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ESTIMATING THE KINETIC PARAMETERS FOR ENZYMATIC DRUG METABOLISM IN THE WHOLE ANIMAL

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Abstract—A method for estimating the Michaelis constant, K_m, and the maximum reaction velocity, V_{max}, for the enzymatic degradation of a parent compound to a metabolite in the intact animal is presented. The technique involves a mathematical analysis which has shown that under specific conditions the peak/plateau blood concentrations of metabolite are related to initial parent compound concentration by the Michaelis–Menten relationship. It has also been shown how these data can be analysed with the "direct linear plot" of Eisenthal and Cornish–Bowden (*Biochem. J.* **139**, 715 (1974)) to yield the enzyme kinetic parameters.

Many drugs exhibit non-linear pharmacokinetics due to saturation of metabolizing enzymes. Estimation of the Michaelis–Menten parameters for drug metabolism is valuable, particularly for individualizing dosage schedules, e.g. phenytoin [2, 3]. These parameters can be derived by curve fitting parent drug plasma concentration data using appropriate integrated rate equations [4]. Estimates may also be obtained from the relationship between dose and the steady-state plasma drug concentration with repeated doses as shown by Ludden *et al.* [2] and Mullen [3] has applied the direct linear plot of Eisenthal and Cornish-Bowden [1] to these types of data. Here, we show that under specific conditions the direct linear plot can be used to estimate the Michaelis–Menten parameters from metabolite blood concentrations after single doses of drug. The method must assume that both parent compound and metabolite have an apparent volume of distribution which is dose independent and that elimination of metabolite is a first order process. These conditions were satisfied for the *O*-demethylation of the radiation sensitizer misonidazole (1-(2-nitroimidazole-1-yl)-3-methoxypropan-2-ol; Ro-07-0582, Roche Laboratories, MI) in the mouse which has been used as the illustration.

THEORY

Consider the system

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2'}{\rightarrow} \underset{E}{+} \overset{P}{\rightarrow} p' \quad \overset{k_3'}{}$$

where S, E and ES are the concentrations of substrate, free enzyme and an enzyme/substrate complex respectively. In this system P represents product which is within a "compartment" (e.g. the vascular system) which can be measured and p' represents product which has been removed from that compartment assuming a first order kinetic process. Two possible back reactions $ES_{k_{-2}} \leftarrow P$ and $P_{k_{-3}} \leftarrow p'$ have been omitted. This simplification is justified if both k_{-2} and k_{-3} are very much smaller in magnitude than the respective rate constants for the forward reactions or if the substrate concentration, S, at time t approximates to that at time zero, S_0. However, the rate constants for $ES \rightarrow P$ and for $P \rightarrow p'$ have been "primed" and written as k'_2 and k'_3 in acknowledgement of the simplification.

The rate equations relevant to the present analysis are

$$\delta ES/\delta t = k_1(S\ E) - k_{-1}ES - k_2'\ ES \qquad (1)$$

$$\delta P/\delta t = k_2'\ ES - k_3'\ P. \qquad (2)$$

When $\delta P/\delta t = 0$ an asymptotic or "plateau" value of P, P_{plat}, will have been reached. Thus, from equation (2) it can be seen that

$$k_2'\ ES = k_3'\ P_{plat}$$

therefore

$$ES = (k_3'\ P_{plat})/k_2'. \qquad (3)$$

Substituting this solution for ES into equation (1) gives

$$\frac{\delta ES}{\delta t} = k_1(S\ E) - \frac{k_3'\ P_{plat}}{k_2'}\{k_{-1} + k_2'\}.$$

Dividing through by k_1 we get

$$\frac{1}{k_1}\frac{\delta ES}{\delta t} = S\ E - \frac{k_3'\ P_{plat}}{k_2'}\left[\frac{k_{-1} + k_2'}{k_1}\right]. \qquad (4)$$

The expression $(k_{-1} + k_2')/k_1$ is the Michaelis constant, K_m, and formal proof of this relationship is given in ref. 5. However, this definition needs some clarification in this particular context. The constant k'_2 should represent the rate of dissociation of complex to yield product plus free enzyme. But, the product concentration is being measured in a "compartment" (in this case blood) which is physically distinct, and separated from the "compartment" (cells) in which the reaction is taking place. If there

is slow diffusion of product from the enzyme across membrane barriers to the site of measurement then the measured K_m is not the true value of the enzyme but an apparent *in vivo* K_m. With this qualification equation (4) reduces to

$$\frac{1}{k_1}\frac{\delta ES}{\delta t} = S\,E - \frac{k_3'\,P_{plat}}{k_2'}K_m. \qquad (5)$$

