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COMPARISON OF THE ACCURACY OF THE SCATCHARD, LINEWEAVER–BURK AND DIRECT LINEAR PLOTS FOR THE ANALYSIS OF STEROID–PROTEIN INTERACTIONS

JOHN T. WOOSLEY and THOMAS G. MULDOON
Department of Endocrinology, Medical College of Georgia, Augusta, GA 30902, U.S.A.

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SUMMARY

Determination of equilibrium dissociation constants and concentration of specific high-affinity binding sites for the interaction between a steroid and its protein receptor has been examined as a function of the graphical method of analysis. Using data from 17β-estradiol association with rat uterine cytosol as a basis for the experimental range of standard deviations encountered, a series of pseudorandom data sets were generated by computer. These data were used to examine the efficacy with which graphical presentations by the method of Scatchard, Lineweaver and Burk, or the direct linear plot would allow accurate determinations of the binding parameters. Of the three methods, the direct linear plot was consistently superior.

INTRODUCTION

Investigations into the nature and extent of interaction between 17β-estradiol and intracellular receptor proteins is presently finding wide application in both basic endocrine research and clinical analysis. Such diversity of usage would be best suited by the employment of a single means of data interpretation which combines the simplicity desired for routine determinations and the accuracy required for experimental reproducibility.

In a recent report [1], we have introduced the use of the direct linear plot method of Eisenthal and Cornish-Bowden[2] to analysis of the specific interaction between 17β-estradiol and its cytoplasmic receptor in the rat uterus. The data presented suggested that the direct linear plot was approximately equivalent to the method of Scatchard[3] or Lineweaver and Burk [4] as a means of estimating the concentration of receptor binding sites and the binding affinity of the interaction, and that its primary advantage lay in its relative simplicity. We now present evidence, based on computer-generated data, that, of the three graphical methods mentioned, the direct linear plot allows the most accurate determination of the binding parameters. With the utilization of a simple program for a programmable desk-top calculator, median values for binding parameters can be estimated from a large number of experimental data points.

METHODS

Theoretical considerations

The binding of 17β-estradiol to intracellular estrogen receptors can be described by an arrangement of the Michaelis–Menten equation, as [5]:

$$B = B_{max} \cdot F/(K_D + F). \quad (1)$$

A plot of bound 17β-estradiol (B) against free 17β-estradiol (F) yields a rectangular hyperbola passing through the origin with asymptotes, $B = B_{max}$ (the total number of 17β-estradiol binding sites) and $F = -K_D$ (the equilibrium dissociation constant) for the estrogen–receptor complex.

Since the relationship between the independent variable, F, and the dependent variable, B, is curvilinear, it is customary to estimate B_{max} and K_D from a linear transformation of equation (1). The most commonly used transformations are those of Scatchard [3]:

$$B/F = [B_{max}/K_D] - [(1/K_D) \cdot B], \quad (2)$$

and of Lineweaver and Burk[4]:

$$1/B = 1/B_{max} + K_D/B_{max} \cdot 1/F. \quad (3)$$

Because equations (2) and (3) are algebraically identical to equation (1), it seems reasonable to expect that either could be used to estimate B_{max} and K_D with equal accuracy. This is certainly the case where B and F can be measured without error. Since, however, experimental data do contain errors, the two equations are not statistically interchangeable. Different implicit assumptions are made concerning the nature of the error and the assignment of different weights to different points on the curve [5]. In equation (3), taking the reciprocal of B tends to place undue emphasis upon the smallest values of B, which are precisely the ones most likely to have the greatest percentage error. In equation (2), B appears on both sides of the equation so that a plot of B/F against B will inevitably show some degree of correlation.

The direct linear plot [2] is statistically more acceptable since far less sweeping assumptions are made concerning the nature of the experimental error. The

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equation describing the direct linear plot is a rearrangement of the Scatchard equation:

$$B_{max} = B + B/F \cdot K_D. \quad (4)$$

In the Scatchard plot, data points are plotted in x,y space (i.e., $B,B/F$ space). In the direct linear plot, lines, representing points in x,y space, are plotted in parameter space (i.e., K_D,B_{max} space). If the data are error-free, all lines intersect at the same point in the first quadrant, providing a single estimate for B_{max} and K_D (Fig. 1). If the data contain error, the intersection of each individual line with another line provides a separate estimate for B_{max} and K_D (Fig. 2). In the latter case, the median intersection point provides the best estimate for B_{max} and K_D [2, 6].

