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An Evaluation of Ways of Using Equilibrium Dialysis to Quantify the Binding of Ligand to Macromolecule

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1. The effect of systematic error (loss of ligand, complex or macromolecule) on three of the experimental designs by which equilibrium dialysis may be used to quantify the interaction of ligand and macromolecule is examined theoretically, and the design that is least sensitive to systematic error is identified. 2. Thirteen methods for fitting the binding isotherm to experimental data are compared by using them to analyse simulated data containing random error, and the most reliable method is identified.

The binding of a ligand to a macromolecule can, in the simplest case, be described by two parameters, namely the dissociation constant of the complex (K) and the number of binding sites on the macromolecule for the ligand (n). The equation relating the concentration of ligand bound (b) by a given concentration of macromolecule (m) to the concentration of free (i.e. unbound) ligand (f) is then:

$$\frac{b}{m}=\frac{n\cdot f}{K+f} \qquad (1)$$

The parameters K and n can be estimated by using equilibrium dialysis. Usually (e.g. Strange *et al.*, 1976) a tracer amount of labelled ligand of known radioactivity is added to the dialysis apparatus together with a known amount of unlabelled ligand (t) and of macromolecule. At equilibrium the concentration of free ligand can be estimated from the radioactivity found in the compartment without the macromolecule, and the concentration of (bound+free) ligand from the radioactivity in the compartment containing the macromolecule. For simplicity, we will assume that the two compartments are of equal size and have a combined volume of v. The concentration of bound ligand can then be deduced by subtracting the concentration of free ligand either from the estimated concentration of (bound+free) ligand or from the average final concentration of the amount of ligand known to have been added; in the latter case the estimate of (bound+free) ligand is not used. Finally eqn. (1) is fitted to these data to give K and n.

There are, however, several different ways of fitting this equation and, if the data contain error, they will give different estimates of K and n. The best method to use in a particular situation may well depend on the sort of error encountered and on what exactly is meant by 'best' (e.g. most accurate, most precise).

Endrenyi & Kwong (1972) have compared nine possible methods, assuming that the estimate of the concentration of free ligand contained random error, and taking the concentration of bound ligand as the difference between the concentrations of total and free ligand. They preferred two of these methods, whether the error was of constant absolute or of constant relative magnitude. One is the linear regression of f on f/b, whereas the other depends on rewriting eqn. (1) in terms of f and t:

$$f^2-\left(\frac{t}{v}-K-\frac{m\cdot n}{2}\right)f-\frac{K\cdot t}{v}=0 \qquad (2)$$

and then fitting eqn. (2) to the values of f and t by a non-linear least-squares regression.

Endrenyi & Kwong's (1972) analysis is incomplete in at least two respects. They did not consider what happens if the amount of radioactive ligand added is also subject to random experimental error, as we have found it to be. Nor did they take into account the effect of systematic error, which would arise if, say, ligand were adsorbed on the surface of the dialysis apparatus; for instance, we noticed that up to 20% of added lithocholate seemed to be lost in this way (Strange *et al.*, 1977). We have therefore extended their analysis by examining the implications of these two possibilities.

Theory, Methods and Results

Experimental designs

We have studied three experimental designs (A, B and C) in which b and f can be determined. In all of them the total amounts of macromolecule and ligand

added are known, and in A and B (but not C) either the specific radioactivity of the added ligand is also known, or the amount of radioactivity added is estimated in a separate experiment. Before describing the designs in detail, we will define the symbols to be used: K = the dissociation constant of complex (e.g. mol/l); n = the maximum number of mol of ligand bound per mol of macromolecule; m = the concentration of macromolecule (e.g. mol/l); b = the concentration of (bound+free) ligand in the half-cell containing the macromolecule (e.g. mol/l); b' = the concentration of (bound+free) radioactive ligand in that half-cell (e.g. d.p.s./l); f = the concentration of free ligand (e.g. mol/l); f' = the concentration of free radioactive ligand (e.g. d.p.s./l); t = the total amount of ligand added (e.g. mol); t' = the total amount of radioactive ligand added (e.g. d.p.s.); s = the specific radioactivity of the added ligand (e.g. d.p.s./mol); $s = t'/t$; v = the combined volume of the two half-cells (e.g. litres).

The three experimental designs may now be described in detail, as follows.

