A Linear Method for Determining Specific Activity of Tracers in Radioimmunoassays

C. S. Chiang

I describe a rapid and objective method for graphically determining specific activity of radioactive tracers by self-displacement. Two sets of results are plotted on the same graph: total/bound counts vs (a) concentration of unlabeled analyte (for the standard curve results) or (b) quantity of radioactivity (for tracer self-displacement results). Because these plots are linear, not only are the difficulties of curve fitting and the need for numerous data points avoided, but also how well the data fit the lines can be assessed by calculating the standard deviation of the slope of the lines. This method minimizes uncertainty between data points, allows easier interpolation, and often yields more precise results than do previously published procedures.

Most ligand binding assays, including radioimmunoassays, require the use of a fixed concentration of tracer (label) to keep assay performance optimal (1, 2). This is especially important in assays where high sensitivity is required and iodinated tracers are used. Because of the short half-life of 125I, new tracers must be prepared at least every two months. Tracer concentration cannot be deduced simply from the amount of radioactivity present; therefore, determination of specific radioactivity is necessary for assuring the quality of the tracer. The most widely used procedure for estimating specific activity of tracers is self-displacement (3, 4).

These procedures are applicable to assays where the binder is relatively homogeneous and binds ligand (unlabeled analyte) and tracer with equal affinity. Although satisfactory for most applications, the published self-displacement procedures (3, 4) involve plots of complex curves to generate results, thus necessitating the use of subjective manual curve-fitting or complex curve-fitting computer programs. Here I describe a graphical method involving only linear plots for determining specific activity of tracers by self-displacement. This method can be easily computerized by using linear regression algorithms only. It is based on the finding that, in ideal binding reactions, the plot of total/bound counts (T/B) vs ligand concentration is linear (5, 6).

Materials and Methods

L-3,3',5'-Triiodothyronine ("reverse" T3, rT3) and [125I]rT3 were obtained from Calbiochem (Behring Diagnostics) and New England Nuclear, respectively. Antibody to rT3 was produced in a rabbit by immunizing the animal with a conjugate of rT3-bovine serum albumin monthly for six months. Barbital, bovine serum albumin, and Polyethylene Glycol 6000 were purchased from Sigma, Miles, and Eastman Kodak, respectively. Antibody, rT3, and [125I]rT3 were incubated in barbital buffer (50 mmol/L, pH 8.6) at 20 °C for 3 h (0.4 mL total volume). Bound and free radioactivity were separated by adding Polyethylene Glycol 6000, centrifuging at 2000 × g for 20 min and aspirating the supernate, which contains the free fraction.

For determining the tracer's specific activity, I used two sets of tubes. The tubes in the first set contained constant quantities of antibody and tracer, with increasing amounts of unlabeled analyte. The tubes in the second set contained the identical quantity of antibody but increasing amounts of tracer; the unlabeled analyte was omitted. I plotted the two sets of results on the same graph: total/bound counts (T/B) vs concentration of unlabeled analyte, and T/B vs quantity of tracer (radioactivity).

Results and Discussion

To best illustrate the utility of this method, I present an
example for determining the specific activity of a radioimmunoassay tracer by this method. Table 1 lists the data for
the first set of tubes (standard curve) and for the second set of tubes (tracer self-displacement). For the first set of data
(standard curve), plot the ratio of total to bound radioactivity (T/B) against the mass of the standards on linear-linear
graph paper, using the top abscissa for "mass per tube" (see Figure 1). For the second set (tracer self-displacement), plot
the ratio of T/B against the total radioactivity, using the same ordinate for T/B but the bottom abscissa for "quantity
of radioactivity."

A linear regression analysis of all the points with T/B = 2
for each set of tubes will generate two regression lines

<table>
<thead>
<tr>
<th>Table 1. Data for Determining the Specific Activity of a Tracer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration, pg/tube</td>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Standard curve data</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>25</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>200</td>
</tr>
</tbody>
</table>

Tracer self-displacement data

18 992 8 210 2.31
35 976 13 111 2.74
81 835 18 231 4.49
184 106 21 520 8.56
348 504 22 189 15.71

For the example shown in Table 1 and Figure 1, the specific activity of rT3 tracer was calculated to be 1257 counts/min
or 1.03 nCi (counting efficiency = 55.5%) per picogram of tracer.

