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STATISTICAL ANALYSES OF ENZYME KINETICS: SIMPLE MICHAELIS-MENTEN AND BI-BI PING-PONG

A.A. HAMBARDZUMYAN

SPC "Armbiotechnology"
arthambardzumyan@gmail.com

Using multivariate linear regression approach, publicly available applications have been developed in Gauss 4.0 package for calculation of the kinetic parameters and their dispersions in the cases of simple Michaelis-Menten type and the bi-bi ping-pong type enzyme kinetics. The methods of derivation of corresponding equations of stationary kinetics, their linearization and derivation of analytic forms of kinetic parameters and their dispersions are presented.

Stationary kinetics – bi-bi ping-pong – multivariate linear regression – kinetic parameters – calculation

Կիրառելով բազմաչափ գծային ռեգրեսիայի մոտեցումը Գաուս 4.0. լեզվով հանրամատչելի ծրագրեր են մշակվել պարզ Միքաելիս-Մենտեն և բի-բի պինգ-պոնգ տիպի ֆերմենտային կինետիկաների կինետիկական պարամետրերի և դրանց դիսպերսիաների հաշվարկման համար: Ներկայացվել են ստացիոնար կինետիկայի համապատասխան հավասարումների դուրս բերման, դրանց գծայնացման և կինետիկական պարամետրերի և դրանց դիսպերսիաների հավասարումների դուրս բերման մեթոդները:

Ստացիոնար կինետիկա – բի-բի պինգ-պոնգ – բազմաչափ գծային ռեգրեսիա – կինետիկական պարամետրեր – հաշվարկում

Используя подход многомерной линейной регрессии, были разработаны публично доступные программы на базе пакета Gauss 4.0. для расчета кинетических параметров и их дисперсий для случаев ферментативной кинетики типа Михаэлиса-Ментен и би-би пинг-понг. Представлены методы вывода соответствующих уравнений стационарной кинетики, их линеаризации и вывода аналитических форм кинетических параметров и их дисперсий.

Стационарная кинетика – би-би пинг-понг – многомерная линейная регрессия – кинетические параметры – расчет

In biochemical research, mostly in enzyme kinetics, the datasets related to stationary kinetics are usually analyzed graphically. More often the double reverse coordinates are used for this purpose, i.e. the dependence of $1/v$ on $1/S$ (the Lineweaver–Burk plot) [13]. But it is this approach that causes the strongest criticism from statistics and gives very unreliable values for K_M and V_{max}. This approach did not allow estimating the weight of the experimental point when tracing the line through the set of points, and also it did not allow estimating the mistakes in determination of the

kinetic parameters. Therefore, the best approach in solving the problems of enzyme kinetics is the statistical approach, namely statistical methods based on linear regression analysis. This approach enables to estimate the kinetic parameters and their errors taking into account the weights of each experimental point [3].

Kinetic parameters of stationary kinetics are easily calculated solving systems of linear differential equations. However, the method of King and Altman, based on graph theory, provides a visual and familiar to the eyes of biochemists formula for the calculation of the initial rates of enzymatic reactions, in which there are clearly distinguished parameters (maximum speed of reaction, the Michaelis constant, inhibition constants, etc.) that are interesting to us [12]. A special case of the mechanism of the enzymatic reaction – bi-bi ping-pong, common in reactions catalyzed by aminotransferases – is well represented in the literature [15].

We intend to present the statistical data processing methods and applications calculating the relevant kinetic parameters of stationary kinetics to the scientific community [3-4]. These approaches have been used in our works concerning the enzyme kinetics [1-2, 5-11, 14].

The aim of this work is to derive equations that allow us calculating the parameters of stationary kinetics and their dispersions for the cases of simple Michaelis-Menten kinetics and the bi-bi ping-pong kinetics, as well as to present the applications that calculate these values in Gauss 4.0.

Materials and methods. The terms and symbols used by Cornish-Bowden in Chapter 10 [3] were used in the present work.

The equation for bi-bi ping-pong reaction rate was derived using Kings and Altmans graph theory [12].

Equations for kinetic parameters and their variances were derived using multivariate linear regression analyses [4].

The applications for calculation of kinetic parameters and their variances were written on the matrix language - Gauss 4.0. (Aptech Systems, Inc.).

