial rates was made. Most procedures for determining kinetic parameters are based on a set of assumptions on data errors, which, in our opinion, is not a sensible starting position since the kinetic researcher has not usually experimental evidence on the sort of error of his data and, as pointed out by STORER *et al.*[9], it may be very hazardous relying on theoretical generalizations.

Each one of the methods for obtaining V and Km is comparatively more sensitive to errors in a certain range of substrate concentrations (e.g., plots of 1/v versus 1/s are strongly affected by errors at low concentrantions, while bad data at high substrate levels specially damage plots of s/v against s). The precision and accuracy of kinetic parameters estimates are very heavily influenced by these «position effects». To minimize this negative influence, we first looked for methods to obtain V and Km scarcely affected by it and chose those more appropriate to balance the effects of errors position, calculating V and Km by combining the estimates obtained by several procedures the concentrations range of maximal sensitivity to errors of each coinciding with the zones at which the influence of bad data is minimal for the rest.

Equations

The relationships between a series of initial rates (v_1, v_2, ..., v_n) and substrate concentrations (s_1, s_2, ..., s_n) can be described by the Michaelis-Menten equation rearranged in the following way:

$$V.s_1 = (v_1.s_1) + (Km.v_1) \tag{1}$$

$$V.s_2 = (v_2.s_2) + (Km.v_2) \tag{2}$$

....

$$V.s_n = (v_n.s_n) + (Km.v_n) \tag{3}$$

By adding all these equations:

$$V.\Sigma s_i = (\Sigma v_i s_i) + (Km.\Sigma v_i) \tag{4}$$

From other rearrangements of the Michaelis-Menten equation can equally be derived:

$$V.\Sigma s_i/v_i = (\Sigma s_i) + (n.\ Km) \tag{5}$$

$$V.\Sigma 1/v_i = n + (Km.\Sigma 1/s_i) \tag{6}$$

$$V.n = (\Sigma v_i) + (Km.\Sigma v_i/s_i) \tag{7}$$

where «n» is the number of observations (s, v).

By combining eqn. (4-7), 63 different ways to estimate V may be obtained; the following two were chosen on the basis of the considerations previously exposed:

$$V = \frac{(\Sigma v_i s_i\ .\ \Sigma v_i/s_i) - (\Sigma v_i)^2}{(\Sigma s_i \Sigma v_i/s_i) - (n.\Sigma v_i)} \tag{8}$$

$$V = \frac{(\Sigma s_i \Sigma v_i/s_i) - (n.\Sigma v_i)}{(\Sigma s_i/v_i\ \Sigma v_i/s_i) - n^2} \tag{9}$$

obtained by resolving eqn. (7) for Km and substituting Km for this value in eqn. (4) and (5), respectively.

By combining eqn. (4-9), 67 methods of Km estimation can be derived. On the same reasons, the following three were selected:

$$Km = \frac{(V \cdot \Sigma s_i) - (\Sigma v_i s_i)}{(\Sigma v_i)} \tag{10}$$

$$Km = \frac{(n.V) - \Sigma v_i}{\Sigma v_i/s_i} \tag{11}$$

derived by rearranging eqn. (4) and (7), respectively, and

$$Km = \frac{(n.\Sigma s_i) - (\Sigma v_i \Sigma s_i/v_i)}{(\Sigma v_i/s_i \Sigma s_i/v_i) - n^2} \tag{12}$$

obtained by resolving eqn. (5) for V and substituting in eqn. (7).

Obviously, eqn. (8-12) can only be applied when one has at least two rate data at different substrate concentrations.

Estimation of V and Km.

V is obtained as the mean of two secondary values:

(a) One calculated by averaging the two primary results from eqn. (8) and (9).