At any time, t, the concentration of free enzyme, E, is given by the relationship

$$E = E_0 - ES$$

where E_0 is the initial enzyme concentration and where ES represents the concentration of enzyme which is bound to substrate. Substituting in equation (5) gives

$$\frac{1}{k_1}\frac{\delta ES}{\delta t} = S(E_0 - ES) - \frac{k_3'\,P_{plat}}{k_2'}K_m. \qquad (6)$$

If we now substitute the relationship for ES given by equation (3) into equation (6), expand then simplify, we get

$$\frac{1}{k_1}\frac{\delta ES}{\delta t} = S\,E_0 - \frac{k_3'\,P_{plat}}{k_2'}(S + K_m).$$

Multiplying through by the factor $k_2'/(S + K_m)$ gives

$$\frac{1}{S + K_m}\frac{k_2'}{k_1}\frac{\delta ES}{\delta t} = \frac{k_2'\,E_0 S}{(S + K_m)} - k_3' P_{plat}. \qquad (7)$$

It will be recognised that $k_2'\,E_0$ is the maximum reaction velocity, V_{max}. Thus, the first term on the right-hand-side of equation (7) is $S\,V_{max}/(S + K_m)$ and is equal to the initial velocity, v, of the Michaelis–Menten equation. Therefore, on rearrangement of equation (7) we get

$$v = \frac{S\,V_{max}}{S + K_m} = k_3' P_{plat} + \frac{1}{S + K_m}\frac{k_2'}{k_1}\frac{\delta ES}{\delta t}. \qquad (8)$$

If $\delta ES/\delta t = 0$, equation (8) reduces to

$$v = k_3'\,P_{plat} = S\,V_{max}/(S + K_m) \qquad (9)$$

where P_{plat} is the measured product concentration when this is constant for a given initial substrate concentration, S, and where k_3' is the rate constant for product loss from the compartment in which P is measured.

The Michaelis–Menten relationship (see equation 9) has been rearranged by Eisenthal and Cornish-Bowden [1] to give the maximum velocity, V_{max}, in terms of the remaining parameters, thus

$$V_{max} = v + (v\,K_m)/S. \qquad (10)$$

For each value of v which is associated with its substrate concentration, S, a line can be plotted which crosses the abscissa at $-S$ and the ordinate at v. With a perfect data set the various lines for each v and S combination will all intersect at a point with (x, y) coordinates of (K_m, V_{max}). This is the "direct linear plot" of Eisenthal and Cornish-Bowden. Substituting $k_3'\,P_{plat}$ for v (equation 9) into equation (10) and dividing by k_3' gives

$$\frac{V_{max}}{k_3'} = P_{plat} + \frac{P_{plat}}{S}K_m.$$

Thus, by using the Eisenthal/Cornish-Bowden method and plotting lines passing through the coordinates $(-S,0)$ and $(0,P_{plat})$ for each S and P_{plat} combination we will find that the intersection of those lines will occur at the point with coordinates $(K_m, V_{max}/k_3')$. If k_3' can be measured the maximum velocity is defined.

RESULTS

The data used in this analysis were derived from a previous study [6] where full experimental details were given. The blood concentrations of the radiation sensitizer misonidazole following intraperitoneal administration of varying doses in mice are shown in Fig. 1. The corresponding data for the *O*-demethylated metabolite desmethylmisonidazole (Ro 05-9963) measured simultaneously are shown in Fig. 2. Peak/plateau concentrations of metabolite (Fig. 2) were obtained by averaging the first four points on the two highest curves (○ and ●). The three highest points (2nd to 4th) were averaged to give the third and fifth peak/plateau values (△ and □), and the fourth (■) was obtained by averaging the two highest points. The direct linear plots of these data against their associated negative values of initial substrate concentrations (intercept values Fig. 1) are shown in Fig. 3. The mean value of the X-axis coordinates at the intersections, which gives K_m, was 852 μM ± 92 μM (2 SE). The mean of the Y-axis coordinates at the intersections was 386 μM ± 20 μM (2 SE). The elimination half-time for desmethylmisonidazole has been shown to be 42 min and was independent of dose [6]. Thus, the loss rate con-