Using the method of Morris (3) to calculate the specific activity of this tracer requires two graph steps. First, the
ratio of bound to free radioactivity (B/F) is plotted vs the mass of the standards (on log scale) on semilog graph paper
for the first set of tubes, and B/F is plotted vs the total radioactivity present per tube (on log scale) on the same
graph paper. Two smooth curves connecting data points for each set of tubes can be constructed by manual or computerized
curve fitting. In step two, corresponding quantities of mass and radioactivity are determined at several B/F values
by interpolation and the corresponding radioactivity vs mass is plotted on linear-linear graph paper. The slope of
this second plot is the specific activity of the tracer. By the method of Morris (3), the specific activity of this rT3 tracer
was calculated to be 1.00 nCi/pg, essentially the same as that calculated above.

The method I describe here is based on the finding by Fernandes et al. (6) that the plot of T/B vs ligand concentration
is linear in a linear coordinate system (6). They showed that the principle of this plot is derived from the mass-action
law and is interconvertible to the logit B/Bo vs log ligand concentration plot, the data-reduction method most widely
used in radioimmunoassays (6). Therefore, this method for specific activity calculation can be successfully utilized in
binding reactions that can be analyzed by the logit-log method.

Both Morris (3) and Gocke et al. (4) used plots of B/F vs log concentration, which are complex curves. Furthermore,
because the horizontal axis is a log rather than a linear coordinate, the difference of concentration between any two
tubes cannot be kept exactly to scale because of the unknown contribution of tracer to total concentration (mass) in
the first set of tubes. That is, \( \log (X + Q_1) - \log (X + Q_2) \neq \log Q_1 - \log Q_2 \), where \( X \) is the concentration of labeled
ligand in the first set of tubes and \( Q \) is the concentration of unlabeled analyte.

Actually, if one adds the labeled ligand concentration to the unlabeled ligand concentration in the first set of tubes, the
slope and shape of the curve will change. Therefore, Morris (3) had to use two graphs to take into account the
concentration of labeled ligand so as to calculate the specific activity correctly.

In the method described here, the plot of T/B vs ligand concentration is linear in a linear coordinate system (the
mathematical derivation is available from the author or the Editorial Office of this journal). Therefore, any amount of
tracer can be accommodated in the first set of tubes while maintaining the differences of concentrations between any
two tubes accurately to scale. In fact, the top horizontal axis of Figure 1 can be labeled as \( X + 50, X + 100, X + 150 \), etc.,
where \( X \) is the concentration of labeled ligand in the first set
of tubes. Because both the plot and the coordinate system are linear, the inclusion of \( X \) does not change the shape or slope of this regression line. In the calculation, the operation \( Q_1 - Q_2 \) cancels out \( X \); i.e., \((X + Q_1) - (X + Q_2) = Q_1 - Q_2\). Therefore, the presence of \( X \) on the label for the horizontal axis can be omitted without affecting the result in any way. This unique feature of the described method makes it possible to use only one graph to calculate the specific activity of tracers.

Another advantage of using linear plots is that, once the two linear regression lines for the two sets of tubes are derived (from data points), the specific activity result will be independent of the arbitrary choices of \( Q_1 \) and \( Q_2 \) (or \( R_1 \) and \( R_2 \)). In other words, the same specific activity will be obtained from any combination of \( Q_1 \) and \( Q_2 \) (or \( R_1 \) and \( R_2 \)). In contrast, if plots composed of complex curves are used, the specific activity result may be influenced by the arbitrary choice of \( Q_1 \) and \( Q_2 \) as well as by the method of curve fitting (e.g., manual vs computerized polynomial).

This method can be easily computerized for computerized analysis of experimental data. Because only linear regression algorithms are needed, the programming is simple and the computer time required is short, even if a slow computer language (e.g., interpreted \texttt{BASIC}) is used. Using the same microcomputer, the same set of data, and two programs written in \texttt{BASIC}, specific activity estimation by this method took considerably less time than by Morris' method involving a curve-fitting routine.

If a computer with appropriate software is not at hand, using this method manually offers several advantages over methods relying on complex curves. Firstly, the two linear regression lines can be determined by arithmetic calculations on a calculator or even by hand: that is, the regression lines are derived objectively. In contrast, manually fitting a curve through a set of data points is always somewhat subjective. Secondly, only one graphical step is needed to generate the final result by this method. Thirdly, results generated by methods relying on complex curves will be subject to more variability because of the many alternative ways of drawing a curve to connect many data points; the method reported here, however, will yield the same result every time because linear regression of a given set of data will always yield the same regression line.

The method described here for calculating specific activity can also be used to calculate the specific activity of enzymatic or fluorescent immunoassay tracers.

In summary, the method described here is objective, rapid, and not prone to variations caused by alternative curves fitted through data points. Regardless of the mode of calculation (manual or computerized), this method minimizes uncertainty between data points and saves time.

References