Design A. The radioactivity of free ligand is counted, and f is calculated from it and either s or t'/t (e.g. $f = f'/s$). The radioactivity of the (free+bound) ligand is counted, and b is calculated from it, f' and s or t'/t [e.g. $b = (b'-f')/s$].

Design B. The quantity f is found as in design A. The radioactivity of (free+bound) ligand is not counted, and b is calculated from f and t, e.g.

$$b = 2\left(\frac{t}{v} - f\right)$$

Design C. The quantities f' and b' are counted as in design A, but the specific radioactivity of the added ligand is estimated as

$$\frac{v\left(f' + \frac{b'}{2}\right)}{t}$$

Then f is calculated from it and f', and b from it, b' and f'.

In practice the variables m, b and f may be subject to both random and systematic errors. The most likely systematic errors are caused by the loss (by adsorption on the surfaces of the dialysis chambers) of free ligand, complex and/or free macromolecule, and they will influence experimental designs A, B and C to different extents. Therefore we shall first describe the effects of these systematic errors on the three experimental designs and identify the least sensitive one. Then we shall evaluate the various mathematical methods for analysing the data obtained in this preferred experimental design, assuming only random experimental error to be present.

Effect of systematic error

The systematic errors which will be considered are the loss of: (*a*) free ligand; (*b*) free macromolecule; (*c*) complex.

Design A. (*a*) Even though free ligand were lost, the values of f and b (calculated from f', b' and s or t'/t) would still be correct. Consequently the estimates of K and n would be unaffected.

(*b*), (*c*) If a fraction α of the macromolecule or complex were lost the estimate of n would be $(1-\alpha)$ of the true value, whereas K would be unaffected.

Design B. (*a*) The effect of losing a fraction α of the total ligand can be deduced as follows. Eqn. (1) can be re-arranged to:

$$f = (m \cdot n)(f/b) - K$$

i.e. the relationship between f and f/b is that of a straight line of slope $m \cdot n$ and intercept $-K$ (this is the plot favoured by Endrenyi & Kwong, 1972).

Now, when a fraction α of the total ligand is lost, eqn. (2) becomes:

$$f^2 - \left\{(1-\alpha)\frac{t}{v} - K - \frac{m \cdot n}{2}\right\} f - K(1-\alpha)\frac{t}{v} = 0 \quad (3)$$

In this instance b is erroneously substituted for $2(t/v - f)$, and eqn. (3) rearranges to:

$$(1-\alpha)f - (2\alpha \cdot K + m \cdot n)(f/b) - 2\alpha(f)(f/b) + (1-\alpha)K = 0$$

This is the equation of a hyperbola, not a straight line (Bowley, 1913). It can be shown that the hyperbola has an intercept of $-K$ and a slope which is greater than $m \cdot n$ (so long as $f/b < (1-\alpha)/2\alpha$). Consequently, if a straight line were fitted to the data, both K and n would be overestimated. Alternatively, the curvature of the plot might be misinterpreted, for instance, in terms of heterogeneous binding sites. If an amount, α, of the ligand were lost, it can be shown in a similar manner that the plot of f against f/b would again be curvilinear, with an intercept of $-K$ and a slope greater than $m \cdot n$.

(*b*) The loss of a fraction α of the macromolecule would not alter K, but the estimate of n would be $(1-\alpha)$ of its true value.

(*c*) The loss of complex would lead to a curvilinear plot of f against f/b [as in (*a*)], together with an error in the estimate of the amount of macromolecule present and hence further error in n.

Design C. (*a*) If a fraction α of the ligand were lost, its specific radioactivity would be underestimated by α, and consequently both f and b would be overestimated by α. So long as α were independent of concentration, the plot of f against f/b would be linear, but the values of n and K derived from it would be overestimated by α. However, if α varied with concentration (for instance, in an inverse manner) the plot would be curvilinear.

(*b*) As for experimental designs A and B, losing a fraction α of the macromolecule would not alter K but would make n be underestimated by α.

(*c*) The loss of complex would lead to the effects described in both (*a*) and (*b*).

These considerations therefore show that, if losses of ligand or of macromolecule cannot be discounted, experimental design A is the one to use. Since Endrenyi & Kwong (1972) have only discussed the effects of random errors on the estimates of K and n derived by using design B, we will now examine their effects on design A.