Generation and treatment of data

A set of error-free data was generated from the Michaelis–Menten equation by setting $K_D = B_{max} = 1$, using an approach similar to that employed by Atkins and Nimmo[7]. In our studies, rather than choosing values of free steroid (substrate in reference 7) and computing bound steroid (velocity in reference 7), the reverse procedure was adopted.

For $B = 0.2$, 0.35, 0.5, 0.65 and 0.8, corresponding values of F were calculated to yield a "perfect" 5-point saturation binding curve. The above range of bound steroid was chosen since it allows the most accurate estimates of B_{max} and K_D [8]. This range encompasses that region of the hyperbolic curve which yields 76% of total theoretically-obtainable information [9], as compared to the range chosen by Atkins and Nimmo[7], which affords 57% of the information. Deranleau[9] has pointed out that true representation of the data from a given experiment can only be accomplished if these data extend over approximately 75% of the saturation curve.

A program was written in BASIC for a Hewlett–Packard computer (Model HP-2000 Access) which generated 100 pseudorandom data sets (containing error in B and F). The data points generated followed a normal distribution about the mean of the "perfect" data set with a standard deviation of our choosing.

Each pseudorandom data set was fitted to the Scatchard and Lineweaver–Burk equations by simple linear regression, and to the direct linear plot by a computer program, a listing of which is available from the authors. This program is based on the approach taken by Airas[10] for a computer solution to the direct linear plot equation as applied to enzyme kinetics.

For each pair of data points (F_i, B_i) and (F_j, B_j):

$$B_i = (B_{max} \cdot F_i)/(K_D + F_i), \quad (5)$$

and:

$$B_j = (B_{max} \cdot F_j)/(K_D + F_j). \quad (6)$$

Rearranging:

$$B_i = B_{max}/(1 + K_D/F_i), \quad (7)$$

$$B_j = B_{max}/(1 + K_D/F_j). \quad (8)$$

Solving for B_{max} in equation (7):

$$B_{max} = B_i + K_D B_i/F_i, \quad (9)$$

and substituting for B_{max} in equation (8):

$$B_j = (B_i + K_D B_i/F_i)/(1 + K_D/F_j). \quad (10)$$

Solving for K_D:

$$K_D = (B_j - B_i)/[(B_i/F_i) - (B_j/F_j)]. \quad (11)$$

By a similar derivation:

$$B_{max} = (F_i - F_j)/[(F_i/B_i) - (F_j/B_j)]. \quad (12)$$

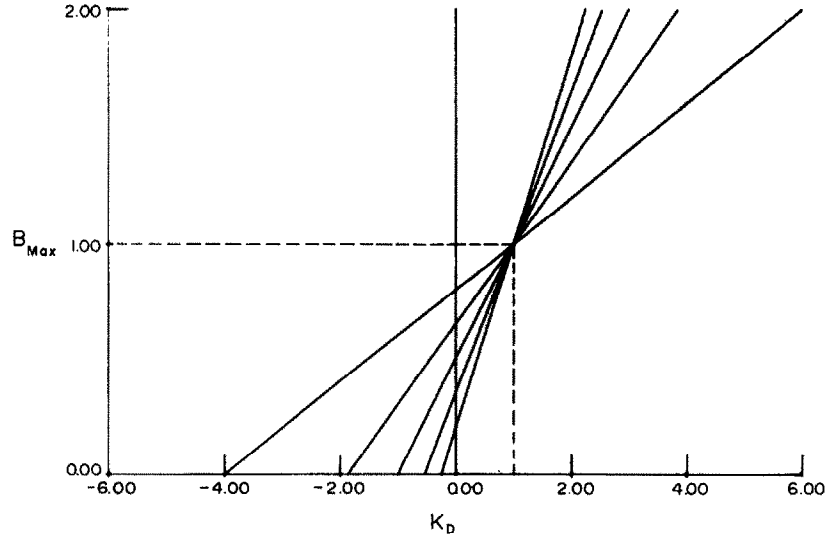

Fig. 1. Direct linear plot of error-free binding data. All lines intersect at a common point under these conditions.