(b) The other estimated by taking the median of the *n(n*-1)/2 primary values of V reached by processing each possible binary combination of the *n* available observations (s_1v_1 with s_2v_2, s_3v_3, ..., s_nv_n; s_2v_2 with s_3v_3, ..., s_nv_n; ...; $s_{n-1}\ v_{n-1}$ with s_nv_n); median is preferred to mean because the distribution of these primary values largely deviates from normality. So operating, the same values are obtained regardless of the method used for processing the pairs of observations. For this purpose eqn. (8) has been applied in the program used for the present work, but for rutine laboratory use may be more convenient some graphical method in order to detect bad rate data or situations in which the experimental results do not conform to Michaelis-Menten equation. Fig. 1 shows how to use the plot of 1/v versus 1/s for estimating this secondary V value from 4 experimental observations.

Km is calculated as the mean of the two secondary values found by the following way:

(a) Averaging the three primary Km obtained from eqn. (10), (11) and (12); for this purpose, the final V value calculated as already indicated must be used in eqn. (10) and (11).

(b) Taking the median of the primary Km values obtained by applying eqn. (12) or any other suitable method to each possible binary combination of observations, as before. How to obtain this secondary Km by means of the 1/v versus 1/s plot is shown in Fig. 1.

Application to more complexes cases

This method can be used as well for calculating the kinetic parameters of bisubstrate reactions: a) From all the observations (A, v) at each of the *m* concentrations of B as «fixed substrate», *m* apparent V and K_m^A are estimated. b) K_s^A is obtained by representing these apparent values as 1/V and $-1/K_m^A$ in a plot of 1/v versus 1/A and drawing the *m* straight lines from each 1/V to the $-1/K_m^A$ corresponding to the same «fixed substrate» concentration; the median of the proyections on the *X* axis from the $m(m-1)/2$ interception points of these lines (Fig. 2) gives an excelent estimate of $-1/K_s^A$. c) By taking as rate data the apparent values of V and as «s» those concentrations of B at which the V^{App} were obtained and reprocessing these data, K_m^B and a first estimate of the maximum velocity of the reaction are calculated. d) K_s^B, K_m^A and a second estimate of the maximum rate can be equally derived from the observations (B, v) at each of the m concentrations of A as «fixed substrate». e) A final value for the maximum reaction rate is calculated by averaging the two estimates obtained in (c) and (d).

By similar procedures it is possible to resolve any case in which the rate equation can be written in the form:

$$v = \frac{V^{app}}{1 + (K_m^{app}/s)} \qquad (13)$$

which is obeyed by most enzyme reactions.

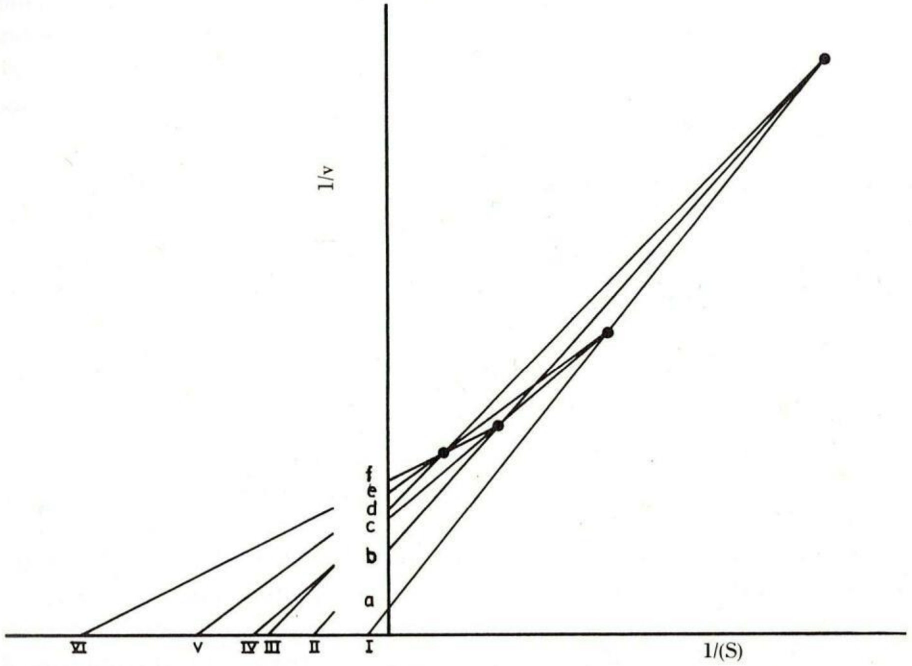

Fig. 1.–Estimation of the «b» secondary values for V and Km from four experimental observations ( ) by means of the 1/v versus 1/s plot. Straight lines joining each point to all others are drawn. This generates six [n(n-1)/2] primary values for 1/V («a» to «f») and other six for −1/Km («I» to «VI»). The medians («c» + «d»)/2 and («III» + «IV»)/ are taken as 1/V and −1/Km, respectively.

