Improved Rearrangement of the Integrated Michaelis-Menten Equation for Calculating In Vivo Kinetics of Transport and Metabolism

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ABSTRACT

A multiple regression form of the integrated Michaelis-Menten equation was developed and evaluated with simulated data having controlled error. Both multiple and traditional linear regression fit errorless data perfectly, but multiple regression is much more stable with regard to accuracy and precision of estimating the Michaelis constant and maximum rate of reaction when data contain error. Bias in determining estimators of kinetic coefficients was -4 and -3% versus -56 and -35% with 10% error in the data. Multiple regression estimates for Michaelis constant and maximum rate of reaction directly as opposed to estimating $1/K_m$ and maximum rate of reaction/Michaelis constant by linear regression. The difference in accuracy in estimating actual Michaelis constant, for example, is 4% versus 227% error with only 10% error in the data. Precision of estimation is approximately the same as precision of the data for multiple regression. For the 800 data sets examined, $R^2$ was always greater than .92 for multiple regression, but frequently was not significant for linear regression. The actual initial concentration was provided for linear regression but calculated by multiple regression with accuracy and precision equivalent to estimation of Michaelis constant and maximum rate of reaction. The multiple regression method has statistical power to determine treatment effects on Michaelis constant and maximum rate of reaction with a practical number of animals.

(Key words: Michaelis-Menten, in vivo kinetics, transport, clearance)

Abbreviation key: $K_m =$ Michaelis constant, $V_{max} =$ maximum rate of reaction.

INTRODUCTION

The integrated Michaelis-Menten or Henri equation (Equation [1]) has the potential to be a useful tool in animal sciences for determining nutrient uptake by tissue, absorption of nutrients from the gut, and organ blood flow and function in vivo. Use of the more common differential form of the equation in vivo requires a separate continuous infusion experiment for each data point (ordered pair of plasma concentration and rate of clearance) to be used in the regression analysis. A single calculation of Michaelis-Menten coefficients, therefore, requires multiple experiments confounded with time, animal variation, or both. Use of the integrated Michaelis-Menten equation allows calculation of kinetic coefficients from a single, bolus injection experiment. In applied animal sciences, in which the cost of individual animals is much greater than for laboratory animals most often used in basic research, the ability to calculate kinetic coefficients from a single, bolus injection experiment is a marked advantage. In situations in
which instantaneous mixing of the compound of interest does not occur, a second marker could be used to correct for the mixing phenomenon. Alternatively, the compound of interest could be infused at a rate that would generate an asymptotic concentration approximately fourfold greater than the expected Michaelis constant ($K_m$). Then, as concentration approaches the asymptote, the infusion should be discontinued and the "decay" curve monitored.

The integrated form of the Michaelis-Menten equation is

$$C(0) - C(t) + K_m \times \ln(C(0)/C(t)) - V_{\text{max}} \times t = 0$$  \[1\]

where $t$ = time, $C(t)$ = substrate concentration at time $t$, and $V_{\text{max}}$ = maximum rate of reaction. Several rearranged forms exist (9, 15, 16) that linearize the equation, but the most commonly used form is Equation [2].

$$\frac{\ln(C(0)/C(t))}{t} = \frac{1}{K_m} [\ln(C(t) - C(0))/t] + \frac{V_{\text{max}}}{K_m}.$$  \[2\]

A simple linear regression of Equation [2] is easy to apply graphically, but it has severe limitations. First, $C(0)$ must be known. However, $C(0)$ rarely is known for in vivo experiments, although it usually is estimated from the first few data points defining the $C(t)$ curve. An error in estimating $C(0)$ will bias each datum in the set. The value of the estimate of $C(0)$ is strongly dependent on the method used in estimation (6). Also, the kinetic coefficients are not estimated directly, i.e., slope = $1/K_m$ and intercept = $V_{\text{max}}/K_m$. A small error in estimating the slope will be magnified in estimating $K_m$. The error structure of $V_{\text{max}}$ is very complex because it not only involves the error associated with estimating the intercept, but also the error of estimating $K_m$.