Results and Discussion. The equation for initial rate of simple **Michaelis-Menten type reaction kinetics** is well documented [https://en.wikipedia.org/wiki/Michaelis-Menten_kinetics] and presented in (1).

$$v = \frac{V_{max}S}{K_M+S} \qquad (1)$$

Where: v is initial rate, V_{max} – maximal rate, S – substrate concentration and K_M – Michaelis constant.

This equation is linearized as follows:

$$\frac{s}{v} = \frac{K_M}{V_{max}} + \frac{1}{V_{max}}s \qquad (2).$$

Denoting: $\frac{s}{v} \to y$, $\frac{K_M}{V_{max}} \to \beta_1$, $\frac{1}{V_{max}} \to \beta_2$, $\frac{1_1}{1_n} \to x_1, \cdots \to x_2$, $\frac{\square_1}{s_n}$ we can describe the linearized equation for rate of the enzymatic reaction (2) together with measurement errors (ε) in vector form:

$$\vec{y} = \vec{X}\vec{\beta} + \vec{\epsilon} \qquad (3),$$

β-s and their variances can be calculated according the Gauss-Markov theorem [4] by following equations:

$$\beta = (X'X)^{-1}X'y, \quad V_{(\beta)} = \sigma_0^2(X'X)^{-1} \qquad (4),$$

where: X' is the transposed matrix X, $(X'X)^{-1}$ – inverse matrix $(X'X)$, $V_{(\beta)}$ – variances of β, and σ_0^2 – experimental variance.

When the measurements are given weights, the equations (4) are converted to (5).

$$\beta = ((wX)'X)^{-1}(wX)'y, \quad V_{(\beta)} = \sigma_0^2((wX)'X)^{-1} = \sigma_0^2 Z^{-1} = \sigma_0^2 U \qquad (5)$$

The experimental variance is calculated from equations (6).

$$\sigma_0^2 = \frac{SS}{N-P}, \qquad SS = \sum we^2 = \sum w_i(y_i - x_i\beta_i)^2 = \sum ud^2 \qquad (6)$$

Where: SS is the sum of the weighted squares, N – number of measurements, P – number of degrees of freedom (for simple Michaelis-Menten type reaction kinetics P=2), e – estimate of ε deviation of y, d – estimate of δ deviation of v, u – weight of d.

$w \approx \frac{v^4}{s^2}$ is accepted in enzymology [3].

The equations for calculating K_M and V_{max} and their dispersions (variances) were derived in [3] and are the followings:

$$V_{max} = \frac{1}{\beta_2}, \qquad K_M = \frac{\beta_1}{\beta_2} \qquad (7),$$

$$V_{(K_M)} = V_{(\frac{\beta_1}{\beta_2})} = \sigma_0^2\left(\frac{M_{11}}{\beta_2^2} - 2\frac{\beta_1 M_{12}}{\beta_2^3} + \frac{\beta_1^2 M_{22}}{\beta_2^4}\right), \; V_{(V_{max})} = V_{(\frac{1}{\beta_2})} = \sigma_0^2\frac{M_{22}}{\beta_2^4} \qquad (8),$$

where: β_1 and β_2 can be calculated by equations (5), σ_0^2 – by equations (6), and M_{ij} – is the (i, j) element of matrix U (5).

The application code for calculating the parameters of Michaelis-Menten kinetics and their variances, written in Gauss 4.0, is presented in fig. 1.

```
A
print "Michaelis_Menten";
n=5; k=2;
fname="D:\\My Documents D\\Data\\Enzyme_activity.xls";
{dat1,names}=import(fname,"a1:b6",3);

B
y=dat1[1:n,1]./dat1[1:n,2];
w=dat1[1:n,2].^4 ./dat1[1:n,1].^2;
x=(dat1[1:n,1]./dat1[1:n,1])~(dat1[1:n,1]);
z=(w.*x)'*x;
u=inv(z);
b=inv(z)*((w.*x)'*y);
Vmax=1/b[2,1]; Km=b[1,1]/b[2,1];
sigma0=(w'*((y-(b'*x')').*(y-(b'*x')')))/(n-k);
s_Vmax=(sigma0*u[2,2]/b[2]^4)^0.5;
s_Km=(sigma0*(u[1,1]/b[2]^2-2*b[1]*u[1,2]/b[2]^3+b[1]^2*u[2,2]/b[2]^4))^0.5;

C
print "Km=" Km~s_Km;
print "Vmax=" Vmax~s_Vmax;
```

Fig. 1. The application code for calculating the parameters of Michaelis Menten kinetics and their variances.
A – data input block in the form of Excel spreadsheet for n=5 and k=2, B – block for calculating kinetic parameters and their standard deviations, C – data output block.