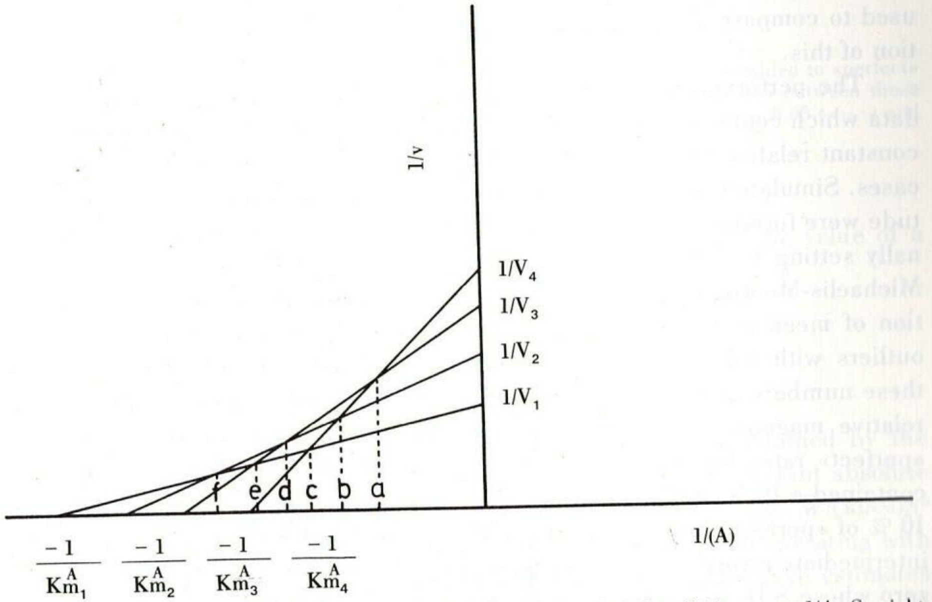

Fig. 2.–Ks estimation in bisubstrate reactions by means of a plot of 1/v versus 1/A. Straight lines from the values of 1/V , on the vertical axis, to the corresponding apparent values of −1/Km are drawn. −1/Ks is obtained as the median of the m(m-1)/2 projections on abscisae («a» to «f») of the intersection points of these lines.

METHODS

To compare the methods tested we have used simulated rate data following Atkins and Nimno (1) except in that:

(a) Five values of «s», instead of seven, have been used for each V and Km estimation since this figure is more closed to the number of observations generally performed. Hundred different V and Km values, instead of fifty, were obtained in each experiment in order to attain a larger population in which smaller bias became significant.

(b) Three substrate concentration ranges (short, 0.25-1.86 Km; medium, 0.25-4 Km; and long, 0.25-19 Km) were used instead of one, since preliminar experiences showed qualitative differences respect to the performance of some of the methods tested at different ranges. Substrate concentrations were varied in a geometrical instead of aritmetical progression, as usual in laboratory experiments.

(c) All the methods tested yielded results whose shape of distribution was leptokurtic-like, with tails of different length; in this situation both the mean and the standard deviation have a very poor value since they are largely affected by a small number of outliers. To make feasible to compare the results obtained by the different methods tested, the highest and lowest 5 % of the estimates were suppressed, leaving a population with a distribution reasonably closed to the Gaussian one.

(d) The coefficient of variation instead of the standard deviation was used to compare the precision of the methods, since it gives a better estimation of this.