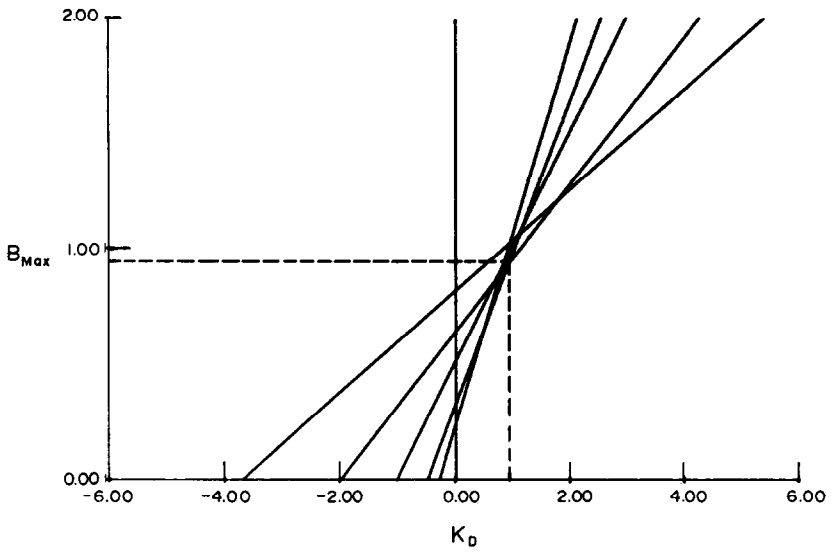

Fig. 2. Direct linear plot of binding data containing error. No single intersection point exists in the first quadrant. The median of the various points of intersection is chosen to yield the best estimate of the binding parameters.

For n data points, there are $\frac{1}{2}n(n-1)$ estimates for K_D and B_{max}. The median (not to be confused with the mean) is chosen as the best value for each parameter [2].

The standard deviations chosen to cluster about the mean of the data set range from 0.03–0.07. This range includes the value of 0.05 which we find to be the average standard deviation for our experimental determination of B and F over the saturation fraction range of 0.2–0.8 (1 = complete saturation of the receptor) (Table 1). As briefly outlined in the footnote to Table 1, these data represented specific receptor binding values collected from assays of rat uterine cytosol receptor concentration as previously described [1].

Table 1. Standard deviations for measurements of bound and free 17β-estradiol concentrations at three values of fractional saturation of uterine receptors

Fractional saturation	Bound steroid ($M \times 10^{11}$)	Free steroid ($M \times 10^{11}$)
0.2	1.443 ± 0.046	1.178 ± 0.046
0.6	4.431 ± 0.048	5.192 ± 0.048
0.8	6.277 ± 0.059	32.674 ± 0.059

Rat uterine cytosol, stored in liquid nitrogen subsequent to the estimation of K_D and B_{max}, was used to determine the standard deviation of bound and free data pairs at the designated values of fractional saturation of receptor, the latter being calculated from the Michaelis–Menten equation. Each value is the mean ± S.D. of 10 measurements of bound and unbound steroid. Duplicate binding experiments were performed in the presence of a 100-fold molar excess of unlabeled 17β-estradiol to correct for nonspecific binding; free hormone was calculated as the difference between total hormone added and that specifically bound. Bound and free steroid were separated by charcoal adsorption methodology.

RESULTS

The means and standard deviations for computer estimates of K_D (Table 2) and B_{max} (Table 3) are shown for the Lineweaver–Burk, Scatchard and direct linear methods of estimation. Standard deviations were computed according to the equation:

$$\text{S.D.} = \sqrt{\left[n\sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i\right)^2\right] \Big/ n(n-1)}. \quad (13)$$

The number of estimates excluded by virtue of falling outside the range $0.05 \leqq K_D$ or $B_{max} \leqq 5$ appear in parentheses following the values of the means.

