Fitting integrated enzyme rate equations to progress curves with the use of a weighting matrix

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A method is presented for fitting the pairs of values product formed–time taken from progress curves to the integrated rate equation. The procedure is applied to the estimation of the kinetic parameters of the adenosine deaminase system. Simulation studies demonstrate the capabilities of this strategy. A copy of the FORTRAN77 program used can be obtained from the authors by request.

INTRODUCTION

The information contained in the progress curve of an enzyme-catalysed reaction theoretically allows a rapid and easy determination of the kinetic parameters of the enzyme, and in consequence the progress-curve method could be an interesting alternative to initial-rate analysis. However, kinetic analysis from progress curves has an important shortcoming: the errors obtained are not independent. This correlated error structure is obvious, since the experimental error in the initial substrate or enzyme concentration is propagated along the whole of the curve, and therefore results obtained from one single progress curve may systematically lead to deviations from the actual parameter values. Also, the displacement in time assignment, due to possible error in the zero time consideration, should be borne in mind. A possible solution is to combine the data from a set of different progress curves, but this necessarily leads to more experimental effort.

The aim of the present paper is to solve the problem of the correlation of errors in order to obtain good parameter estimates from a small number of curves. The method has been applied to the determination of the kinetic parameters of commercial adenosine deaminase. Since inosine appears with time, data must be fitted to the suitable inhibition model. Thus the method allows simultaneous calculation of V , $K_{m(\text{adenosine})}$ and $K_{i(\text{inosine})}$. The method consists of fitting the pairs product formed–time to the following integrated rate law:

$$V \cdot t = ([\text{adenosine}]_0 - [\text{adenosine}]) \cdot (1 - K_m/K_i) + K_m \cdot [\text{adenosine}]/K_i \cdot \ln([\text{adenosine}]_0/[\text{adenosine}])$$

employing the weighting matrix devised by Markus & Plesser (1981). This weighting matrix has been used successfully by Canela & Franco (1986) for fitting data pairs of initial velocities and substrate, both derived from progress curves, to the rate law. A FORTRAN77 program suitable for this purpose is available from the authors on request.

THEORY AND COMPUTATIONAL METHODS

The method consists in fitting the pairs of data product formed–time to the integrated rate law by using a FORTRAN77 version of the non-linear-regression program previously developed by Canela (1984). In order to correct the correlations, a weighting matrix is used that includes the factor of propagation of error due to the possible mistaken estimation of initial

concentration of substrate. A complete description of the method has been published previously (Markus & Plesser, 1981; Canela & Franco, 1986). Since the integrated rate law is a transcendental equation, the subroutine for evaluating the current value of the product formed at each time t is based on the half-interval search method; after 15 fixed searches the product concentration value reached was sufficiently close to the exact solution of the equation.

The question of correlation of errors was dealt with differently by Markus & Plesser (1981) and by Duggleby & Nash (1989). Markus & Plesser (1981) assume that the initial substrate concentration had a known value subject to a certain error (case of errors in the control variables; Fedorov, 1972). This error is propagated along the progress curve and is proportional to the derivative of the actual substrate concentration (at each time) with respect to the initial substrate concentration. These authors then incorporate this effect in the variance–co-variance matrix (U) according to the following expression:

$$U = I\sigma^2 + HH'$$

where I is the unit matrix, σ^2 denotes the experimental variance, H_{ij} is equal to $g_{ij}\sigma_{aj}$ and H' stands for the transpose of the matrix H . g_{ij} is assumed to be equivalent to the derivative of the function with respect to the initial concentration of the j th substrate at the i th observation. σ_{aj} are the standard deviations of the errors Δa_j .

MATERIALS AND METHODS

Adenosine and inosine were purchased from Merck (Darmstadt, Germany). Adenosine deaminase (EC 3.5.4.4) was obtained from Boehringer Mannheim (Mannheim, Germany). All other products were the best grade available from Merck. De-ionized water further purified with the Millipore Milli Q system was used throughout.

The adenosine deaminase was assayed with adenosine as substrate, with the product inosine when it was required. The reaction was monitored at $30 \pm 0.1^\circ\text{C}$ by following the disappearance of adenosine at 265 nm in a Hewlett–Packard 8450A UV/VIS spectrophotometer provided with a thermostatically controlled cell holder (89100A temperature controller) and a 7470A plotter. The absorbance data were continuously recorded and stored in the memory of the spectrophotometer. Decrements in absorbance were converted into adenosine consumed by using a molar absorption coefficient of $8.33 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. Time zero was considered to be the moment when 0.1 ml of enzyme was added to 2.9 ml of the substrate solution. This was possible, as this

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Table 1. Description of the simulation

For the six curves the initial values of substrate and product concentration were simultaneously perturbed according to a normal distribution, the mean of which was the actual value of the corresponding substrate or product concentration. In all cases the coefficient of variation was 2.5% or (equivalently) the standard deviation was $0.025 \times \text{mean}$. This was repeated 1000 times, and the means of these values are listed. It was tested that the random numbers were Gaussian in distribution by the calculation of variance, skewness and kurtosis (results not shown).