The performance of the procedures tested was compared by using rate data which contained three types of errors: of constant absolute magnitude, of constant relative magnitude and intermediate, with and without outliers in all cases. Simulated activity data incorporating errors of constant absolute magnitude were formed from «perfect», i.e. error-free, rates (obtained by conventionally setting V = Km = 1 and calculating «v» for the different «s» from the Michaelis-Menten equation) at which numbers taken at random from a population of mean zero and S.D. = 0.05 were added; when indicated, a 10 % of outliers with S.D. = 0.125 were included by randomly choosing a 10 % of these numbers and multiplying them by 2.5. Activities with errors of constant relative magnitude (C.V. = 10 %) were produced by multiplying a 10 % of «perfect» rates by numbers of mean one and S.D. = 0.1; populations which contained a 10 % of outliers with C.V. = 25 % were obtained by multiplying a 10 % of «perfect» rates by numbers of mean one and S.D. = 0.25. Rates with intermediate errors were formed by adding to «perfect» data numbers of mean zero whose S.D. was taken from the continuous line of Fig. 3 which arbitrarily represents an intermediate case between errors of constant absolute magnitude of S.D. = 0.05 and of constant relative magnitude with C.V. = 5 %; populations with outliers were obtained by multiplying by 2.5 the value of a 10 % of numbers chosen at random.

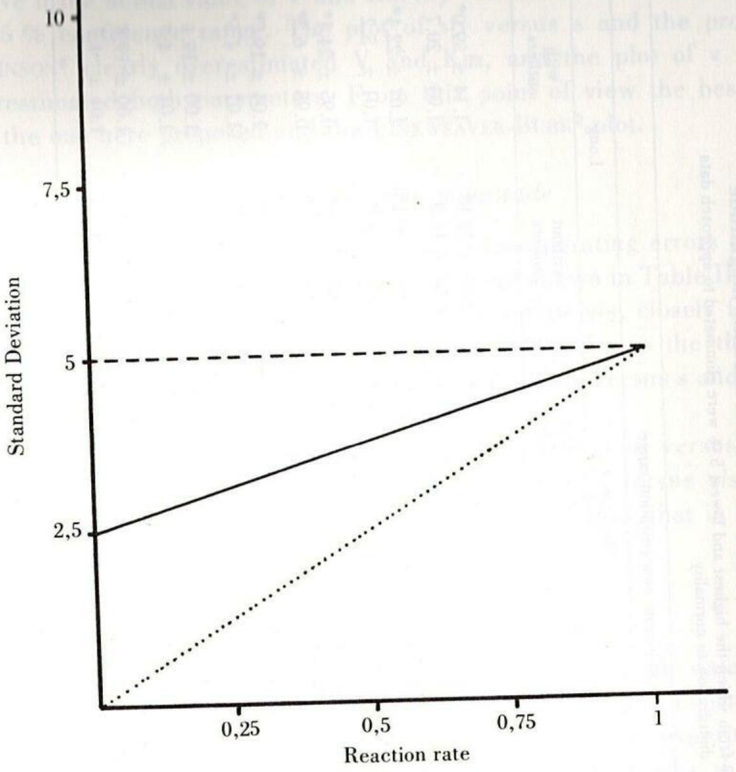

Fig. 3.–Arbitrary line (——) used for obtaining the S.D. o random numbers added to «perfect» data to form rates with intermediate errors. This line is the geometrical median between those data representing the S.D. of rate errors of constant absolute magnitude with S.D. = 0.05 (– – –) and constant relative magnitude with C.V. = 5 % (....).

RESULTS

Operation with data errors of constant absolute magnitude

Table I sumarizes the results of V and Km estimates obtained by the different methods compared from rates containing errors of constant absolute magnitude, with and without outliers. As it can be seen, the Wilkinson[5] method and the one here described were the most precise when operating with this kind of error. Eisenthal and Cornish-Bowden[7] procedure gave estimates of reasonably good precision. The three linear transformations yielded results of lower precision, the Lineweaver-Burk[2] plot being by far the least precise.

