The effects of systematic errors on the analysis of irreversible enzyme inhibition progress curves

Peter GRAY

Materials Research Laboratory, Defence Science and Technology Organisation, P.O. Box 50, Ascot Vale, Victoria 3032, Australia

The effects of errors in K_m and V_{max} on the determination of the kinetic constants for the irreversible inhibition of acetylcholinesterase were determined. The results show that small errors in the values of K_m and V_{max} give rise to large errors in the fitted parameters k_2 and K_d . The problem may be minimized for multiple progress curves by selecting an appropriate number and distribution of inhibitor concentrations.

INTRODUCTION

Irreversible or slowly reversible inhibitors of acetylcholinesterase are of commercial, medical and military interest. Organophosphorus compounds and carbamates are used as insecticides (Aldridge & Reiner, 1972; Wang & Murphy, 1982). Carbamates are used as pretreatment drugs for nerve-agent poisoning (Green, 1983) and for the treatment of Alzheimer's disease (Giacobini *et al.*, 1987), glaucoma and myasthenia gravis (Taylor, 1980). The chemical-warfare agents known as the nerve agents are also organophosphorus anticholinesterases (Robinson, 1971).

The mechanism by which these compounds inhibit acetylcholinesterase is written, by analogy with substrate hydrolysis, as follows (Liu & Tsou, 1986):

where EH is the enzyme, IX is the inhibitor and EH·IX is the Michaelis complex. The inhibition reaction is described by the dissociation constant ($K_d = k_{-1}/k_{+1}$), the unimolecular rate constant k_{+2} and the re-activation rate constant k_{+3} . For some inhibitors, such as the nerve agents, k_{+3} is much smaller than k_{+2} and no re-activation is observed over the time course of most experimental studies. However, for di-isopropyl phosphorofluoridate and some carbamates and organophosphorus compounds significant spontaneous re-activation does occur (Aldridge & Reiner, 1972; Berry, 1971; Dawson & Poretsky, 1985; Hovanec & Lieske, 1972; Lanks & Seleznick, 1981; Lieske *et al.*, 1976).

Several different methods have been used to determine the values of k_{+2} and K_d . These methods have been evaluated and a non-linear-regression method has been developed to overcome the deficiencies of some of the methods (Gray & Duggleby, 1989). The non-linear-regression method involves fitting the following equation (Liu & Tsou, 1986; Tsou, 1965a,b) to a family of progressive inhibition curves:

$$[P] = [P]_{\infty}(1 - e^{-A \cdot [IX] \cdot t}) \quad (2)$$

where $[P]$ and $[P]_{\infty}$ are the product concentrations at time t and $t = \infty$ respectively. The apparent rate constant for the formation of the inhibited enzyme, A , is defined as follows:

$$A = \frac{k_{+2}/K_d}{1 + [S]/K_m + [IX] \cdot K_d} \quad (3)$$

where K_d is the inhibitor dissociation constant, $[S]$ is the substrate concentration and K_m is the Michaelis constant (Tsou, 1965a,b; Tian & Tsou, 1982).

$[P]_{\infty}$ is defined as follows (Tsou, 1965a,b; Tian & Tsou, 1982):

$$[P]_{\infty} = \frac{V_{max} \cdot [S] \cdot K_d}{k_{+2} \cdot [IX] \cdot K_m} \quad (4)$$

This method was shown to provide accurate values of k_{+2} and K_d with the use of perfect data to which random errors had been added and with the use of accurate values of V_{max} and K_m (Gray & Duggleby, 1989). However, in practice there will always be some error associated with these experimentally determined parameters and in $[S]$ and $[IX]$. This method and the methods used by other workers require the use of a number of progress curves with a range of inhibitor concentrations (Forsberg & Puu, 1984; Gray & Duggleby, 1989). However, there is evidence to suggest that some of these inhibitors exert allosteric effects (Friboulet *et al.*, 1986; Main, 1979; Nishioka *et al.*, 1976; Watts & Wilkinson, 1977). The existence of these effects would invalidate the analysis of multiple inhibition curves. It is therefore desirable to be able to determine k_{+2} and K_d from a single inhibition curve.