We used the presented multivariate linear regression analyses and corresponding application code for calculation of the kinetic parameters and their variances for *Rhodotorulla auriantiaca* KM-1 phenylalanine ammonia-lyase and L-phenylalanine system [7]. We obtained K_M=1.75±0.44 mM and V_{max}=3.01±0.43 units/mg, which fit well with the same parameters obtained from inhibition analyses.

We used this approach in studying the substrate specificity of free enzyme and the shift of K_M after covalent immobilization of the recombinant L-aminoacylase of *Geobacillus stearothermophilus* on silochrome C-80 [5]. It was shown that the enzyme exhibits higher affinity to N-acetyl-L-alanine with comparison to N-acetyl-L-valine and N-acetyl-L-methionine (K_M=0.56±0.08, 1.31±0.80 and 1.43±0.64, correspondingly) and that in the result of immobilization, the Michaelis constant of N-acetyl-L-methionine increased more than twice.

We also used the multivariate linear regression analyses approach in characterization of D-aminoacylase of newly isolated strain – *Rhodococcus armeniensis* AM6.1 [11]. The K_M-s for 9 N-acetyl-D- and N-acetyl-DL-amino acids were calculated. For N-acetyl-D-leucine and N-acetyl-DL-tyrosine, which exhibit substrate inhibition, Michaelis constant calculated using this method fit well with the same parameter obtained from analyses of substrate inhibition.

The bi-bi ping-pong type reaction kinetics is also well documented [3, 15]. The equation for initial rate of such type reaction kinetics, derived using King and Altman approach, is presented in (9).

$$v = \frac{V_{max}S_1S_2}{K_{M2}S_1+K_{M1}S_2+S_1S_2} \qquad (9)$$

Where: v is initial rate, V_{max} – maximal rate, S_1 and S_2 – concentration of substrate 1 and 2, K_{M1} and K_{M2} – Michaelis constants for substrate 1 and 2.

This equation is linearized as follows:

$$\frac{S_1S_2}{v} = \frac{K_{M2}}{V_{max}}S_1 + \frac{K_{M1}}{V_{max}}S_2 + \frac{1}{V_{max}}S_1S_2 \qquad (10)$$

Denoting:

$$\frac{S_1S_2}{v} \to y,\ S_1 \to x_1,\ S_2 \to x_2,\ S_1S_2 \to x_3,\ \frac{K_{M2}}{V_{max}} \to \beta_1,\ \frac{K_{M1}}{V_{max}} \to \beta_2,\ \frac{1}{V_{max}} \to \beta_3,$$

we can describe the linearized equation for rate of the enzymatic reaction (10) together with measurement errors (ε) in vector form (3) as in the case of simple Michaelis-Menten kinetics. Here also β-s and their variances can be calculated according the Gauss-Markov theorem [4] by equations (4). When the measurements are given weights, the equations (4) are converted to (5). Here also the experimental variance is calculated from equations (6).

$$\sigma_0^2 = \frac{SS}{N-P}, \quad SS = \sum we^2 = \sum \mathrm{w_i}(\mathrm{y_i} - \mathrm{x_i}\beta_\mathrm{i})^2 = \sum \mathrm{ud}^2 \qquad (6)$$

Where: SS is the sum of the weighted squares, N – number of measurements, P – number of degrees of freedom (for bi-bi ping-pong type reaction kinetics P=3), e – estimate of ε deviation of y, d – estimate of δ deviation of v, u – weight of d.

The estimation of *w* can be done by the following way:

From $v = \frac{V_{max}S_1S_2}{K_{M2}S_1+K_{M1}S_2+S_1S_2} + \delta$

and $\frac{S_1S_2}{v} = \frac{K_{M2}}{V_{max}}S_1 + \frac{K_{M1}}{V_{max}}S_2 + \frac{1}{V_{max}}S_1S_2 + \varepsilon$,

we can easily derive: $\delta = \frac{vV}{K_{M2}S_1+K_{M1}S_2+S_1S_2}\varepsilon \approx \frac{v^2}{S_1S_2}e$ (11),

and using $u \sim \frac{1}{v}$ internal condition of Cornish-Bowden [3] we can obtain:

$$SS = \sum ud^2 = \sum \frac{1}{v}\left(\frac{v^2}{S_1 S_2}\right)^2 e^2 = \sum \frac{v^3}{S_1^2 S_2^2} e^2 = \sum we^2, \text{ or } w = \frac{v^3}{S_1^2 S_2^2} \qquad (12)$$