Effect of random error

In experimental design A, the radioactivity of free ligand is counted, and its concentration is calculated either from the known specific radioactivity of the added ligand (s) or from t'/t, which is an estimate of it. The radioactivity of the (free+bound) ligand is also counted, and b is calculated from b', f' and s (or t'/t), i.e.

$$f = f'/s \quad (4)$$

and

$$b = (b' - f')/s \quad (5)$$

The effect of random experimental error on the results given by the various methods for fitting eqn. (1) to these data can be assessed by analysing simulated experiments in the way described by Atkins & Nimmo (1975) and Nimmo & Atkins (1976). In this instance the simulated data were chosen to be similar to those actually observed by Strange *et al.* (1976, 1977). Consequently the set of 'perfect' (i.e. error-free) data was simulated by setting $K = 100$, $n = 1$, $m = 30$ and $f' = 21000$, and then using eqns. (1), (4) and (5) to calculate b' at a series of ten values of s. These values of s ranged from 1400 to 52.5, and were such that the corresponding values of f (which ranged from 15 to 400) were roughly geometrically spaced, and those of b' varied between approximately 26500 and 22300.

Two types of 'real' data were simulated, the first containing independent random errors in each of the experimentally determined variables m, f' and b' but not s, and the second in all four of these variables. They were formed from the perfect set by using a series of normally distributed pseudo-random numbers of known mean and S.D., generated by the Edinburgh Regional Computing Centre program Random. The errors in f', b', s and m were set respectively to 1%, 1%, 0 or 1%, and 5%, these being roughly equal to those observed in practice. The ten pairs of values of b/m and f were then calculated from f', b', s and m by using eqns. (4) and (5). Fifty different sets of real data of each type were constructed in this way.

Eqn. (1) was fitted to the data (i.e. the values of b/m and f) for each simulated experiment by using the unweighted version of each of the ten methods tested. These were the methods of (1) Cohen (1968), (2) Eisenthal & Cornish-Bowden (1974), (3) Merino (1974) and (4) Wilkinson (1961), and the linear regressions of (5) m/b on $1/f$, (6) $1/f$ on m/b, (7) $f \cdot m/b$ on f, (8) f on $f \cdot m/b$, (9) b/m on $b/f \cdot m$, and (10) $b/f \cdot m$ on b/m. The arithmetic means of the values given by each pair of linear regressions were also calculated, so that in effect a total of thirteen methods were compared [(11): mean of (5), (6); (12): mean of (7), (8); (13): mean of (9), (10)]. The programs were run on an ICL 4-75 computer at the Edinburgh Regional Computing Centre.

The results are shown in Table 1. It should be borne in mind that a good method ought to give

Table 1. *Estimates of K and n determined by* 13 *methods*

Values are: means ± S.D. (numbers of data sets successfully analysed), median. The perfect values are $K = 100$, $n = 1.00$. A data set was considered to have been successfully analysed if $0 > K > 200$ and $0 > n > 5$.

	Error in m, f' and b' only		Error in m, f', b' and s	
Method	K	n	K	n
1. Cohen (1968)	90±31 (49), 87	0.96±0.26 (50), 0.94	90±31 (49), 87	0.97±0.26 (50), 0.93
2. Eisenthal & Cornish-Bowden (1974)	68±30 (49), 66	0.83±0.27 (50), 0.77	68±29 (49), 65	0.83±0.27 (50), 0.77
3. Merino (1974)	74±41 (36), 74	0.90±0.42 (42), 0.83	83±50 (36), 72	0.90±0.44 (45), 0.82
4. Wilkinson (1961)	77±33 (28), 74	1.21±0.61 (39), 0.99	77±33 (28), 73	1.17±0.62 (40), 0.99
5. m/b versus $1/f$	83±28 (49), 79	0.86±0.20 (49), 0.83	83±28 (49), 79	0.87±0.20 (49), 0.83
6. $1/f$ versus m/b	106±29 (48), 103	1.03±0.23 (49), 1.01	106±30 (48), 103	1.03±0.23 (49), 1.02
7. $f \cdot m/b$ versus f	84±54 (41), 75	0.86±0.39 (49), 0.82	84±54 (41), 77	0.86±0.39 (49), 0.83
8. f versus $f \cdot m/b$	59±39 (31), 51	0.65±0.32 (49), 0.55	54±39 (31), 50	0.65±0.33 (49), 0.55
9. b/m versus $b/f \cdot m$	63±24 (48), 63	0.81±0.23 (50), 0.79	63±24 (48), 63	0.81±0.23 (50), 0.80
10. $b/f \cdot m$ versus b/m	137±34 (36), 144	1.43±0.47 (49), 1.32	137±33 (36), 143	1.43±0.47 (49), 1.32
11. mean of (5), (6)	96±30 (49), 90	0.95±0.20 (49), 0.92	96±30 (49), 91	0.95±0.21 (49), 0.92
12. mean of (7), (8)	65±48 (41), 62	0.76±0.35 (49), 0.68	65±48 (41), 62	0.76±0.35 (49), 0.68
13. mean of (9), (10)	114±35 (46), 109	1.12±0.29 (49), 1.09	114±35 (46), 110	1.13±0.29 (49), 1.08