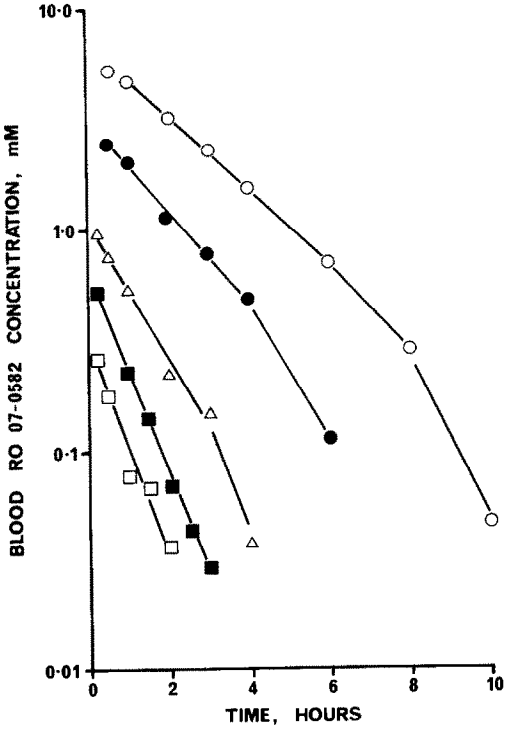

Fig. 1. Time course of plasma misonidazole concentrations after intraperitoneal injection of five different doses.

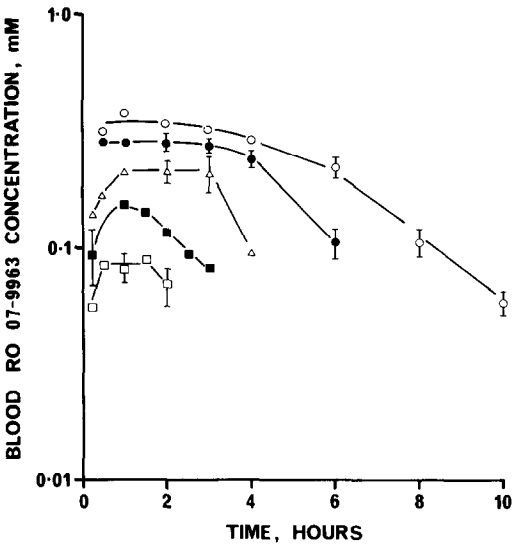

Fig. 2. Time course of plasma desmethylmisonidazole concentrations measured simultaneously with the data in Fig. 1.

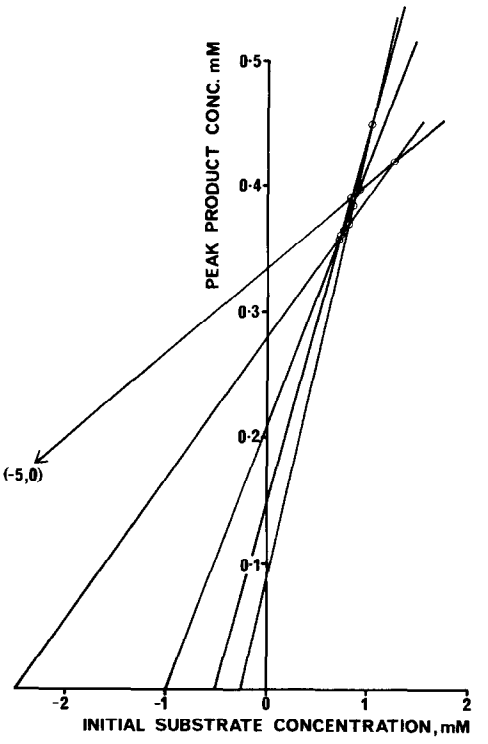

Fig. 3. Direct linear plot of Eisenthal and Cornish-Bowden for peak product concentration (ordinate) vs initial dose administered.

stant, k_3', is $0.0166\,\text{min}^{-1}$ and hence $V_{max} = 6.4\,\mu\text{moles min}^{-1}$.

In equation (9) it was shown that the initial reaction velocity, v, is equal to $k_3'\,P_{plat}$. Lineweaver and Burk [11] have presented equation (9) in reciprocal form and by plotting $1/P_{plat}$ versus $1/S$ a line with slope $k_3'\,K_m/v_{max}$ will be obtained which cuts the Y-axis at k_3'/V_{max}. Also, when $1/P_{plat}$ is equal to zero the X-axis intercept will be $-1/K_m$. Eadie–Hofstee derivative plots (refs 9 and 10) were also used to analyse these data and the results of these various analytical methods are shown in the table after correcting for the rate constant for product loss, k_3', from the vascular compartment.