The equations for calculating K_{Mi} and V_{max} and their variances are the followings:

$$K_{M1} = \frac{\beta_2}{\beta_3},\ K_{M2} = \frac{\beta_1}{\beta_3},\ V_{max} = \frac{1}{\beta_3} \qquad (13)$$

$$V_{(K_{M1})} = V_{(\frac{\beta_2}{\beta_3})} = \sigma_0^2\left(\frac{M_{22}}{\beta_3^2} - 2\frac{\beta_2 M_{23}}{\beta_3^3} + \frac{\beta_2^2 M_{33}}{\beta_3^4}\right),\ V_{(K_{M2})} = V_{(\frac{\beta_1}{\beta_3})} =$$

$$\sigma_0^2\left(\frac{M_{11}}{\beta_3^2} - 2\frac{\beta_1 M_{13}}{\beta_3^3} + \frac{\beta_1^2 M_{33}}{\beta_3^4}\right),\ V_{(V_{max})} = V_{(\frac{1}{\beta_3})} = \sigma_0^2 \frac{M_{33}}{\beta_3^4} \qquad (14),$$

where: β_1, β_2 and β_3 can be calculated by equations (5), σ_0^2 – by equations (6), and M_{ij} – is the (i, j) element of matrix U (5).

The application code for calculating the parameters of bi-bi ping-pong kinetics and their variances, written in Gauss 4.0, is presented in fig. 2.

```
A
print "GAT, ping-pong bi-bi";
n=16; k=3;
fname="D:\\My Documents_D\\ALT.xls";
{dat1,names}=import(fname,"a1:c17",2);

B
y=dat1[1:n,1].*dat1[1:n,2]./dat1[1:n,3];
w=dat1[1:n,3].^3 ./dat1[1:n,1].^2 ./dat1[1:n,2].^2;
x=(dat1[1:n,1])~(dat1[1:n,2])~(dat1[1:n,1].*dat1[1:n,2]);
z=(w.*x)'*x;
u=inv(z);
b=inv(z)*((w.*x)'*y);
Km2=b[1,1]/b[3,1]; Vmax=1/b[3,1]; Km1=b[2,1]/b[3,1];
sigma0=(w'*((y-(b'*x')').*(y-(b'*x')')))/(n-k);
s_Km2=(sigma0*(u[1,1]/b[3]^2-2*b[1]*u[3,1]/b[3]^3+b[1]^2*u[3,3]/b[3]^4))^0.5;
s_Vmax=(sigma0*u[3,3]/b[3]^4)^0.5;
s_Km1=(sigma0*(u[1,1]/b[3]^2-2*b[2]*u[2,3]/b[3]^3+b[2]^2*u[3,3]/b[3]^4))^0.5;

C
print "Km2"  Km2~s_Km2;
print "Km1"  Km1~s_Km1;
print "Vmax" Vmax~s_Vmax;
```

Fig. 2. The application code for calculating the parameters of bi-bi ping-pong kinetics and their variances.
A – data input block in the form of Excel spreadsheet for n=16 and k=3, B – block for calculating kinetic parameters and their standard deviations, C – data output block.

We used the presented multivariate linear regression analyses and corresponding application code for calculation of the Michaelis constants and their variances for *Brevibacteium flavum* valine:pyruvate aminotransferase [6]. We obtained K_M=1.53±0.10 mM for L-alanine, K_M=0.52±0.15 mM for L-valine, K_M=0.41±0.13 mM for pyruvate and K_M=0.23±0.01 mM for 2-ketoisovaleriate.

We also used the multivariate linear regression analyses approach for calculation of K_M for L-phenylalanine, L-glutamic acid, L-aspartic acid, and the corresponding keto acids, as well as V_{max} for the following pairs of substrates: L-phenylalanine-2-ketoglutarate, L-phenylalanine-oxaloacetate, L-glutamic acid-phenylpyruvate, and L-aspartic acid-phenylpyruvate for aminotransferases PAT1, PAT2, and PAT3 of *Erwinia carotovora*, catalyzing transamination of phenylpyruvate [14].

Thus, using multivariate linear regression approach, publicly available applications have been developed for the calculation of the kinetic parameters and their dispersions for the cases of simple Michaelis-Menten type and the bi-bi ping-pong type enzyme kinetics.

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