These limitations of Equation [2] can be circumvented by rearrangement of Equation [1] into a multiple regression form (Equation [3]).

$$C(t) = [C(0) + K_m \times \ln(C(0))] - K_m \times \ln(C(t)) - V_{\text{max}} \times t.$$  \[3\]

In this form, $C(t)$ is regressed on $\ln(C(t))$ and $t$; $C(0)$ appears only in the intercept term, eliminating the need for a priori knowledge of $C(0)$. The $K_m$ and $V_{\text{max}}$ are estimated directly as negative slope 1 and negative slope 2, respectively, of the regression. The theoretical foundation of Equation [3] is in a later section of this paper.

The objective of this paper was to employ computer simulation in order to compare the accuracy and precision of estimating Michaelis-Menten coefficients using Equation [3] with those of the traditional simple regression rearrangement and those of a direct nonlinear regression of the integrated Michaelis-Menten equation.

MATERIALS AND METHODS

Experiment 1

The differential form of the Michaelis-Menten equation was incorporated into a simulation program (4) to generate a data set without experimental error. The simulation modeled a 28-L reaction volume with a $K_m$ of 100 µmol/L and a $V_{\text{max}}$ of 1900 µmol/min after addition of 11,200 µmol of substrate. Although only simple Michaelis-Menten kinetics is considered presently, the data used are a subset of a simulation of blood flow distribution in cattle (12) with removal of indocyanine green by the liver (10). Ordered pairs, [t, C(t)], generated from the simulation were 0, 400.000; 1, 346.501; 2, 294.802; 3, 245.319; 4, 198.592; 5, 155.316; 7, 82.668; 10, 20.169; 15, .823; and 20, .028.

Additional data sets were generated by adding random noise to the errorless values of the integrated Michaelis-Menten equation. Noise equaled error level $\times$ calculated datum $\times$ random number. Error levels were .005, .01, .02, .04, .05, .08, and .10. Random numbers were selected by the NORMAL function of SAS (13), which generates random numbers from a normal population with a mean of 0 and standard deviation = 1. One hundred data sets were generated for each value of error. Each data set was fitted to both the simple regression model, Equation [2], and the multiple regression model, Equation [3], using PROC GLM of SAS (14). Accuracy of estimators of $K_m$ and $V_{\text{max}}$ for each fit was calculated as (observed $-$ actual)/actual, keeping in mind that simple regression produces direct estimates $1/K_m$ and $V_{\text{max}}/K_m$. Actual values
were regression coefficients obtained from fitting curves to errorless data, and observed values were the respective regression coefficients after fitting curves to data with added noise. A perfect estimate yields an accuracy of zero. Precision was determined as the standard deviation of the accuracy of the estimators. The distribution frequency and significance of bias of the accuracy values were evaluated using PROC UNIVARIATE of SAS (14), which test whether the mean is equal to zero and whether the population is distributed normally. The rate in deterioration of accuracy and precision with increasing error was determined by linear regression (14). The C(O) was estimated for multiple regression fits from the intercept value using the iterative routine listed in Figure 1. The actual value of C(O) was provided for the simple regression, but the datum at t = 0 per se was not included in either regression. The units for V_max for both regressions are micromoles per (minute x liters) and can be converted to conventional units by multiplying by reaction volume or as can be determined by multiple regression for in vivo experiments, multiplying by dose of substrate divided by C(O).

Experiment 2

The multiple regression method also was compared with a more modern nonlinear fitting routine suggested by Fernley (5) for the integrated Michaelis-Menten equation. Because the method of Fernley (5) was unsatisfactory (see Results and Discussion section), Equation [3] was fitted directly by the Marquardt algorithm using PROC NLIN of SAS (14). Two groups containing 100 data sets each were generated for curve fitting. One group contained 10% relative error only, as described in Experiment 1. The other group contained the same relative error (same seed was used in the random number generator) plus constant error (constant error = .5 x random number selected from a normal distribution with a mean of zero and standard deviation of 1). Data were evaluated with four paired t comparisons using PROC MEANS of SAS (14). The comparisons were 1) multiple regression versus nonlinear regression within data sets with only relative error, 2) multiple regression versus nonlinear regression within data sets with both relative and constant error, 3) relative error versus relative error plus constant error within multiple regression, and 4) relative error versus relative error plus constant error within nonlinear regression.

