Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves

De Lean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. Am. J. Physiol. 235(2):E97-E102, 1978 or Am. J. Physiol.: Endocrinol. Metab. Gastrointest. Physiol. 4(2):E97-E102, 1978. - Physiological and pharmacological studies of hormones, drugs, and neurotransmitters often generate families of sigmoidal dose-response curves. Optimally efficient data analysis should involve simultaneous description of all curves, rather than fitting each one individually. We have developed a general computerized method to describe the dose-response curves in terms of basal and maximal responses, &e, and curve shape or steepness. This facile method permits rigorous statistical analysis, provides a basis for pooling of information from separate experiments, and allows one to test which characteristics are shared by various curves.

Dose-response curves from bioassays, radioreceptor assays, radioimmunoassays (RIA), and DNA-RNA hybridization are typically smooth, symmetrical, and sigmoidal (S-shaped) when the dose is portrayed on a logarithmic scale. Usually, these curves may be equally well described by the Gaussian cumulative distribution (probit analysis) or by a logistic model (15, 29). The latter has advantages of mathematical simplicity and has been widely used for bioassay, radioimmunoassay, and related techniques (2, 4, 5, 7, 10, 12-14, 20, 27-29, 31, 33, 35-37). The general form of the logistic function may be expressed as

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

where \( Y \) is the response; \( X \) the arithmetic dose; \( a \), the response when \( X = 0 \); \( d \), the response for "infinite" dose; \( c \) is the ED\textsubscript{50}, i.e., the dose resulting in a response halfway between \( a \) and \( d \); and \( b \) is a "slope factor" that determines the steepness of the curve.\(^2\)

\(^1\) Reversal of the roles of \( a \) and \( d \) reverses the sign of \( b \). The parameters \( a \) and \( d \) may be alternatively defined as the minimum and the maximum of the response range, respectively. With the initial definition (responses at zero and infinite dose, respectively), \( b \) is always positive. In the alternative definition, the sign of \( b \) depends on whether the response is increasing (\( b \) positive) or decreasing (\( b \) negative) with increasing dose \( X \).

\(^2\) The slope factor, \( b \), corresponds to the slope of the logit-log plot, when \( X \) is portrayed in terms of natural logarithms

This equation has been used as the basis for analysis of dose-response curves, individually. When two or more dose-response curves have been constructed, the usual practice has been to characterize each one separately and then to compare the slopes and potencies, e.g., in terms of the ratios of the ED\textsubscript{50}'s. However, this

\[ dY/d \ln(X) = (d - a)b/4 \quad \text{when } X = c \]

or

\[ d \logit{(Y - d)/(a - d)}/d \ln(X) = \pm b \]

The logistic equation is mathematically analogous to the Hill equation used for ligand binding and enzyme kinetics. The parameter \( b \) has the same mathematical form as the Hill coefficient, \( n_H \). However, \( b \) cannot be interpreted in the same thermodynamic terms as \( n_H \) except under very special circumstances. In the proper application of the Hill equation, \( X \) refers to free ligand concentration, whereas in most applications of the logistic equation (including those in this report), \( X \) indicates total ligand concentration (29). The finding of a \( b \) value greater than unity may indicate positive cooperativity or a host of other interpretations. For instance, the presence of a significant threshold and spare receptors will elevate the \( b \) value (24). Whether true cooperativity is involved is a moot point. In another commonly occurring case, heterogeneity of binding sites is reflected in an \( n_H \) of less than unity. Usually (though not always), the \( b \) value measured when \( Y = (a + d)/2 \) will also be less than unity. However, extensive simulation studies have indicated that the \( b \) value may be far removed from the true \( n_H \) (unpublished observations). Hence, slopes of logit-log plots of RIA's and RRA's should not be referred to, nor interpreted as Hill coefficients. Instead, these should be simply referred to as \( b \) values, or logit-log slopes.
modified by Marquardt and Levenberg (18, 22). Selection of shared parameters is made interactively and the general curve fitting model is automatically modified to accommodate the constraints. In cases in which there is nonuniformity of variance, the program permits use of weighting, by use of either a linear, parabolic, or power–function relationship between the variance ($\sigma^2$) of $Y$ and the $Y$ level.$^{5,6}$ Goodness of fit is evaluated on the basis of the residual variance, by use of the "extra sum of squares principle," which is only approximate when applied to nonlinear models (11). Any constraint (parameter sharing) will increase the sum of squares of the residuals but will also decrease the effective number of parameters estimated. If the gain in the number of degrees of freedom (number of data points minus number of estimated parameters) counterbalances the gain in the sum of squares of residuals, the $F$ test will be small (around 1), indicating the appropriateness of the constraints used. Randomness of the residuals (deviations of observed from predicted responses) is tested by evaluation of the number of "runs" of positive or negative residuals (1, 11). The data points are expected to be randomly distributed above and below the fitted curve if the model is appropriate. Significant nonrandomness of the signs of the residuals indicates an inappropriate fit.