The aim of the present study was to investigate the effects of systematic errors on the determination of k_{+2} and K_d from single and multiple progressive inhibition curves.

METHODS

Calculation of progress curves

Nishioka *et al.* (1976) derived the following more general equation, which describes the progressive inhibition in the presence of substrate when $k_{+3} \neq 0$.

$$[P] = \left(\frac{D \cdot k_{+3} \cdot t}{C} \right) + \left(\frac{B \cdot D}{C^2} \right) (1 - e^{-C \cdot t}) \quad (5)$$

where

$$A = 1 + \frac{K_d}{[IX]} + \frac{K_d \cdot [S]}{K_m \cdot [IX]} \quad (6)$$

$$B = k_{+2}/A \quad (7)$$

$$C = B + k_{+3} \quad (8)$$

$$D = \frac{V_{max} \cdot K_d \cdot [S]}{A \cdot K_m \cdot [IX]} \quad (9)$$

and

$$[P]_{\infty} = \frac{D \cdot k_{+3} \cdot t}{C} + \frac{B \cdot D}{C^2} \quad (10)$$

Eqn. (5) reduces to eqn. (2) when $k_{+3} = 0$.

Fig. 1. Inhibition of acetylcholinesterase by di-isopropyl phosphorofluoridate

The di-isopropyl phosphorofluoridate concentrations were 0.55 mM (upper curve), 1.66 mM (middle curve) and 2.77 mM (lower curve). Substrate concentration was 0.75 mM. The error bars represent twice the standard deviations determined from five replicates.

Simulated progress curves were calculated by using eqn. (5) with $k_{+3} = 0$. Errors were added in order to approximate the experimental observations. In Fig. 1 is shown a series of progressive inhibition curves resulting from stopped-flow measurement of the inhibition of acetylcholinesterase by di-isopropyl phosphorofluoridate. The individual data points are the means with standard deviations. These data show that the standard deviations are quite small and relatively constant along the curve. The average standard deviation of 66 such measurements over a range of inhibitor and substrate concentrations was $0.0766 \mu\text{M}$. Errors were incorporated in the simulated data by choosing a random number from a normal distribution with mean zero and standard deviation $0.0766 \mu\text{M}$ and adding it to the calculated value.

Progressive inhibition curves were calculated by using the values of K_d ($4.0 \mu\text{M}$) and k_{+2} (0.92 s^{-1}) for the inhibition of electric-eel acetylcholinesterase by soman (Gray & Dawson, 1987). The value of V_{max} ($0.5 \mu\text{M} \cdot \text{s}^{-1}$) was chosen so that approx. 10% of the substrate was used at the lowest inhibitor concentration used. A substrate concentration of 1 mM and a K_m of $50 \mu\text{M}$ used. No correction was made for the use of substrate.

Multiple progress curves were generated by using inhibitor concentrations in the range $0.1 K_d$ to $1.0 K_d$. The reaction time was the time to reach 95% of $[P]_{\infty}$, and 25 points were calculated for each curve. In order to calculate standard deviations at each point, ten replicate curves were used. Single curves were also 95% complete, but, however, 50 data points were determined.

Data analysis

Non-linear-regression analysis was carried out as described in Gray & Duggleby (1989). The two- and three-dimensional plots were prepared by using Sygraph (Systat, Evanston, IL, U.S.A.).

The data were scaled to micromolar to minimize round-off errors (Motulsky & Ransnas, 1987). The data points were weighted by using the standard deviation calculated from ten replicate progressive inhibition curves. In order to study the effects of systematic errors in the constants K_m , V_{max} , and $[S]$, progressive inhibition curves were calculated by using the erroneous values and non-linear regression was carried out with the use of the true values. Better results were obtained if the parameter $[S]/K_m$ was fitted instead of K_m alone (eqn. 3).