Experiment 3

This experiment was conducted to determine the power of the multiple regression rearrangement to detect differences in K_m and V_max because of treatment effects and, in a cursory way, to determine whether selection of sampling times used caused the poor performance of the traditional linear regression rearrangement. Power was determined by establishing a population of 400 data sets with 5 or 10% relative error. Within the population were four subsets of 100 each with actual respective K_m and V_max values of 1) 100 and 1900; 2) 90 and 1900; 3) 100 and 2090; and 4) 110 and 1710. Error-free values for each of the subsets were generated by SYSL (4). Relative error was added as described in Experiment 1. Estimates of K_m and V_max were determined by multiple and linear regression techniques. Means and variance of the estimates were calculated within relative error and regression groups. Power to detect a difference between two treatment means at P < .05 was deter-
Figure 2. Comparison of accuracy and precision of estimators and R² of fitting the integrated Michaelis-Menten equation to linear (○) and multiple regression (●) models with varying magnitude of relative error in the data. a) R², b) accuracy of Michaelis constant (Kₘ) estimator, c) accuracy of maximum rate of reaction (Vₘₐₓ) estimator, and d) accuracy of initial substrate concentration ([C(0)]) estimator. Accuracy is defined as (observed estimator - actual value)/actual value. Precision is defined as the standard deviation of accuracy and is indicated by the vertical line connecting accuracy value to the horizontal tick. Positive and negative halves of the standard deviation are shown for multiple and linear regression models, respectively. The C(0) is not estimatable by linear regression; hence, both the plus and minus ranges of the standard deviation are shown for multiple regression.
Figure 3. Comparison of the frequency distribution $R^2$ and accuracy of estimators of Michaelis constant ($K_m$), maximum rate of reaction ($V_{max}$), and initial substrate concentration ($C(0)$) resulting from fitting data with 10% relative error to linear (open bars) and multiple regression (solid bars) models of the integrated Michaelis-Menten equation.

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mined for multiple regression using FINV and PROBF functions of SAS (13). The noncentrality parameter used was

\[(\text{avg1} - \text{avg2})^2 \times \text{rep}/(2 \times \text{var})\]

where avg1 = mean of treatment 1, avg2 = mean of treatment 2, rep = number of animals per treatment, and var = variance of the population.

To eliminate the possibility that selection of sampling times compromised the performance of the linear regression rearrangement, 100 data sets with no times deleted (i.e., samples at 1-min intervals from 1 to 20 min rather than nine selected sampling times) and with 5% relative error were fitted by linear regression and compared with the multiple regression fits of 9 data points per data set with 5% relative error.

RESULTS AND DISCUSSION

Experiment 1

Both linear and multiple regression models fit errorless data perfectly, demonstrating their mathematical identity. Reliability, R^2, of the simple regression deteriorated rapidly as error increased (Figure 2a). At 10% error, over half of the simple regression fits had R^2 less than .44, the value necessary for a significant regression with these data sets, whereas fitting the same data to the multiple regression model always had R^2 of .92 or greater (Figure 3a).

The mean accuracy of estimators for $K_m$, $V_{max}$, and C(0) for the multiple regression (Figure 2, b, c, and d) remained relatively constant as error in the data increased. Slopes of regressing accuracy on percentage of error in data were -.4, -.3, and -.3 for the respective estimators. Units for these slopes are percentage imprecision/percentage error in data.

In contrast, the simple regression estimators for $K_m$ and $V_{max}$ (Figure 2, b and c) had a strong negative bias (-5.9 and -3.7% bias per percentage of error, respectively). The accuracy of -56% for the inverse of $K_m$ with 10% noise in the data corresponds to 227% error in calculation of $K_m$ compared with 4% error determined by the multiple regression model. Accuracy of the $V_{max}$ estimator deteriorated at only approximately half the rate of the estimator of $K_m$, but the calculation of $V_{max}$ will include the error of its estimator as well as the error associated with $K_m$. The accuracy values were not distributed normally for either estimator (Figure 3, b and c). The precision of the estimators decreased from 2.2 to 3.5 times the rate at which noise in the data increased. For the data presented, the exact value of C(0) was provided. When error was added to C(0), the reliability of the regression deteriorated much more rapidly, and negative values for $K_m$ and $V_{max}$ often were observed, although there were occasional data sets in which the error in C(0) compensated for noise in the data and actually improved the fit. The C(0) cannot be estimated objectively from the data with the simple regression model.

