Automated Computer Analysis for Enzyme-Multiplied Immunological Techniques

David Rodbard1 and S. W. McClean2

The dose/response curve in enzyme-multiplied immunnoassays (EMIT®) and related techniques may be described by the same "four-parameter logistic function" as has proven useful in radioimmunoassays, immunoradiometric assays (IRMA), and two-site immunoradiometric ("sandwich") assays. This function provides an empirical description of the dose/response curve by use of an objective least-squares regression analysis, with the advantages of computerization and automation. This facilitates further statistical analyses, quality control, estimation of precision for an unknown, and improved, objective estimation of the sensitivity or minimal detectable dose.

Several methods have been developed for the computer analysis of radioimmunoassay and immunoradiometric assay dose/response curves (1–3). The enzyme-multiplied immunoassay technique (EMIT),3 presently used for quantitation of phenytoin, phenobarbital, and a variety of other drugs, is based on the same chemical and mathematical principles that underlie radioimmunoassays and immunoradiometric assays, i.e., competition between labeled antigen and unlabeled antigen for a limited number of antibody combining sites. In the case of enzyme-multiplied immunoassays, the label is an enzyme rather than a radioactive isotope (5–9). A detailed mathematical analysis of the kinetics of enzyme-multiplied immunoassays will be given elsewhere (4). Here, we report on a simple, practical method for computerized statistical analysis of dose response curves from EMIT.

The commercial supplier of reagents for EMIT recommends use of a hand-drawn graph of the response variable, called "EMIT Units" vs. the logarithm of the concentration of standard preparation, or else the use of a special nonlinear graph paper, the nature of which is not defined. Thus, many persons are not aware of the similarity (or even identity) of the statistical problems involved in the analysis of EMIT dose/response curves with the dose/response curves of conventional radioimmunoassays.

The Four-Parameter Logistic Model

Dose/response curves that are smooth, symmetrical, and sigmoidal in appearance when plotted vs. logarithm of dose may often be described by the four-parameter logistic function given as follows:

\[ y = \frac{a - d}{1 + (X/c)^b} + d \]  

(1)

Here, \( y \) represents the response variable, \( a \) represents the response variable at zero dose of the standard preparation, \( d \) represents the response corresponding to an infinite dose of the test preparation, \( c \) represents the "ED\(_{50}\)"—i.e., the dose level that results in a predicted response halfway between \( a \) and \( d \)—and finally \( b \) is a factor that describes the steepness of the dose/response curve. Generally, in radioimmunoassays, \( b \) will be close to unity, i.e., +1. This will correspond to a hyperbolic or nearly hyperbolic dose/response curve when response is plotted vs. arithmetic dose, i.e., dose or concentration of standard on a linear scale. The parameter \( b \) corresponds to the slope of a "logit-log" plot (2). It appears that the typical dose/response curves for several EMIT systems will obey this type of dose/response curve (5–9).

We have previously developed (1, 3) computer programs for a nonlinear least-squares regression analysis of data with use of equation 1. This program utilizes a Newton–Gauss iterative routine. It automatically provides initial estimates for parameters \( a, b, c, \) and \( d \). Weighted regression may be used if necessary or desirable (10). The principles underlying this program have been described (1, 3, 10) and the programs are available (11). This program provides a detailed statistical analysis of dose/response curves and provides estimates of potencies for unknowns.

Methods

We used the EMIT (Syva Corp., Palo Alto, Calif. 94304) assays for phenytoin, phenobarbital, and primidone. The methods were adapted to the use of a Perkin-Elmer KA-150, a fully mechanized enzyme rate analyzer. Details are presented elsewhere (12).

To adapt the EMIT reagents (Syva Corp.) to the Perkin-Elmer KA-150, we did not prepare them according to the manufacturer's directions; however, the

---

1 Reproduction Research Branch, National Institute of Child Health and Human Development, NIH, Bethesda, Md. 20024.
2 Clinical Chemistry Service, Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, Md. 20024.
3 EMIT is a registered trademark of Syva Corp.
4 Received July 29, 1976; accepted Sept. 3, 1976.
relative proportions of antibody, substrates, and enzyme-conjugated drug remained the same. The preparation and use of these reagents is described elsewhere (12).