Sum-of-squares plots were derived by generating an exact data

set by using the true values of k_2 , K_d , K_m and V_{max} , and calculating the sum of squares as follows:

$$\text{SSQ} = \sum ([P]_{\text{true}} - [P])^2 \quad (11)$$

The appropriate parameters were then adjusted and the process was repeated. A total of 20 adjustments were made for each parameter resulting in a 20×20 matrix of sum-of-squares values.

Experimental

The stopped-flow studies were carried out with the use of the materials and techniques described in Gray & Dawson (1987). Di-isopropyl phosphorofluoridate was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

In Fig. 2 is shown the effect of errors in V_{max} and K_m on the fitted parameters k_{+2} and K_d calculated from a family of progress curves. Errors in V_{max} and K_m of a few per cent resulted in very

Fig. 2. Effects of errors in (a) V_{max} , and (b) K_m on the fitted values of k_{+2} and K_d

The true values of V_{max} and K_m are $0.5 \mu\text{M} \cdot \text{s}^{-1}$ and $50 \mu\text{M}$ respectively. The true values of k_{+2} and K_d are indicated by the broken lines. The error bars indicate twice the standard errors of the fitted parameters. The parameters were calculated from a family of five progress curves spaced equally on a $1/[X]$ scale from $0.1 K_d$ to $0.5 K_d$. Each curve was the average of ten replicates.

Fig. 3. Effect of inhibitor concentration on the values of the fitted parameters

The true values of k_{+2} and K_d are shown by the broken lines. The error bars represent twice the standard errors of the fitted parameters. The true values of $V_{\max}$ and K_m were used to construct the progress curves.

large errors in k_{+2} and K_d . For example, an error of +5% in $V_{\max}$ resulted in an error of 296% in K_d and k_{+2} . Similarly a -5% error in K_m produced errors of 52% in K_d and 44% in k_{+2} . In addition, the errors were not symmetrical about the true value. A +5% error in $V_{\max}$ resulted in a much larger error in K_d than did a -5% error. The reverse was true for errors in K_m . The same effects were observed with single progress curves. Moreover, the errors in the fitted parameters varied with inhibitor concentration (Fig. 3), increasing with decreasing inhibitor concentration.

The reason for the ill-conditioned nature of the equation is seen in Fig. 4. Fig. 4(a) shows the sum-of-squares surface plotted in the parameter space defined by k_{+2} and K_d . Instead of a clear minimum, a long trough runs diagonally through the k_{+2}/K_d plane. Even on the expanded scale (Fig. 4b) the minimum is not obvious.

One approach to solution of this problem is to consider $V_{\max}$ and K_m as additional unknown parameters to be estimated. The sum-of-squares surface in the $V_{\max}/K_m$ parameter space also shows a trough rather than a distinct minimum (Fig. 5). This suggests that it may not be feasible to determine k_{+2} , K_d , $V_{\max}$ and K_m simultaneously. However, 'structure' may be added to the data by increasing the number of members of the family of progress curves and selecting an appropriate distribution of inhibitor concentrations.

Table 1 shows the effect of these adjustments on the determination of the four parameters. In any given distribution of inhibitor concentrations, linear, $1/[IX]$ or $\ln[IX]$, the error in the fitted parameters decreased as the number of progress curves was raised from five to seven. The best conditions to use are either seven or nine curves with either a $1/[IX]$ or $\ln[IX]$ distribution. The standard errors of $[S]/K_m$ are high in all cases.

This approach does not produce satisfactory results for single progress curves. Attempting to fit k_{+2} , K_d and $V_{\max}$, assuming the true value of K_m resulted in very large standard errors of k_{+2} and K_d (Table 2).

