The Calculation of Errors and the Choice of Sampling Times in the Determination of Irreversible Disposal Rates *in vivo* by Isotope Experiments

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In well-known methods of estimating rates of irreversible disposal (utilization) *in vivo* the rates are calculated from the areas to infinity under specific radioactivity–time (S – t) or quantity-of-label–time (q – t) curves obtained by measurements on samples of plasma after intravenous injection of labelled substrate. The errors in the calculated rates are mostly those of the estimates of the areas. These errors are of two kinds: random, caused by the variances of the values of S or q , and systematic, caused by differences between the curves used to interpolate between these values and the true curves. A rigorous method is given for calculating the random errors from the variances of the values of S or q , and is applied to choosing the best times to sample the plasma from small animals from which few plasma samples can be taken. A procedure for estimating systematic errors is also given. Programs in BASIC language to carry out the calculations are deposited as Supplementary Publication SUP 50058 (5 pages) at the British Library (Lending Division), Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms given in *Biochem. J.* (1975) 145, 5.

Rates of irreversible disposal (utilization) of plasma-borne substrates can be determined by injecting isotopically labelled substrates intravenously and following their disappearance from the plasma. The rates are simple functions of the areas to infinity under the specific radioactivity–time (S – t) curves or quantity of plasma label–time (q – t) curves (Gurpide *et al.*, 1963; Mann & Gurpide, 1966; Shipley & Clark, 1972). The method is often applicable even when the metabolic state is not steady, as is usually the case in conscious animals (Heath & Barton, 1973).

The errors in the estimates of the rates are almost entirely those of the areas under the curves, which in turn depend on the variances of the values of S or q and the method of interpolation between these values. Interpolation produces systematic errors, because the curves used to link neighbouring points differ from the true curves. These errors can be made small (see below), and in comparable experiments tend to be similar in sign and magnitude, so that in comparisons of rates obtained in animals in different metabolic states, e.g. fed and starved, random errors are much more important. These are solely dependent on the variances of S or q .

Heath & Barton (1973) proposed an approximate method for calculating the random errors from these variances. Their method can, however, sometimes be seriously misleading, and the purpose of the present paper is first to present a more rigorous, Gaussian method, the principles of which are well expounded by, among others, Deming (1964). Secondly, a

consideration of the propagation of errors from data points to the calculated area leads to suggestions on experimental design and the choice of sampling times.

The procedure is the same for S – t and q – t curves, and only the latter are used in examples.

Basic Procedure

The procedure to be described is applicable whenever the area (A) under the curve can be expressed explicitly in terms of the values of the points defining it, in this case the experimental values of q at the known sampling times. A suitable method for calculating the areas is considered below. It will be assumed that there are no errors in the n sampling times, $t(1)$ to $t(n)$, but that the corresponding values of q , $q(1)$ to $q(n)$, come from populations with standard errors $\sigma(1)$ to $\sigma(n)$. Denoting the standard error of A by σ_A , and noting that the errors in values of q at different sampling times are independent of each other, standard theory (Deming, 1964) gives:

$$\sigma_A^2 = \sum_{i=1}^n \left[\frac{\partial A}{\partial q(i)} \cdot \sigma(i) \right]^2 \quad (1)$$

In practice, values of q fall during an experiment, so that $q(n)$ is a small fraction of $q(1)$. Consequently the coefficients of error of the values of q , e.g. $c(i)$ of $q(i)$, are more constant than the standard errors, and it is convenient to work in them. Then, as $\sigma(i) = q(i) \cdot c(i)$

by definition, the coefficient of error (c_A) of the area is given by:

$$c_A^2 = \frac{\sigma_A^2}{A^2} = \sum_1^n \left[\frac{q(i)}{A} \cdot \frac{\partial A}{\partial q(i)} \cdot c(i) \right]^2 = \sum_1^n \left[\frac{\partial \ln A}{\partial \ln q(i)} \cdot c(i) \right]^2 \quad (2)$$

Values of $\partial \ln A / \partial \ln q(i)$ are readily calculated numerically on rewriting it in terms of small finite differences, i.e.