TABLE I

Performance of the method with rates containing errors of constant absolute magnitude

Values are means ± C.V. (%) of 100 estimations from which the highest and lowest 5 % were eliminated to approach data distribution to normality

METHOD		Substrate concentration range					
		Short		Medium		Long	
		without outliers	with outliers	without outliers	with outliers	without outliers	with outliers
1/v versus 1/s	Km	0.99 ± 131.0	0.86 ± 150.2	1.20 ± 76.7*	1.11 ± 82.4	1.15 ± 52.9*	1.14 ± 59.0*
	V	0.98 ± 71.6	0.91 ± 77.9	1.08 ± 39.5	1.04 ± 41.4	1.04 ± 18.9	1.03 ± 20.7
s/v versus s	Km	1.21 ± 36.1*	1.27 ± 48.9*	1.10 ± 22.3*	1.12 ± 27.6*	1.06 ± 19.1*	1.07 ± 22.4*
	V	1.09 ± 18.6*	1.11 ± 24.0*	1.03 ± 8.6*	1.04 ± 10.0*	1.01 ± 4.9	1.01 ± 5.2
v versus v/s	Km	0.73 ± 36.7*	0.63 ± 46.6*	0.90 ± 26.6*	0.85 ± 29.9*	0.98 ± 22.4*	0.95 ± 24.1*
	V	0.84 ± 17.3*	0.78 ± 21.3*	0.94 ± 10.3 *	0.92 ± 11.7*	0.99 ± 5.4*	0.98 ± 6.2*
Wilkinson	Km	1.08 ± 30.6*	1.09 ± 37.5*	1.05 ± 18.7*	1.06 ± 22.9*	1.04 ± 15.9*	1.04 ± 19.7*
	V	1.05 ± 16.5*	1.06 ± 20.4*	1.02 ± 8.2*	1.03 ± 9.6*	1.01 ± 4.1*	1.01 ± 4.5*
Eisenthal and Cornish-Bowden	Km	0.85 ± 33.5*	0.76 ± 37.2*	0.99 ± 22.3	0.94 ± 25.2*	1.02 ± 17.6	1.00 ± 19.4
	V	0.95 ± 14.7*	0.91 ± 16.1*	1.01 ± 8.1	0.99 ± 9.1	1.01 ± 4.4	1.01 ± 4.7
Proposed method	Km	0.88 ± 29.5*	0.80 ± 33.5*	0.99 ± 22.1	0.95 ± 24.7	1.02 ± 17.4	1.00 ± 19.8
	V	0.97 ± 14.4	0.95 ± 16.1*	1.00 ± 8.3	1.00 ± 9.4	1.01 ± 4.3	1.01 ± 4.7

* Mean out of the 95 % confidence range.

The criterium used to compare the accuracy of the methods tested was to observe if the actual value of V and Km (by convention 1) was included inside the 95 % confidence range. The plot of s/v versus s and the procedure of WILKINSON[5] clearly overestimated V and Km, and the plot of v versus v/s underestimated both parameters. From this point of view the best methods were the one here proposed and the LINEWEAVER-BURK[2] plot.

Operation with errors of constant relative magnitude

Km and V estimates obtained with data incorporating errors of constant relative magnitude, with and without outliers, are shown in Table II. The most precise procedure resulted to be the plot of v versus v/s, closely followed by our method. That of EISENTHAL and CORNISH-BOWDEN[7] was the third in this respect. The method of WILKINSON[5] and the plots of s/v versus s and 1/v versus 1/s were clearly less precise.

As before, the method of WILKINSON[5] and the plot of s/v versus s overestimated both V and Km; the linear transformation of v versus v/s tended to underestimate both parameters, altough in a lower degree that in the case of errors of constan absolute magnitude.

Operation with intermediate errors

Table III shows the Km and V values estimated from rate data containing errors intermediate between those of constant absolute and constant relative magnitude, a type of error which may be very frequent in experimental data according to STORER et al.[9]. The most precise method was the one here described, with slight but constant advantage over the EISENTHAL and CORNISH-BOWDEN procedure which was the second in precision. The procedure of WILKINSON[5] and s/v versus s plot operated reasonably well except when the velocity data contained outliers. The plot of v versus v/s and, in a higher degree, that of 1/v versus 1/s were the least precise.