The mean results for V_{max} and K_m given in the table were used to predict the peak plasma concentration of metabolite, P_{plat}, from the initial admin-

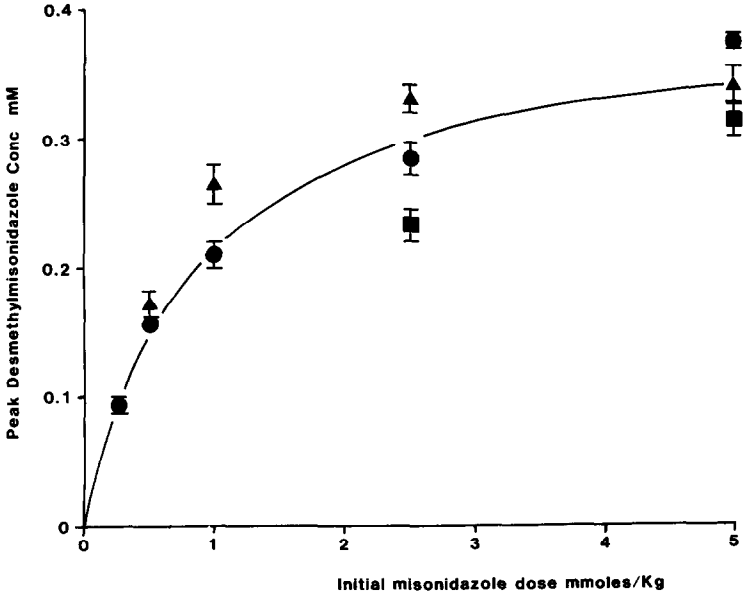

Fig. 4. Predicted peak plasma desmethylmisonidazole concentration vs initial misonidazole dose. The curve was calculated by equation (9) from the mean values of V_{max} and K_m given in the table. The data points are from three independent experiments.

Table 1. Results of the various methods of analysing the experimental data to give K_m and V_{max} for the conversion of misonidazole to desmethylmisonidazole in the whole mouse

Analysis method	K_m (μM)	V_{max} (μmoles min^{-1})
Direct linear	852	6.40
Lineweaver–Burk	762	6.47
Eadie–Hofstee	847	6.79
Mean	820	6.55

istered doses of misonidazole via equation (9). The result is shown in Fig. 4. The data points from three independent experiments were then added. The agreement is good.

DISCUSSION

The method of obtaining the enzyme kinetic data presented in this paper can be used in any system in which the initial concentration of substrate and the time course of product concentration can be measured even if the latter is being lost from the system. Both V_{max} and K_m can be estimated as long as the rate constant for product loss can be determined. However, a number of qualifications must be considered as it is evident from equation (8) that the method can only be rigorously applied if $\delta ES/\delta t$ is zero, i.e. if "steady-state" conditions prevail. When using *in vitro* preparations it is usually possible to control the experiment so that these conditions are met by using high substrate concentrations. However, with *in vivo* systems an upper limit on substrate concentration will frequently be imposed by toxicity. Furthermore, the relative magnitudes of the rate constants for production and loss of product are important in determining how soon a plateau in product concentration is reached. If the rate constant for loss, k_3', is greater than that for production, k_2', a plateau in product concentration will be reached relatively "early" in the reaction before significant substrate depletion occurs. Thus, the substrate concentration when the product concentration is constant will approximate to the initial substrate concentration and $\delta ES/\delta t$ will tend to zero.

This is difficult to demonstrate in the intact animal in which various complications may arise. These include loss of substrate via other metabolic routes and excretion pathways and the possible retention of substrate in "depot" compartments which do not contain the metabolizing enzymes and each will contribute to non steady-state conditions. Depot, slowly equilibrating, compartments containing no enzyme will initially give rise to an increase in the effective substrate concentration in those compartments which do contain enzyme. At later times there will be a slow and continuous release from depot compartments into those containing enzyme as the concentration of substrate in the latter falls. This factor, however, will tend to be minimised when the agent has an even whole body distribution as is the case with misonidazole [7]. Moreover, excretion of both substrate and product may be an active process dependent on enzyme action which may add further complications as excretion may be rate limited. Studies with injected desmethylmisonidazole have shown that it has a half-life of 42 min which is independent of dose [6]. This, together with the high urinary recovery of desmethylmisonidazole indicates first-order elimination kinetics mainly via renal clearance. We feel that the renal clearance of misonidazole is also likely to be first-order, but this has not yet been demonstrated.