EXAMPLES

We shall illustrate the utility and versatility of this approach to data analysis, by means of four examples: 1) RIA estimation of relative potency, in this case of an iodinated antigen, to obtain a measure of specific activity; 2) comparison of agonists and antagonists in a neurotransmitter radio-receptor assay system; 3) in vitro bioassay of human chorionic gonadotrophin (hCG) and several of its deglycosylated derivatives; 4) DNA-RNA hybridization analysis.

1) Radioimmunoassay potency estimation. A simple

The weighting coefficients ($a_w$, $a_Y$) are estimated in a preliminary analysis of replicates, as described (30). Commonly, the "within-dose" variance is significantly smaller than the "between-dose" variance around the regression. Accordingly, in general we advise use of the means of replicates as input for the regression program, provided sufficient data are available to ensure convergence. Otherwise, a biased underestimate of the residual variance may be obtained and statistics for selecting among competing models may be biased. When within-dose and between-dose variances are comparable, then each observation should be entered.
example of simultaneous fitting of two sigmoidal curves is shown in Fig. 1, A and B. The potency estimate of labeled luteinizing hormone releasing hormone (\(^{125}\)I-labeled LHRH) relative to native LHRH was measured by RIA. The dose–response (bound/total ratio for labeled hormone or B/T) curve for the labeled hormone tested is compared to the standard RIA dose–response curve. In this application, the relative potency is identical with the specific activity in terms of radioactive counts per picogram of LHRH. Unconstrained curve fitting for the labeled hormone or B/T) curve for the labeled hormone tested is compared to the standard RIA dose–response curve. In this application, the relative potency is identical with the specific activity in terms of radioactive counts per picogram of LHRH. Unconstrained curve fitting for the labeled hormone used in an RIA assay.

The parameters shared between the two curves are indicated in the first column. The \(F\) test for the effect of the constraints on the residuals is indicated in column two. The number of sign runs of the residuals for each curve is indicated in columns three and four. The total number of observations is 7 and 6 for native LHRH (curve 1) and labeled hormone (curve 2), respectively. For each curve, the expected range of residuals is indicated in parentheses. Fig. 1, A and B correspond to lines 1 and 4 of this table. Based on these statistical tests, we infer that both curves share common values for \(b\) and \(d\), and \(a = 0\).

In contrast, each of the four curves for agonists (Fig. 2B) has a slope factor \((b)\) significantly lower than unity: 0.68 ± 0.06, 0.45 ± 0.04, 0.40 ± 0.03, and 0.40 ± 0.03. We infer that the curves for the agonists are not parallel because the additional constraint of parallelism results in a deterioration of goodness of fit, with a significant increase in the average scatter around the curves (increased \(F\) test value) and a significant nonrandomness of the residual signs (Table 3). This lack of parallelism for the agonists is mainly due to the first curve (morphine), which is steeper than the other three curves.
TABLE 2. Tests for goodness of fit for various models for Fig. 2A

<table>
<thead>
<tr>
<th>Parameters Shared</th>
<th>F Test</th>
<th>Run Tests for Curves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(1, 4, 10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2, 3, 9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3, 2, 6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4, 2, 6)</td>
</tr>
<tr>
<td>a, d</td>
<td>1.00</td>
<td>7</td>
</tr>
<tr>
<td>a, b, d</td>
<td>1.57</td>
<td>7</td>
</tr>
<tr>
<td>a, d, and b = 1</td>
<td>1.39</td>
<td>7</td>
</tr>
</tbody>
</table>

Simultaneous unconstrained curve fitting did not converge; therefore the least constrained case (a and d shared for all curves) was used as a basis for the F tests in column two. The total number of observations were 12, 11, 7, 7 for haloperidol (curve 1), chlorpromazine (curve 2), phentolamine (curve 3), and propanolol (curve 4), respectively. For each curve, the expected range (<i>P > 0.05</i>) of values of the number of sign runs is indicated in parentheses. The curves shown in Fig. 2A correspond to line two of this table. The tests shown were nonsignificant (<i>P > 0.05</i>); therefore, one may infer that all curves have a common a, d, and b, and that b = 1.