$$\frac{\partial \ln A}{\partial \ln q(i)} \approx \frac{q(i) \cdot \delta A(i)}{\bar{A} \cdot \delta q(i)} \quad (3)$$

where $\bar{A}$ is the mean value of A , and is calculated from the mean values of q . The mean value of $q(i)$ is then multiplied by $1 + \Delta$, where Δ is a small fraction, and A is recalculated to give $\bar{A} + \delta A(i)$. Then, as $\Delta = \delta q(i)/q(i)$, substitution in eqn. (3) gives:

$$\frac{\partial \ln A}{\partial \ln q(i)} \approx \frac{\delta A(i)}{\bar{A} \cdot \Delta} \quad (4)$$

The remaining $n-1$ terms are then calculated similarly, multiplying each time only the relevant value of q by $1 + \Delta$. The calculation is completed by inserting the resulting values and the experimental estimates of $c(i)$ into eqn. (2).

The method is equally applicable to any of the curves met in isotopic work, and, in fact, to any curve whose area can be expressed as an explicit function of the values of the points. The applications given below are, however, confined to disappearance curves, for which methods of minimizing systematic errors have been more fully explored.

Calculation of Areas

For the estimation of areas under disappearance curves the interpolation method of Corney & Heath (1970) is nearly always applicable. Exceptions are curves in which the slopes do not become continuously less with time after injection, as for example, some curves from labelled proteins in severely burnt patients (Heath, 1970). In other cases 'true' disappearance curves are very closely approximated by single exponential functions between neighbouring points, so that, in the notation used by Heath & Barton (1973):

$$q(i+1) = q(i) \cdot \exp \{-g(i, i+1)[t(i+1) - t(i)]\} \quad (5)$$

where the exponential coefficient is given by:

$$g(i, i+1) = \{\ln [q(i)/q(i+1)]\} / [t(i+1) - t(i)] \quad (6)$$

The zero-time value of q , $q(0)$, is obtained by back-extrapolation:

$$q(0) = q(1) \cdot \exp [g(1, 2) \cdot t(1)] \quad (7)$$

and the area is calculated by:

$$A = \frac{q(0) - q(2)}{g(1, 2)} + \sum_2^{(n-2)} \frac{q(i) - q(i+1)}{g(i, i+1)} + \frac{q(n-1)}{g(n-1, n)} \quad (8)$$

Random errors are then calculated by using eqns. (2) and (4).

A program in BASIC language has been written for calculating areas by eqns. (5)–(8) and errors by eqns. (2)–(4) (Supplementary Publication SUP 50058).

Choice of Sampling Times

The problems in the choice of sampling times in single-injection experiments are most acute with small animals, because either when a complete q - t curve is obtained from each animal, blood loss must be minimized, or, when only a single sample can be obtained from each animal, it is desirable to minimize the number of animals used. In either case it is best to sample at as few time-points as possible consistent with keeping systematic errors within acceptable limits. The above methods can be used to aid the choice of sampling times in such situations.

It is convenient first to consider the statistical requirements of minimizing random and systematic errors when the choice of times is not restricted and the coefficients of error, values of $c(i)$, are the same at each time. We consider below what modifications are required when these conditions cannot be met.

A possible procedure, based on that described by Corney & Heath (1970) and Heath & Barton (1973), is as follows. We assume that the general shape of the curve to be measured is known roughly from trial experiments or from published data. These data are fitted roughly to a curve of the form:

$$q = \sum_1^m B_i \exp. (-g_i t) \quad (9)$$

involving m terms in suitably chosen constants B_i and g_i . This curve is used to simulate the anticipated experimental results. The area (A_t) under it is known exactly:

$$A_t = \sum_1^m B_i / g_i \quad (10)$$

so that the consequences of a choice of n time-points in an experiment designed to measure the area by the methods given above can be assessed, and the effects of varying the time-points on both systematic errors and on the propagation of random errors can be studied. It is assumed that the optimum choice of time-points in the simulation is close to the optimum choice in the final experiment. In practice, a guess is first made as to the number and position of time-points, and corresponding values of q are calculated (eqn. 9). The systematic error is given by the

discrepancy between the area A calculated by application of eqns. (3)–(8) and that given by eqn. (10) (A_t). The method of calculating areas represents each segment of the semilogarithmic curve, $\log q-t$, between time-points by straight lines. Systematic errors mostly arise where the true $\log q-t$ curve shows obvious curvature, and this is exaggerated by the bi- or tri-exponential functions which are all that can be fitted to the limited number of data points from a trial experiment. Consequently the value of $(A-A_t)/A_t$ tends to over-estimate the systematic error, and is a suitable measure of it for the present purpose.