Standards that span the therapeutic range and include the lower toxic concentrations are not sufficient to define the curve above the "ED50" point. Because solubility in serum at very high concentrations can be a problem with phenobarbital, we chose to obtain a measure of the response for infinite dose in a way other than preparation of additional standards. Reagent A contains antibody and substrates and reagent B contains glucose-6-phosphate dehydrogenase (EC 1.1.1.49) conjugated to the drug. The same concentrations of substrates are contained in the phenobarbital, phenytoin, and primidone reagents (R. Bastiani, Syva Corp., personal communication). By using reagent A from the phenobarbital kit with reagent B of the phenytoin kit we measured the maximum activity possible for the glucose-6-phosphate dehydrogenase bound to phenytoin. In the absence of the antibody specific for phenytoin all of the enzyme-drug was free and therefore active. The response for "infinite dose" for phenobarbital was obtained in a similar way.

The KA-150 printed the enzyme activity of each sample on a paper tape. The enzyme activities for the standards and unknowns were then entered at a terminal into the IBM 370 facility at NIH, Division of Computer Research and Technology.

Results

Figure 1A shows a typical dose/response curve for primidone; Figure 1B shows a typical dose/response curve for phenobarbital. The ranges of three replicate observations for each dose are shown by vertical line; the computer-generated fit is shown by a smooth curve, together with its 95% confidence limits (± Student's t times the estimate of the standard deviation of an observation around the curve). Figure 2 shows the same curves on "logit-log" graph paper, when the response has been "normalized" by using the "a" and "d" values determined by the four-parameter logistic model. Figure 3 shows the representative values for a, b, c, d, and the RMS error (square root of the residual variance) for 17 assays for primidone. The parameters are quite stable between assays, although there is a small but significant degree of "heterogeneity" of results from assays performed on different days or on using different batches of reagents. Similar results were obtained for the assays for the other drugs. Charts such as this are useful for quality control purposes (3). The computer program (11) also provides an objective, quantitative estimate of the minimal detectable dose within each assay (not shown).

Discussion

The present results indicate that the "four-parameter logistic function" may be used as an empirical description for enzyme-multiplied immunoassay techniques. Thus, a single program is sufficient to handle

Fig. 1. A. Typical dose/response curve for EMIT assay for primidone
Note the smooth symmetrical sigmoidal curve. The 95% confidence limits for an observation around the line are shown
B. Typical dose/response curve for phenobarbital assay

Fig. 2. A. Same dose/response curve as in Figure 1A, except shown on "logit-log" coordinates with use of the values of a and d obtained by a fit utilizing equation 1
B. Same dose/response curve as shown in Figure 1B, except portrayed on logit-log coordinate systems with 95% confidence limits and values of a, b, c, and d, corresponding to those in Figure 1B
both radioimmunoassays and these other specialized immunological techniques. Indeed, this program may also be used for several other purposes, including several types of bioassays, antibody dilution curves, radial immunodiffusion tests, etc. (1).

In order to use this program, we must provide the computer with sufficient information regarding the level of the maximal or plateau response for “infinite” dose. This information may be obtained in several ways. First, the experimentalist may simply run a few tubes with an extremely high dose level, possibly 10- to 50-fold higher than the highest dose commonly recommended by the commercial supplier of the reagents for this type of assay. Thus, one obtains one or several observations on the upper plateau of the dose/response curve. Alternatively, the response for infinite dose may be estimated by analyzing the contents of some test-tubes in which the addition of antibody has been omitted, but are otherwise treated the same as the other tubes on the dose/response curve. In this way, we obtain a measure of the “maximal” response that would occur if all of the antibody were totally saturated or “neutralized” by means of a massive excess of the ligand. Unfortunately, the commercial packaging of the reagents for this test does not now permit the individual laboratory to analyze a tube by omitting the antiserum. One approach to avoid this problem, is to use the antibody–substrate “cocktails” provided for the phenobarbital test to measure the “maximal” response in the phenytoin assay. Likewise, one may use the antibody–substrate cocktail for the phenytoin assay to obtain a measure of the maximal response in the phenobarbital assay system, because the phenytoin assay system does not (or should not) contain any antibodies to the phenobarbital ligand. In this type of analysis it is implicitly assumed that the substrates used for these two types of tests are identical in nature and concentration; at present this depends on the manufacturer. Accordingly, it would be desirable to induce the manufacturer to provide the substrate reagent without the antibody already present, or else to utilize the first approach outlined above—namely, to run several standards at extremely high concentrations. The ligands (phenobarbital, phenytoin, etc.) are readily available in pure form and are quite inexpensive. However, the solubility of the anticonvulsants is such that a concentration 10- to 50-fold higher than standard the highest customary dosage would be impossible to achieve without altering the pH.