A second approach is to use a method that does not involve the use of $V_{\max}$. Such a method is the Apparent-Rate-Constant Method described by Liu & Tsou (1986). A plot of $1/(A \cdot [IX])$ versus $1/[IX]$ (eqn. 3) allows k_{+2} to be determined as the reciprocal of the intercept on the ordinate and K_d from the slope, where

Fig. 4. Plot of the sum-of-squares surface in the k_{+2}/K_d parameter space

Five inhibitor concentrations linearly spaced between 0.1 and $1.0K_d$ were used. In (a) points were calculated by using 5% increments in each parameter. (b) shows the area around the true minimum and was generated by using 1% increments in k_{+2} and K_d . The dot (●) indicates the true minimum.

slope = $K_d k_{+2}(1 + [S]/K_m)$. In my application of this method, A was determined by fitting eqn. (2) to the data and k_{+2} and K_d were calculated by using eqn. (3). In this case the best results were achieved by reparameterizing eqn. (3) and determining K_a instead of K_d . The value of K_d was then calculated as $1/K_a$.

In Table 3 are shown the results of these calculations made

Fig. 5. Plot of the sum-of-squares in the $K_m/V_{\max}$ parameter space. The dot (●) marks the true minimum.

Table 1. Effect of number and distribution of progress curves on the value of the fitted parameters k_{+2} and K_d

Each progress curve was the average of ten replicates. The standard errors of the fitted parameters are given in parentheses. The true values of the parameters are $k_{+2} = 0.92 \text{ s}^{-1}$, $K_d = 4.0 \text{ μM}$, $V_{\max} = 0.5 \text{ μM} \cdot \text{s}^{-1}$ and $[S]/K_m = 20$.

No. of curves	Fitted parameter	Inhibitor distribution		
		[IX]	1/[IX]	ln [IX]
5	$k_{+2} (\text{s}^{-1})$	0.909 (0.054)	0.924 (0.085)	0.907 (0.063)
	$K_d (\text{μM})$	3.988 (0.877)	4.006 (0.798)	3.905 (1.801)
	$V_{\max} (\text{μM} \cdot \text{s}^{-1})$	0.499 (0.056)	0.499 (0.005)	0.499 (0.011)
	$[S]/K_m$	19.845 (4.948)	20.069 (4.084)	20.210 (9.441)
7	$k_{+2} (\text{s}^{-1})$	0.942 (0.043)	0.890 (0.053)	0.921 (0.045)
	$K_d (\text{μM})$	3.959 (1.283)	3.888 (0.379)	4.029 (0.746)
	$V_{\max} (\text{μM} \cdot \text{s}^{-1})$	0.499 (0.008)	0.500 (0.003)	0.500 (0.004)
	$[S]/K_m$	20.751 (6.809)	19.902 (2.104)	19.877 (3.651)
9	$k_{+2} (\text{s}^{-1})$	0.901 (0.034)	0.915 (0.046)	0.920 (0.039)
	$K_d (\text{μM})$	3.839 (1.472)	3.970 (0.465)	3.987 (0.564)
	$V_{\max} (\text{μM} \cdot \text{s}^{-1})$	0.499 (0.009)	0.499 (0.003)	0.499 (0.004)
	$[S]/K_m$	20.448 (7.968)	20.055 (2.399)	20.066 (3.072)

with the same inhibitor concentrations used for Table 1. In this case the best results were achieved with seven points distributed linearly between $0.1K_d$ and $1.0K_d$. This method was also quite robust with respect to errors in K_m . Errors of 10% in K_m resulted in errors of approx. 10% in k_{+2} and K_d with standard errors of approx. 5%. With this particular data set, the curve-fitting program did not converge to a solution when attempts were made to fit k_{+2} , K_d and $[S]/K_m$ simultaneously.