Even though the misonidazole levels are decreasing rapidly (Fig. 1) it is probable, even at the lowest dose, that $S_0 \gg E_0$ and that substrate depletion is not significant. If this is true the analysis is valid as a first approximation as $\delta ES/\delta t$ will tend to zero during the time that the plateau in product concentrations are reached. In order to test the hypothesis rigorously

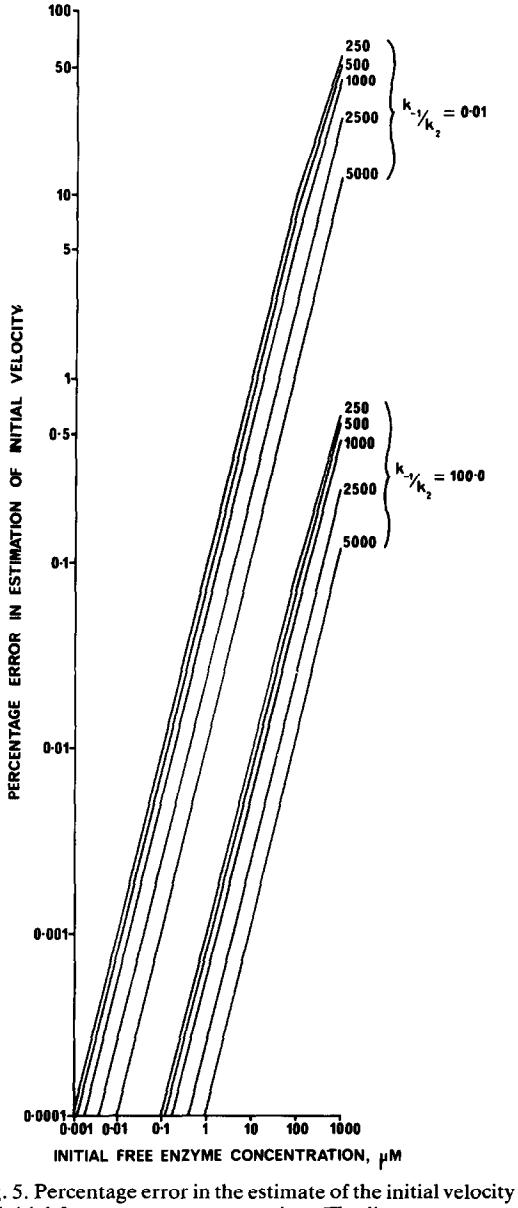

Fig. 5. Percentage error in the estimate of the initial velocity vs initial free enzyme concentration. The lines were computed for the five misonidazole doses shown (μmoles/kg), and for two ratios of k_{-1}/k_2, namely 0.01 and 100.0.

we must assume that $\delta ES/\delta t$ is not zero when $\delta P/\delta t = 0$, and attempt to ascertain the magnitude of the error induced by the last term in equation (8). A computer simulation was carried out with $K_m = 852\,\mu M$ and $V_{max} = 6.4\,\mu$moles min^{-1}. The initial enzyme concentration, E_0, was varied within the range 1000–0.001 μM by an order of magnitude per step. For each value of E_0 the rate constant k_2' can be calculated from $V_{max} = k_2'^* E_0$. The ratio k_{-1}/k_2' was varied from 100:1 to 1:100, again by steps of one order of magnitude. As K_m is known the values of k_{-1} and k_1 can be calculated for given values of k_2' and for given values of the ratio k_{-1}/k_2'. The simulations were performed using a numerical integrating algorithm (DO2EBF) from the NAG mark 7 computer library, Dec. 1978. This routine uses Gear methods [8] which involve variable order variable step techniques to integrate "stiff" differential equation systems which are characterised by very large differences between the values of the rate constants. The percentage error in the estimate of the initial velocity was calculated by comparing the output from equations (8) and (9), i.e. with and without the term involving $\delta ES/\delta t$. These results are given in Fig. 5 which shows the absolute percentage error plotted against E_0 for five initial substrate concentrations and for the two extremes of the ratio k_{-1}/k_2', i.e. 100:1 and 1:100. When k_{-1} is two orders of magnitude greater than k_2' an error of <1% was obtained for $E_0 = 1000\,\mu M$ even at the lowest substrate concentration. However, when the ratio was reversed (k_2' 100 times greater than k_{-1}) very considerable errors (>5%) can be produced by this method of analysis if the initial enzyme concentration exceeds about 50 μM. As yet no formal curve fitting procedures have been employed as our current model is obviously a gross oversimplification of the biological reality, but the family of curves generated for the ratio $k_{-1}/k_2' = 0.01$ (i.e. with the greatest error in Fig. 5) were simply not compatible with the experimental data. In all cases the predicted peak in product concentration occurred considerably later than observed experimentally. Thus, we believe the method of analysis reported here to be valid as a first approximation if it can be assumed that the "whole body" concentration of *O*-demethylating enzymes does not exceed about 50 μM and that k_2' does not exceed k_{-1} by more than two orders of magnitude. Furthermore, the method may be useful for determining the "whole animal" enzyme reaction kinetic parameters for any agents which comply with the overall conditions considered in this communication.

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