Experiment 2

The Fernley form of the equation could not distinguish between Michaelis-Menten and first-order kinetics; i.e., $K_m$ and $V_{max}$ were correlated completely for most data sets; hence, comparisons with the multiple regression seemed to be unjustified. Fitting Equation [3] directly by nonlinear regression yields the same estimates for $K_m$ and $V_{max}$ as multiple regression (Table 1). The slight differences between the means resulted from failure of the nonlinear regression to converge for 4 of the data sets with relative error only and for 10 of the data sets with both relative and constant error. An obvious advantage of multiple regression is that it will always provide estimates. Also, multiple regression does not require initial estimates for fitting, and the well-founded statistical equations could be incorporated easily into the same electronic spreadsheets used for data collection and storage.

Addition of constant error to the data to simulate analytical error did not alter the esti-
Figure 4. Power of the multiple regression rearrangement of the integrated Michaelis-Menten equation to detect treatment differences in Michaelis constant \( (K_m) \) and maximum rate of reaction \( (V_{max}) \) with 5 and 10% relative error in the data. The numbers, 5, 10, 15, 20, and 25, identifying the power curves, indicate the percentage difference in treatment means. a) \( K_m \) with 5% data error, b) \( K_m \) with 10% data error, c) \( V_{max} \) with 5% data error, and d) \( V_{max} \) with 10% data error.
TABLE 1. Comparison of multiple regression and nonlinear regression of the integrated Michaelis-Menten equation in fitting data with relative error only or both relative and constant error.1

<table>
<thead>
<tr>
<th>Model2</th>
<th>Mean estimate</th>
<th>Paired comparisons3</th>
<th></th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Contrast</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
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<td>1 vs. 3</td>
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</tr>
<tr>
<td>2</td>
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<td>2 vs. 4</td>
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<tr>
<td>4</td>
<td>1903</td>
<td>3 vs. 4</td>
<td>1.23</td>
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</table>

1Actual values for Michaelis constant (K<sub>m</sub>) and maximum rate of reaction (V<sub>max</sub>) are 100 and 1900.
2Models are 1) multiple regression with relative error only, 2) multiple regression with both relative and constant error, 3) nonlinear regression with relative error only (4 of 100 data sets failed to converge), and 4) nonlinear regression with both relative and constant error (10 of 100 data sets failed to converge).
3Using a paired t test, differences in estimates by data set were compared for the indicated models.

Experiment 3

Power of the multiple regression rearrangement to detect differences between treatment means for K<sub>m</sub> and V<sub>max</sub> is shown in Figure 4. When treatment differences are greater than the coefficient of variation found in the population, approximately 10 animals per treatment are adequate to detect a treatment effect (P < .05). Power was not calculated for linear regression because of the tremendous increase in variance with the linear regression technique.

Table 2 shows the estimates of K<sub>m</sub> and V<sub>max</sub> for the subsets within the population of 400 data sets and the population variance. For linear regression, the estimators, 1/K<sub>m</sub> and V<sub>max</sub>/K<sub>m</sub>, were converted to K<sub>m</sub> and V<sub>max</sub>, respectively. Using 9 data points per data set, multiple regression accurately estimated K<sub>m</sub> and V<sub>max</sub> with both 5 and 10% relative error in the data. Although the variance was large for the multiple regression method, it is unlikely that the method per se added significantly to the variance of the population because accurate and nearly unbiased estimates were calculated. Fitting 9 data points per data set with 5% relative error to the linear regression model markedly overestimated both coefficients and increased the variance by approximately 400- and 45-fold for K<sub>m</sub> and V<sub>max</sub>, respectively. Increasing the number of data points per data set to 20 improved the performance of the linear regression model, but it still was inferior to the multiple regression model using only 9 data points per data set. Thus, selection of sampling times did not favor the multiple regression model inappropriately. With 9 data points per data set and 10% relative error, the linear regression model generated negative values for estimates of K<sub>m</sub> or V<sub>max</sub> for 71 of the 400 data sets (data not shown); with 5% relative error, 6 of 400 data sets generated negative values for estimates of K<sub>m</sub> or V<sub>max</sub>.