Product	Actual value (μM)	Mean simulated value (μM)
Curve 1		
Adenosine	54.7	54.7
Inosine	197	198
Curve 2		
Adenosine	55.3	55.3
Inosine	200	200
Curve 3		
Adenosine	55.3	55.4
Inosine	200	201
Curve 4		
Adenosine	156	156
Inosine	20.0	20.0
Curve 5		
Adenosine	156	155
Inosine	20.0	20.1
Curve 6		
Adenosine	156	156
Inosine	20.0	19.9

Table 2. Estimates of the kinetic parameters of commercial adenosine deaminase

Experimental values were obtained, and the experimental concentrations of inosine formed and times of the six progress curves of Table 1 were fitted to the integrated rate law, by using the experimental initial concentrations of products. From each progress curve seven points were collected at equal time intervals between 0 and 95% of the reaction completion. The values given in the simulated cases were obtained from the means of 1000 simulated values generated by fitting the data as above, but varying randomly the initial concentration of products according to a normal distribution as described in Table 1. Standard deviations correspond to standard deviations of the means of the 1000 parameter values.

	Value	Standard deviation
Experimental		
V	13.2 $\mu\text{mol/min}$	
$K_m(\text{adenosine})$	12.8 μM	
$K_i(\text{inosine})$	286 μM	
Simulated weighted		
V	13.5 $\mu\text{mol/min}$	2.77
$K_m(\text{adenosine})$	10.6 μM	3.56
$K_i(\text{inosine})$	175 μM	72.1
Simulated unweighted		
V	14.3 $\mu\text{mol/min}$	1.14
$K_m(\text{adenosine})$	27.3 μM	13.3
$K_i(\text{inosine})$	6.82 μM	0.91

spectrophotometer allows stirring of the reaction mixture without delay. Each reaction mixture consisted of adenosine and 0.01 unit of adenosine deaminase in 50 mM-sodium phosphate buffer,

Fig. 1. Parameter estimates of the fit of data from an exact progress curve corresponding to a Michaelis–Menten equation with $V = 2.0 \mu\text{mol/min}$ and $K_m = 30 \mu\text{M}$

The actual initial substrate concentration was 100 μM . The different points correspond to the fit of the same data but including erroneous values for the initial substrate concentration. (a) V estimates; (b) K_m estimates. $\blacklozenge$, Without weighting matrix; $\square$, with weighting matrix.

pH 7.4. Stability of the enzyme was confirmed by Selwyn's (1965) test. For each progress curve seven points were collected at equal time intervals between 0 and 95% of the reaction completion.

RESULTS

We have studied the transformation of adenosine into inosine catalysed by adenosine deaminase in the presence of inosine. This reaction is well characterized: it is irreversible, and inosine inhibits competitively with respect to adenosine. To characterize the system adenosine/adenosine deaminase/inosine, which follows a Michaelis–Menten equation with a term for competitive inhibition, we have selected the six progress curves indicated in Table 1. The parameters calculated with the use of the weighting matrix for adenosine deaminase were (Table 2) $V = 13.2 \mu\text{mol/min}$, $K_m(\text{adenosine}) = 12.8 \mu\text{M}$ and $K_i(\text{inosine}) = 0.286 \text{ mM}$.

In order to test the applicability of the method, we chose the parameter estimates obtained experimentally and have generated six progress curves assuming errors in the initial substrate and product concentrations. Errors were generated by perturbing these initial concentrations for all six progress curves at once according to a normal distribution, the mean of which was the actual value and the coefficient of variation was 2.5% of the actual value of the corresponding concentration ($\sigma = 0.025$). Then we have obtained the simulated estimates of the parameters fitting the data of the six progress curves. This procedure was repeated 1000 times, and the results of the 1000 fittings using the

weighted and the unweighted method are presented in Table 2. It is obvious that the estimates of the parameters obtained with the weighted method are very close to the experimental ones. In contrast, those obtained with the unweighted method are worse for K_m and K_i and also very spread out. The conditions of the simulation are given in Table 1.

In order to illustrate the capabilities of the method we constructed an exact progress curve using a Runge-Kutta program of integration of differential equations (Franco & Canela, 1984). It corresponds exactly to the curve of a Michaelian enzyme of parameters $V = 2.0 \mu\text{mol/min}$ and $K_m = 3.0 \mu\text{M}$, and for an initial concentration of substrate of 0.10 mM. Data were then fitted assuming different mistaken values for the initial substrate concentration. Results obtained with the weighted and the unweighted methods are compared and are shown in Fig. 1. It is obvious that the weighted pattern is notably better than the unweighted one. Note that, with the weighted method, only very high errors in the estimation of initial substrate concentration introduce meaningful modifications in the parameter estimates. The estimates are remarkably good even when the greatest concentration of product observed is 0.095 mM and the initial concentration of substrate is set to the clearly ridiculous value of 0.025 mM.

DISCUSSION

The results shown in this paper illustrate the usefulness of the method of weighting proposed by Markus & Plesser (1981). The simulation results shown in Table 2 suggest that sensitivity of the parameter values with respect to error in initial concentrations of substrates and products with the weighted structure is lower than that obtained with no considerations about errors in initial concentration of compounds. Also, the results displayed

in Fig. 1 indicate the low sensitivity of the parameter estimates to changes in the initial concentration of substrate and product when the weighted pattern is used. When no weighting is included in the regression, errors became significantly high when relatively low displacements of the initial substrate concentration are introduced. Bias was higher when the assumed value of initial substrate concentration was greater than the actual value (Fig. 1).

There is an alternative method proposed by Duggleby & Nash (1989) that deals with each progress curve by use of a first-order auto-regressive model. In this model the residual of the observation for the period t is a function of the residual of the previous period ($t - 1$) plus a random error. Although the results are very interesting, those authors have indicated that further refinement to their work is necessary. Upon further improvement, this alternative method should be compared with that employed here. In our opinion, both approaches are theoretically correct and their combination could be the best solution, although further investigation should be carried out in order to find their correlations and discrepancies and to establish the best methodology to deal with progress curves.

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