With respect to accuracy, the plot of s/v versus s and the method of WILKINSON[5], as usually, overestimated and the plot of v versus v/s tended again to underestimate both V and Km; the method we propose gave the most accurate answers.

DISCUSSION

The results displayed in Tables I, II and III evidence that, all together, our method operated better than the other tested. It was the most precise: the plot of v versus v/s was slightly better in precision when errors were of constant relative magnitude, but yielded poor answers with all other sort of errors; when these were of constant absolute magnitude, the WILKINSON[5] method gave Km values perhaps a little more precise, but slightly worst

TABLE II

Performance of the proposed method with errors of constant relative magnitude

Values are means ± C.V. (%) of 100 estimations from which the highest and lowest 5 % were eliminated to approach data distribution to normality

		Substrate concentration range					
		Short		Medium		Long	
		without outliers	with outliers	without outliers	with outliers	without outliers	with outliers
1/v versus 1/s	Km	1.08 ± 35.4	1.10 ± 41.8*	1.04 ± 26.2	1.04 ± 29.9	1.01 ± 19.5	1.01 ± 22.2
	V	1.04 ± 21.8	1.05 ± 26.0	1.01 ± 14.1	1.02 ± 16.6	1.00 ± 8.7	1.00 ± 10.4
s/v versus s	Km	1.10 ± 26.9*	1.13 ± 30.8*	1.07 ± 22.6*	1.09 ± 25.7*	1.06 ± 30.5	1.07 ± 32.8*
	V	1.05 ± 16.6*	1.06 ± 18.1*	1.03 ± 11.7*	1.03 ± 12.2*	1.01 ± 9.1	1.02 ± 9.2
v versus v/s	Km	0.92 ± 24.3*	0.88 ± 29.7*	0.97 ± 17.9	0.96 ± 20.6*	1.00 ± 13.8	0.99 ± 15.7
	V	0.96 ± 14.4*	0.93 ± 16.3*	0.99 ± 9.7	0.98 ± 10.6*	1.00 ± 6.2	1.00 ± 6.9
Wilkinson	Km	1.11 ± 30.2*	1.13 ± 33.2*	1.08 ± 23.6*	1.09 ± 27.7*	1.07 ± 19.6*	1.08 ± 23.3*
	V	1.06 ± 17.8*	1.07 ± 19.1*	1.04 ± 11.4	1.04 ± 11.9*	1.02 ± 6.9*	1.02 ± 7.3*
Eisenthal and Cornish-Bowden ...	Km	1.02 ± 25.5	0.98 ± 30.1	1.05 ± 18.3*	1.03 ± 22.7	1.05 ± 14.3*	1.04 ± 16.2*
	V	1.02 ± 15.7	1.00 ± 18.2	1.03 ± 10.4*	1.03 ± 11.3*	1.02 ± 7.3*	1.02 ± 7.7*
Proposed method	Km	1.01 ± 24.7	0.97 ± 29.2	1.04 ± 18.1	1.02 ± 20.8	1.04 ± 15.3*	1.04 ± 17.1
	V	1.02 ± 15.0	1.00 ± 17.0	1.02 ± 10.2*	1.02 ± 11.0	1.02 ± 7.0*	1.01 ± 7.5

* Mean out of the 95 % confidence range.

TABLE III

Performance with rates containing intermediate errors

Values are means ± C.V. (%) of 100 estimations from which the highest and lowest 5 % were eliminated to approach data distribution to normality