Table 2. Results of fitting k_{+2} , K_d and $V_{\max}$ to single progress curves

Each progress curve was the average of ten replicates. The inhibitor concentrations are equally spaced on a linear scale between $0.1K_d$ and $1.0K_d$. The standard errors of the fitted parameters are given in parentheses. The true values of the parameters are $k_{+2} = 0.92 \text{ s}^{-1}$, $K_d = 4.0 \text{ μM}$ and $V_{\max} = 0.5 \text{ μM} \cdot \text{s}^{-1}$.

[Inhibitor]	Fitted parameters		
	$k_{+2} (\text{s}^{-1})$	$K_d (\text{μM})$	$V_{\max} (\text{μM} \cdot \text{s}^{-1})$
0.10 K_d	0.827 (0.701)	3.587 (3.057)	0.501 (0.0025)
0.25 K_d	0.680 (0.411)	2.941 (1.808)	0.502 (0.0039)
0.40 K_d	0.807 (0.566)	3.499 (2.511)	0.501 (0.0077)
0.55 K_d	0.810 (0.674)	3.498 (3.000)	0.503 (0.013)
0.70 K_d	0.632 (1.398)	2.708 (6.291)	0.506 (0.033)
0.85 K_d	0.929 (1.251)	4.041 (5.661)	0.499 (0.034)
1.00 K_d	0.930 (1.155)	4.019 (5.228)	0.501 (0.036)

Table 3. Effect of number and distribution of progress curves on the determination of k_{+2} and K_d by the Apparent-Rate-Constant method

Each progress curve was the average of ten replicates. The standard errors of the fitted parameters are given in parentheses. The true values of the parameters are $k_{+2} = 0.92 \text{ s}^{-1}$ and $K_d = 4.0 \text{ μM}$.

No. of curves	Fitted parameter	Inhibitor distribution		
		[IX]	1/[IX]	ln [IX]
5	k_{+2}	0.854 (0.059)	0.858 (0.042)	0.846 (0.044)
	K_d	3.705 (0.263)	3.705 (0.180)	3.676 (0.189)
7	k_{+2}	0.905 (0.046)	0.888 (0.016)	0.774 (0.021)
	K_d	3.937 (0.202)	3.861 (0.224)	3.356 (0.090)
9	k_{+2}	0.799 (0.046)	0.959 (0.079)	0.942 (0.072)
	K_d	3.472 (0.205)	4.167 (0.347)	4.098 (0.319)

DISCUSSION

Previously we have shown that, by using perfect data to which random errors had been added, non-linear-regression analysis provided accurate estimates of the values of k_{+2} and K_d (Gray & Duggleby, 1989). This accuracy depended in turn on accurate values of K_m and $V_{\max}$. In practice, however, there will always be errors associated with these constants. In the present study the determination of k_{+2} and K_d has been shown to be very sensitive to errors in K_m and $V_{\max}$, particularly $V_{\max}$. The reason for this sensitivity lies in the ill-conditioned nature of the equation that describes the inhibition process. Because of the shape of the sum-of-squares surface (Figs. 4 and 5) any distortion provided by small errors in K_m and $V_{\max}$ may move the minimum a considerable distance. Similar behaviour has been observed with other enzyme kinetic problems (Bates *et al.*, 1987). The problem is resolved reasonably well by treating K_m and $V_{\max}$ as fitted parameters and by selecting appropriate inhibitor concentrations to generate a family of progress curves.

The Apparent-Rate-Constant method is less satisfactory because there is insufficient structure in the data to allow simultaneous determination of k_{+2} , K_d and K_m (as $[S]/K_m$). Therefore the results are still influenced by errors in K_m . Another potential problem with this analysis arises from the nature of eqn. (5). The change in the value of A with inhibitor concentration is small. With the constants studied A varied by only 4.3% as the

inhibitor concentration was raised from $0.1K_d$ to $1.0K_d$. The reason for this is the domination of the denominator of eqn. (3) by the value of $[S]/K_m$. $[S]$ is usually much higher than K_m in order to avoid interference by substrate depletion. Errors in the values of A may then give rise to significant errors in the determination of k_{+2} and K_d .