In addition, assuming each point has an equal coefficient of error, c , the values of c_A^2 and of the individual terms in eqn. (2) may also be calculated, the latter representing the contributions made to the variance in A by errors in the individual values of q . By repeating the calculation with different sampling times one soon finds how they affect the systematic and random errors, and suitable sampling times can be chosen to minimize both.

The minimum number of time-points required is that which decreases the systematic error to an acceptable value. It has been found that systematic errors could be decreased to about 2% by using suitable times with only four points per curve for labelled glucose, albumin and fibrinogen (Corney & Heath, 1970), five for non-esterified fatty acids (Cunningham, 1973) and seven for ketone bodies (R. N. Barton, personal communication).

The minimum value of the random error, c_A , is $c/\sqrt{n}$, and is that when times are chosen to make the error on each point contribute equally to c_A , i.e. all values of $\partial \ln A(i)/\partial \ln q(i)$ are equal. It is, however, found that sampling times can deviate from these optimum values by at least 20% with very little effect on c_A , although the errors on the points then make very unequal contributions.

The choice of sampling times is sometimes restricted, e.g. because the metabolic state under study cannot be maintained to the last optimum time, to avoid interference from tailing (Heath & Barton, 1973), or because the optimum first sampling time may be impracticably short. In such cases times can still be chosen that will minimize c_A subject to such restrictions, although c_A will then, of course, be greater than $c/\sqrt{n}$.

A program to aid these trial computations is given in Supplementary Publication SUP 50058.

Serial-sampling experiments

The above procedure is directly applicable to serial-sampling experiments, i.e. those in which a complete curve is obtained from each animal. For such experiments the precision of the analytical method for determining S or q will have been found before the

experiment is carried out, and $c(i)$ is thus simply the coefficient of error of a single determination using this method, and should be the same at all sampling times. Thus for each animal the values of $\bar{A}$ and c_A will be obtained. For a group of animals the individual values of $\bar{A}$ are measured and the variance is calculated in the usual way. Comparison by the F test between the variance and the values of c_A then shows whether the apparent difference between animals represents biological variation or the random errors in the values of $\bar{A}$. Comparisons between groups of animals are of course carried out by using the group means and variances.

Single-sampling experiments

In single-sampling experiments one mean curve is obtained for a group of animals and $c(i)$ must be obtained by replication, since it mostly reflects biological variation. In practice the coefficient of deviation increases with time after injection, so that $c(i)$ must be found by replication at each time-point. There is no simple recipe in such cases. The basic procedure can, however, be used to choose sampling times that decrease systematic errors to acceptable values, and, as the experiment proceeds and some idea of the values of the coefficients of deviation is obtained, to decide at which time-points further replication will most decrease c_A .

Discussion

The Gaussian method described is easy to use, and has not been applied before to this problem. One method that has been suggested (see, e.g., Hetenyi & Norwich, 1974) involves obtaining a least-mean-squares fit of data to eqn. (9), and calculating the errors in the estimate of the area from the errors in the parameters. This requires much more data, and, as the errors in the parameters are usually large and always highly correlated, involves a much more complex and less-accurate computation. It does not show how each individual value of q contributes to the total error, which is important in choosing sampling times.

Strictly the Gaussian method is only precise when the errors or the partial differentials are small, whereas in practice values of c may exceed 5%. There is no simple rigorous check of eqn. (2) in such a case, but an approximate one was carried out by assigning Δ a value of 0.1 in trial calculations, and by comparing the results with those obtained with a value of 0.0001. It was found that c_A was altered by under 1% of c_A , and the contributions of individual terms by under 10%. The method therefore seems to be applicable to curves with quite large errors, but this may not be

the case when values of q are closely spaced so that successive values are similar.

Heath & Barton (1973) gave an approximate method for estimating both c_A and the contributions made to it by errors in individual values of q when values of $c(i)$ were unequal. As they stated, that method tended to under-estimate c_A . Much more importantly, the present method shows that it greatly under-estimated the error from the last value of q and over-estimated that from the penultimate value, and is consequently a poor guide to sampling times or to the number of replicates required at each time.

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