TABLE 3. Tests for goodness of fit for various models for Fig. 2B

<table>
<thead>
<tr>
<th>Parameters Shared</th>
<th>F Test</th>
<th>Run Tests for Curves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(3, 9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9, 3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3, 9)</td>
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<td>(9, 3)</td>
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<tr>
<td>None</td>
<td>1.00</td>
<td>5</td>
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<tr>
<td>All a, d</td>
<td>2.19</td>
<td>7</td>
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<tr>
<td>All a, d, and b = b*</td>
<td>1.94</td>
<td>7</td>
</tr>
<tr>
<td>All a, d, and b = b*</td>
<td>2.16</td>
<td>5</td>
</tr>
<tr>
<td>All a, b, d</td>
<td>6.88*</td>
<td>7*</td>
</tr>
</tbody>
</table>

The number of observations was 10 for apomorphine (curve 1), dopamine (curve 2), epinephrine (curve 3), and norepinephrine (curve 4). When all a's, b's, and d's are set equal, the F test is highly significant (<i>P < 0.005</i>), and the run tests (indicated by an asterisk) reach the level of significance (<i>P = 0.05</i>). Thus, we reject the hypothesis that all curves are parallel and share the same limits a and d.

These latter curves may be constrained to be parallel (same b). Consideration of the nonparallelism of displacement curves for agonists and antagonists may lead to new insights into the mechanisms of interaction of these agents with their specific receptor(s) and permit classification of compounds.

3) Biossay of partial agonists. In the bioassay of a family of agonists and partial agonists, the basal response level (a) may remain the same but the maximal response (d) will be smaller for the partial agonists. However, the curves may reveal the same "steepness" (same b). Appropriate evaluation of the potency estimate of the partial agonists can best be obtained by simultaneous constrained curve fitting. Figure 3 shows Leydig cell adenosine 3',5'-cyclic monophosphate accumulation in response to varying doses of hCG and two related partial agonists. The curves have been forced to share a common a and b. The additional constraint of a common c (ED<sub>50</sub>) does not significantly alter the goodness of fit of the curves. Thus, these three curves would be nearly superimposable if their responses were normalized to 100% of their respective maximum.

4) DNA-RNA hybridization. Whereas parameters b and c usually reflect intrinsic properties of the system, parameters a and d often vary between experiments, depending on experimental conditions. Simultaneous curve fitting may be used as an elegant and efficient method for pooling information from several experiments while minimizing problems of between-experiment variability in some parameters. Figure 4 shows DNA-RNA hybridization curves for ovalbumin mRNA purified from chick oviduct and total mRNA from chick oviducts treated with estrogen. Here the RNA samples were repeatedly assayed in different experiments with varying d values (maximum binding capacity). The d...
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FIG. 4. Hybridization to cDNA of purified mRNA for ovalbumin (c), and of total mRNA from chicks treated with estrogens for 4 (A) or 18 days (C). Each mRNA preparation was assayed in 3 different experiments. For curve fitting, parameter $a$ was set at zero and $b$ was common to all curves. Parameter $c$ was shared between individual curves for same mRNA preparation. Parameter $d$ (extrapolated maximum percentage of initial mRNA associated at infinite time and concentration) varies between experiments ($82 \pm 7$, $58 \pm 4$, $56 \pm 4$, $47 \pm 4$) and was pooled only for curves within the same experiment. Resulting curves are shown normalized to their respective $d$. Data of S. E. Harris, ref. 19.