For single progress curves the problem has not been solved. The effect of errors in V_{max} and K_m renders the non-linear-regression analysis unworkable. The alternative method of Hart & O'Brien (1973) is also subject to error (Gray & Duggleby, 1989).

The present study has examined only one point in the k_{+2}/K_d parameter space. It may be of value to carry out this type of analysis to determine the best experimental conditions whenever dealing with different values of k_{+2} and K_d .

REFERENCES

- Aldridge, W. N. & Reiner, E. (1972) *Enzyme Inhibitors as Substrates*, North-Holland Publishing Co., Amsterdam and London
- Bates, J. H. T., Bates, D. A. & Mackillop, W. (1987) *J. Theor. Biol.* **125**, 237–241
- Berry, W. K. (1971) *Biochem. Pharmacol.* **20**, 3236–3238
- Dawson, R. M. & Poretski, M. (1985) *Biochem. Pharmacol.* **34**, 4337–4340
- Forsberg, A. & Puu, G. (1984) *Eur. J. Biochem.* **140**, 153–156
- Friboulet, A., Goudou, D. & Rieger, F. (1986) *Neurochem. Int.* **9**, 323–328
- Giacobini, E., Becker, R., Elble, R., Mattio, T., McIlhany, M. & Scarsella, G. (1987) in *Neurobiology of Acetylcholine* (Dun, N., ed.), pp. 85–102, Plenum Press, New York
- Gray, P. J. & Dawson, R. M. (1987) *Toxicol. Appl. Pharmacol.* **66**, 409–419
- Gray, P. J. & Duggleby, R. G. (1989) *Biochem. J.* **257**, 419–424
- Green, A. L. (1983) *Biochem. Pharmacol.* **32**, 1717–1722
- Hart, G. J. & O'Brien, R. D. (1973) *Biochemistry* **12**, 2940–2945
- Hovanec, J. W. & Lieske, C. N. (1972) *Edgewood Arsenal Spec. Publ. (U.S. Dep. Army) EASP 1100–11*
- Lanks, K. W. & Seleznick, M. J. (1981) *Biochim. Biophys. Acta* **660**, 91–95
- Lieske, C. N., Clark, J. H., Lowe, J. R., Horton, G. L., Jewell, D. K. & Daasch, L. W. (1976) *Edgewood Arsenal Tech. Rep. (U.S. Dep. Army) EB-TR-76113*
- Liu, W. & Tsou, C. L. (1986) *Biochim. Biophys. Acta* **870**, 185–190
- Main, A. R. (1979) *Pharmacol. Ther.* **6**, 579–628
- Motulsky, H. J. & Ransnas, L. A. (1987) *FASEB J.* **1**, 365–374
- Nishioka, T., Kitamura, K., Fujita, T. & Nakajima, M. (1976) *Pestic. Biochem. Physiol.* **6**, 320–337
- Robinson, J. P. (1971) *The Rise of CB Weapons*, Almqvist and Wiksell, Stockholm
- Taylor, P. (1980) in *Pharmacological Basis of Therapeutics* (Gilman, A. G., Goodman, L. S. & Gilman, A., eds.), pp. 100–119, Macmillan Publishing Co., New York
- Tian, W.-X. & Tsou, C.-L. (1982) *Biochemistry* **21**, 1028–1032
- Tsou, C. L. (1965a) *Shengwu Huaxue Yu Shengwu Wuli Xuebao* **5**, 398–408
- Tsou, C. L. (1965b) *Shengwu Huaxue Yu Shengwu Wuli Xuebao* **5**, 409–417
- Wang, C. & Murphy, S. D. (1982) *Toxicol. Appl. Pharmacol.* **66**, 409–419
- Watts, P. & Wilkinson, R. G. (1977) *Biochem. Pharmacol.* **26**, 757–761

Received 11 April 1990/24 July 1990; accepted 30 July 1990