If the values for “a” and “b” are known, then equation 1 may be rearranged in the linearized form, given by equation 2

$$\logit \left( \frac{y - d}{a - d} \right) = \log_a \left( \frac{d - y}{y - a} \right) = \alpha - \beta \log(X)$$  

(2)

where, a and d have the same meaning as in equation 1, $\alpha$ and $\beta$ are the intercept and slope of a linear regression between the logit of the normalized or transformed response variable $y$ and the logarithm (using either natural or common logarithms) of dose. This is commonly referred to as the “logit-log” method (2, 3). Thus, if values of a and d, and accordingly the difference ($d - a$) are known to be very stable from assay to assay, then one may utilize a linearized form of the dose/response curve. Indeed, the special graph paper supplied by Syva is really logit-log graph paper that has been truncated (R. S. Schneider, R. Cook, Syva Corp., personal communication). Thus, the manufacturers have found that the difference ($d - a$) is approximately 550 units in certain assays under certain conditions, and the logit-log graph paper has been scaled accordingly. The dose/response curves shown in Figure 1 are replotted on logit-log coordinates in Figure 2.

By use of the four-parameter logistic approach, or by means of the logit-log approach, one avoids some of the “mysteries” of the EMIT assay, “EMIT Units”; and EMIT graph paper. Indeed, the entire body of available statistical theory that applies to bioassay and radioimmunoassay is directly applicable to EMIT systems with no significant modification. This should provide a rational approach to these assays.

In the assay systems studied here, the value for the exponent b in equation 1 or the slope $\beta$ in equation 2 was very low, about 0.5 to 0.8. Because of this low b value, the assay system could potentially be utilized over nearly four orders of magnitude. However, this wide range of the assay is obtained at the price of precision: the precision of potency estimates for unknowns would be improved if the steepness of the dose/response curve were to increase, if the scatter of the points around the curve were to remain the same. Attempts to optimize the sensitivity of these assays by computer simulation techniques will be reported elsewhere (4).

In a preliminary error analysis to analyze the relationship between $\sigma_y^2$ and $\bar{y}$, i.e., the relationship between the variance of the response and the position on the dose/response curve, we noted that there was remarkably good “uniformity of variance” for EMIT assays.
Accordingly, an unweighted regression may be used when utilizing equation 1. This is unlike the case in most radioimmunoassays (10) and immunoradiometric assays (1). However, owing to the severe nonuniformity of variance introduced by the logit transformation, it is highly desirable to use a weighted regression when utilizing equation 2 (2). This is especially true if one wishes to utilize the computer program to predict the precision for a potency estimate at any point along the dose/response curve. It is possible that nonuniformity of variance may become significant in other EMIT assay systems or in other laboratories under other conditions. In this case, a systematic analysis of $\sigma^2$ vs. $y$ becomes desirable. Although these analyses involve no calculations more advanced than standard deviation, variance, and least-squares regression analysis, they should be computerized and automated, so that they can be used routinely (10).

The method used here has some limitations relative to use of an empirical relationship (e.g., linear) between $y$ and $\log(X)$. The curve-fitting routine may fail to converge. This is likely to occur if one uses too few "points" on the dose/response curve, or if the range of doses is too narrow. The remedy for this is obvious—run more standards over a wider range of dose, including zero and "infinite" dose.

It is likely that the statistical and computer analyses used here will also be applicable to related types of immunoassays utilizing other methods for monitoring the ratio of bound to free antigen or antibody (13), and to techniques such as radial immunodiffusion. Although the computer programs used here (11) were designed for large, high-speed computers, they have already been translated from FORTRAN into BASIC, for use on smaller computers and the larger desk-top calculators.4

---

4 Information on computer programs is available on request to the authors.

We thank Mr. R. Cook and Dr. R. S. Schneider of Syva Laboratories for providing information about the assay system. V. B. Faden provided assistance in computer programming.

References