		Substrate concentration range					
		Short		Medium		Long	
		without outliers	with outliers	without outliers	with outliers	without outliers	with outliers
1/v versus 1/s	Km	1.16 ± 55.2*	1.17 ± 63.8*	1.08 ± 37.6	1.08 ± 48.2	1.05 ± 27.6	1.05 ± 31.5
	V	1.09 ± 33.2*	1.09 ± 38.3*	1.03 ± 18.7	1.02 ± 20.7	1.01 ± 9.7	1.01 ± 11.4
s/v versus s	Km	1.09 ± 22.7*	1.12 ± 27.7*	1.05 ± 16.0*	1.07 ± 19.2*	1.03 ± 16.9*	1.06 ± 16.8*
	V	1.05 ± 12.2*	1.06 ± 14.3*	1.02 ± 7.1*	1.02 ± 7.8	1.01 ± 4.7	1.01 ± 4.9
v versus v/s	Km	0.91 ± 25.1*	0.85 ± 28.6*	0.97 ± 18.3	0.94 ± 20.0*	1.00 ± 14.6	0.99 ± 17.3
	V	0.94 ± 13.0*	0.91 ± 14.9*	0.98 ± 8.4*	0.97 ± 8.7*	1.00 ± 4.3	0.99 ± 4.7
Wilkinson	Km	1.06 ± 22.9*	1.08 ± 27.3*	1.04 ± 16.0*	1.05 ± 20.3*	1.04 ± 12.5*	1.04 ± 15.4
	V	1.04 ± 12.2*	1.04 ± 14.0*	1.02 ± 6.9*	1.02 ± 7.4*	1.01 ± 3.8*	1.01 ± 4.1*
Eisenthal and Cornish-Bowden ...	Km	0.99 ± 23.6	0.94 ± 25.4*	1.03 ± 16.4	1.00 ± 17.8	1.03 ± 13.2*	1.02 ± 14.6
	V	1.01 ± 11.6	0.98 ± 13.3	1.02 ± 6.8*	1.01 ± 7.3	1.01 ± 4.1*	1.01 ± 4.4
Proposed method	Km	0.99 ± 22.4	0.94 ± 25.5*	1.02 ± 15.6	1.00 ± 17.3	1.03 ± 12.9	1.02 ± 14.5
	V	1.00 ± 11.4	0.99 ± 12.8	1.01 ± 6.6	1.01 ± 7.1	1.01 ± 4.0*	1.01 ± 4.3

* Mean out of the 95 % confidence range.

estimates of V and it performed poorly with errors of constant relative magnitude; the most frequently used procedure for estimating V and Km, the double reciprocal plot, was by far the less precise one, confirming previous reports (1, 5, 10-12). Furthermore, it was very accurate: on 18 simulated experiments performed, the true value of V and Km only deviated out of the 95 % confidence range in four cases, which advatageously compares with all other methods; with the plot of 1/v versus 1/s only two V and six Km actual values fell out of this interval, but it has to be considered that the poor precision of this plot determines a very much large 95 % confidence limit. Finally, it was the most versatile, being little affected by outliers and performing very satisfactorily in all situations, even with a short substrate concentration range; all other methods tested, except that of Eisenthal and Cornish-Bowden[7], lack versatility and produce very poor results in some of the explored circumstances.

An objection to our procedure could be its somewhat higher complexity than the usual ones, but the widespread availability of simple electronic computing devices minimizes this disadvantage. In fact, making use of a programable pocket calculator the whole operation of V and Km estimation from six observations takes only a few minutes.

SUMMARY

A new procedure is described for calculating kinetic parameters of reactions which obey Michaelis-Menten and derived equations. The performance of this method at several substrate concentration ranges was compared with that of the plots of 1/v versus 1/s, s/v, versus s and v versus v/s and with the procedures of Wilkinson and Eisenthal & Cornish-Bowden by using simulated rate data containing errors of constant absolute magnitude, constant relative magnitude and intermediate, with and without outliers in each case. The method proved to be excelent in accuracy and precision in all the explored situations.

RESUMEN

Se describe un nuevo procedimiento para calcular los parámetros cinéticos de las reacciones que obedecen a ecuaciones del tipo de la de Michaelis-Menten y se compara su rendimiento, a varios rangos de concentraciones de sustrato, con el de las representaciones de 1/v en función de 1/s, s/v frente a s y v en función de v/s y con el de los métodos de Wilkinson y de Eisenthal y Cornish-Bowden; a este último objeto se utilizaron datos experimentales simulados en un ordenador, conteniendo errores de magnitud absoluta constante, de magnitud relativa constante e intermedios. El método proporcionó excelentes resultados en todas las circunstancias investigadas.

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