parameter estimates which: (1) are close to the perfect values of $K = 100$ and $n = 1.00$; (2) have a small S.D.; (3) are symmetrically distributed (i.e. the mean should equal the median); (4) contain few outliers (i.e. the number of data sets successfully fitted should be 50). Exactly which of these attributes is the most desirable will depend on the reasons for estimating the parameters in the first place. Nevertheless, Table 1 shows that on all counts method (6) and to a lesser extent method (11) performed well, whereas the other 11 methods tested were unsatisfactory in one way or another. Also, the parameter estimates changed very little when random error was introduced into s, the specific radioactivity of the ligand. Consequently precise determination of this quantity is relatively unimportant.

Discussion

In the first section of the present paper we showed how systematic errors (the loss of ligand, complex or macromolecule) influence three of the experimental designs by which equilibrium dialysis may be used to quantify the interaction between ligand and macromolecule. The most satisfactory design is the one which the radioactivity in both compartments of the dialysis chamber and that of the added ligand are all counted (design A), because the parameter estimates are not affected by loss of ligand. On the other hand, loss of ligand alters the estimates given by designs C and especially B, the design analysed by Endrenyi & Kwong (1972). Further, it could also turn plots that should be linear into curves, with the result that the data would be misinterpreted qualitatively as well as quantitatively. Binding experiments in which complex is separated from free ligand by precipitation or filtration may be misinterpreted in a similar manner (Swillens & Dumont, 1975).

We then compared 13 methods for analysing the data given by design A, and concluded that the regression of $1/f$ on m/b was the best because it gave estimates that were accurate, precise and symmetrically distributed. It is noteworthy that this regression is equivalent to the inverse of the notorious double-reciprocal plot of enzyme kinetics [B. Woolf, unpublished work, quoted by Haldane & Stern (1932); Lineweaver & Burk, 1934], and that the methods of Eisenthal & Cornish-Bowden (1974) and Wilkinson (1961) fared badly, even though they are the best ones to use for fitting the Michaelis–Menton equation (Atkins & Nimmo, 1975). Presumably the discrepancy arises because in enzyme kinetics one of the variables (substrate concentration) is genuinely 'independent' in the statistical sense (i.e. fixed by the observer and effectively free from error), whereas in equilibrium dialysis both of the derived variables f and b are subject to experimental errors whose magnitudes vary in different ways with ligand concentration and which are, moreover, correlated with one another. For example, at the lowest concentration of ligand that we considered, an error in f' of 1% led to errors of 1% and −3.8% in f and b respectively, and an error of 1% in b' gave rise to an error of 4.8% in b. At the highest concentration of ligand the comparable values are 1%, −16.7% and 17.7% respectively.

The relatively complex nature of the relationship between errors in the derived variables f and b and the experimentally determined ones f' and b' raises the whole question of whether or not our conclusions have general validity. It is clear that the qualitative effects of systematic error are independent of the precise values of the binding parameters, and consequently so too is our conclusion that design A is the best. On the other hand, the best way of analysing the data presumably depends to some extent on the relationship between the errors in f and b, and hence on the magnitudes of the binding parameters, the concentrations of ligand and macromolecule used, and the nature of the random error encountered. Even so, we cannot explain why the regression of $1/f$ on m/b worked so well in our situation. Consequently we suggest that, if reasonable parameter estimates are required, the most reliable analytical method should first be identified empirically in the sort of way we have described.

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