In summary, the linear regression model cannot accommodate more than approximately 2% relative error in data, whereas the multiple regression model provides a robust method for evaluating data with the integrated Michaelis-Menten equation, providing reliable estimates kinetic coefficients with up to 10% relative error in data. The C(0) was estimated objectively from the data, K<sub>m</sub> and V<sub>max</sub> were esti-
TABLE 2. Effect of magnitude of relative error and the number of data points per data set on the accuracy and variance of estimating Michaelis constant ($K_m$) and maximum rate of reaction ($V_{max}$) by multiple and linear regression of the integrated Michaelis-Menten equation.¹

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<th>Subset</th>
<th>Coeff²</th>
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<th>LR, 4 5% error</th>
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<td>10% error</td>
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<td>99</td>
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<td>1675</td>
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<th>Variance</th>
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</table>

¹A population of 400 data sets with 100 data sets per each of the subsets was used.
²Michaelis-Menten coefficient (coeff).
³Multiple regression.
⁴Linear regression.
⁵Six data sets that provided negative values for estimates of $K_m$ or $V_{max}$ were deleted from the analysis.

The technique provides adequate statistical power to detect treatment effects with a logistically practical number of animals. These advantages are particularly valuable for measuring Michaelis-Menten coefficients in in vivo systems. The multiple regression rearrangement currently is being used to measure the effects of age on intestinal absorption and to measure kidney and liver function to determine the effects of feed processing on either destroying or generating toxicants in feeds.

REFERENCES
APPENDIX

Theoretical Foundation for Equation [3]

A random variable, \( T \), is defined in Equation [4] by substituting \( Y \) for \( C(t) \) in Equation [3] and defining \( Y \) as \( C(t) + \text{error} \).

\[ T = Y - \left[ C(0) + \frac{K_m}{1 + Y} \right] + \frac{V_{\text{max}} t}{Y} \]  

or

\[ T = Y - B_0 - B_1 \ln(Y) - B_2 t \]  

Error is defined as \( C(t) \times EL \times Z \), where \( Z \) is \( N(0, 1) \), and \( EL = .005, .01, .02, .04, .05, .08, \) and \( .10 \); then, \( Y = C(t)[1 + EL \times Z] \). Therefore

\[ T = [C(t) - B_1] (EL)Z - B_2 t \]  

because \( \ln(Y) = \ln [C(t) (1 + EL \times Z)] \) is approximated by \( \ln C(t) + EL \times Z \).

Accepting the approximation, it safely can be assumed that the mean of \( T \) is approximately \(-B_2 t\), and its variance is equal to

\[ \sigma_T^2 = [C(t) - B_1]^2 EL^2. \]  

Because \( B_1 \) is negative, \( C(t) - B_1 \) always is positive.

The resulting least squares regression of \( C(t) \) on both \( \ln(C(t)) \) and \( t \) require the minimization of \( S(B) \) in Equation [8] by appropriately adjusting the parameters \( B_0 \), \( B_1 \), and \( B_2 \).

\[ S(B) = \sum_{i=1}^{n} [C(t) - B_1]^2 [\ln(Y) - B_0 - B_1 \ln(Y) - B_2 t]^2, \]  

where \( n \) is the number of observations. For numerical precision, taking derivatives of \( [C(t) - B_1]^2 \) can be ignored. The estimation of the parameters then reduces to an iterative linear regression using weights proportional to the variance of \( T \). This is begun by setting \( C(t) - B_1 = 1 \) in Equation [8] and calculating the estimates of \( B_0 \), \( B_1 \), and \( B_2 \). The value of \( C(t) \) and \( B_1 \) then are introduced into \( [C(t) - B_1]^2 \) of Equation [8], and values of \( B \) are calculated repeatedly until convergence is realized. This is the approach of Mounter and Turner (8) when fitting the unintegrated Michaelis-Menten equation or that of Britt and Luecke (3) for implicit equations in general. For more complex forms, the reader is referred to Box and Cox (2) and Barnham and Drane (1). Ruppert et al. (11) used the Box-Cox approach to fit several well-known transformations of the Michaelis-Menten equation.

The inverse of the information matrix derived from Equation [8] will produce estimates of the covariance matrix for the estimates of the \( B \). Their standard errors and correlations come from that same matrix (7).

We have demonstrated that, for standard deviations as great as 10% of \( C(t) \), the weighting function in Equation [8] need only be set to 1. That is, set

\[ [C(t) - B_1]^2 = 1. \]