TABLE 4. Analysis of goodness of fit for various models for Fig. 4

<table>
<thead>
<tr>
<th>Parameters Shared</th>
<th>$F$ Test</th>
<th>Confidence Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a = 0$</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>$a = 0$; and $c$ for a given preparation</td>
<td>1.79</td>
<td>$P = 0.25$ (NS)</td>
</tr>
<tr>
<td>$a = 0$; $b$, $d$, and $c$ for a given preparation</td>
<td>8.55*</td>
<td>$P &lt; 0.005$</td>
</tr>
<tr>
<td>$a = 0$; $b$, $c$ for a given preparation, and $d$ within the same experiment</td>
<td>2.18</td>
<td>$P = 0.1$ (NS)</td>
</tr>
</tbody>
</table>

Each mRNA preparation was assayed once in three different experiments for a total of nine curves. Use of constraints was intended for pooling curves from the different experiments. The $F$ tests for some representative cases are shown together with their approximate confidence levels. The sign run tests were not contributory because of the small number of observations (four) per individual curve. The curves shown in Fig. 4 correspond to line four of this table. The asterisk indicates statistical significance.

values were pooled for all curves within the same experiment while constraining the $c$ values ($C_{90(1/2)}$) to be equal for all curves for the same substance. The additional constraint of parallelism (common $b$) did not alter the goodness of fit. Forcing all the $d$ values to be equal in addition to the constraints for $b$ and $c$ resulted in significant degradation of the goodness of fit (Table 4). In Fig. 4, the data from each of the nine original curves are shown normalized to 100% of the $d$ values for their corresponding experiment.

DISCUSSION

The four-parameter logistic equation seems to appropriately describe, within experimental accuracy, most symmetrical sigmoidal dose–response curves. In those cases in which sigmoidal curves are significantly asymmetrical, the logistic model could be extended by incorporating one or two additional asymmetry parameters (26). For a complex titration curve involving multiple classes of binding sites with widely disparate $K_{i}$s, one may use a summation of logistic terms. This has been applied to DNA–RNA hybridization data (23).

The present program may be used to calculate "parallel line potency estimate" for in vivo bioassays, without need for truncation to a central linear segment or use of logit transform of the response (17). Truncation of the response curves results in systematic loss of information by neglecting the end parts of the curves, whereas the popular use of the logit–log linear regression relies on independent estimates of the limiting values $a$ and $d$, which cannot be readjusted during the fitting process. Simultaneous curve fitting with the four-parameter logistic model uses the available experimental data most efficiently and allows for a greater flexibility in adjusting to varying experimental conditions.

Waud (37–39) has pioneered the use of computer analysis of families of dose response curves. He has applied simultaneous curve fitting based on a three-parameter logistic equation for estimating dissociation constants of agonists and antagonists assayed by pharmacological "null" methods. The computer programs that he developed are most appropriate for that specific purpose. The data analysis that we describe, being more general, may not be as efficient in such specialized cases because we do not specify any underlying relationship between the $ED_{50}$'s of the curves as for "null" methods applied to the case of competitive antagonists.

The four-parameter logistic equation often represents a significant improvement over the three-parameter version because the base-line level (or the background or the nonspecific level) is included among the parameters instead of being considered as a perfectly known constant (20, 29). Provision for weighting may be essential when the range of observed responses is quite large, resulting in unavoidable nonuniformity of variance of the response metamer (30). Flexibility in the choice of the shared parameters and multiple statistical tests for goodness of fit constitute the major advances of the program described here.

The use of constrained simultaneous curve fitting for testing the equality of parameters is preferable to testing the identity of parameters estimated from curves fitted individually. The standard errors and confidence limits of parameter estimates in nonlinear regression are only approximate, and any conclusion regarding the equality of corresponding parameters is only approximate. In contrast, simultaneous constrained curve fitting permits testing for equality of parameters by inspecting the consequences of forcing them to be equal.

Most investigators still use simple graphic methods and subjective visual curve fitting. Perhaps this has been justifiable: computerized curve fitting of one curve at a time may fail to converge on correct values or even converge at all. An experienced experimentalist will automatically employ constraints (forcing the curves to assume desired characteristics based on an underlying model or previous results). Now, the present computer program should retain the advantages of the subjective methods, but also provide objective estimates of the reliability of the parameters.

In conclusion, we have described a simple computerized method for efficient data analysis of families of
dose–response curves. The method proved to be extremely versatile and generally applicable to many different types of bioassays and binding assays, and other physiological or pharmacological dose response curves. The program (ALI.FIT) is readily adaptable to larger desk-top computers and is available on request.

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REFERENCES