DISCUSSION

Taking into consideration both the number of times parameter estimate means are judged not different from the theoretical value of one, and the number of cases in which estimates of K_D and B_{max} that are clearly not within the limits of probability are excluded from consideration, analysis of data by the direct linear plot is decidedly superior to analysis by either the method of Scatchard or that of Lineweaver and Burk using simple linear regression. The present analysis differs from that presented by Atkins and Nimmo[7] in two important respects: error has been included in both variables (B and F), rather than in only one (velocity); and our range of values covers a broader region of the hyperbolic saturation curve. These differences have resulted in a significantly different conclusion. Atkins and Nimmo conclude that the direct linear plot is the best method unless error is of constant absolute magnitude; our results indicate that, even when the error is of constant magnitude,

Table 2. Estimates of K_D from computer-generated data according to the Scatchard, Lineweaver–Burk and direct linear plot methods

Standard deviation of generated data	Scatchard	Lineweaver–Burk	Direct linear
0.030	1.085 ± 0.388 (0)†	1.116 ± 0.503 (1)	0.987 ± 0.205 (0)*
0.035	1.079 ± 0.382 (0)	1.055 ± 0.545 (3)*	0.984 ± 0.229 (0)*
0.040	1.139 ± 0.486 (0)	1.133 ± 0.746 (5)*	0.997 ± 0.317 (0)*
0.045	1.142 ± 0.508 (0)	1.190 ± 0.854 (3)	0.954 ± 0.309 (0)*
0.050	1.213 ± 0.675 (0)	1.068 ± 0.767 (10)*	0.919 ± 0.311 (0)
0.055	1.238 ± 0.720 (1)	1.102 ± 0.676 (10)*	0.936 ± 0.332 (0)*
0.060	1.195 ± 0.659 (1)	1.156 ± 0.921 (6)*	0.947 ± 0.364 (0)*
0.065	1.360 ± 0.757 (5)	1.244 ± 0.993 (16)	0.923 ± 0.367 (0)
0.070	1.128 ± 0.694 (5)*	0.912 ± 0.759 (12)*	0.851 ± 0.430 (0)

* Mean K_D is not different from the theoretical value of one at $P < 0.05$.
† Numbers in parentheses are the number of times within the 100 estimates that K_D falls outside the range $0.05 \leqq K_D \leqq 5$. These values are excluded from consideration in the determination of the mean ± S.D. of K_D.

Table 3. Estimates of B_{max} from computer-generated data according to the Scatchard, Lineweaver–Burk and direct linear plot methods

Standard deviation of generated data	Scatchard	Lineweaver–Burk	Direct linear
0.030	1.034 ± 0.141 (0)†	1.048 ± 0.230 (1)	0.997 ± 0.069 (0)*
0.035	1.035 ± 0.143 (0)	1.035 ± 0.302 (2)*	0.997 ± 0.076 (0)*
0.040	1.054 ± 0.187 (0)	1.153 ± 0.628 (1)	0.998 ± 0.105 (0)*
0.045	1.060 ± 0.194 (0)	1.099 ± 0.450 (2)	0.982 ± 0.102 (0)*
0.050	1.092 ± 0.248 (0)	1.188 ± 0.776 (5)	0.978 ± 0.111 (0)*
0.055	1.123 ± 0.332 (0)	1.044 ± 0.485 (8)*	0.977 ± 0.124 (0)*
0.060	1.113 ± 0.310 (0)	1.089 ± 0.497 (5)*	0.982 ± 0.125 (0)*
0.065	1.199 ± 0.407 (3)	1.192 ± 0.616 (12)	0.981 ± 0.123 (0)*
0.070	1.131 ± 0.425 (2)	1.043 ± 0.592 (7)*	0.952 ± 0.157 (0)

* Mean B_{max} is not different from the theoretical value of one at $P < 0.05$.
† Numbers in parentheses are the number of times within the 100 estimates that B_{max} falls outside the range $0.05 \leqq B_{max} \leqq 5$. These values are excluded from consideration in the determination of the mean ± S.D. of B_{max}.

the direct plot still gives the most accurate and least biased estimates of K_D and B_{max}.

Fitting data to the Scatchard or Lineweaver–Burk plots using a weighted linear regression [5] would be expected to produce better parameter estimates than simple linear regression. However, this assumes a greater knowledge of a binding system than most investigations permit. For this reason, simple linear regression has been the method employed almost universally for determining values of steroid–receptor binding parameters, and it is used in this paper as a basis for comparison of the Scatchard and Lineweaver–Burk analyses with the direct linear plot. It should be kept in mind that, when lines are fitted to these plots using simple linear regression analysis, the following assumptions are made: (a) there is no error in the independent variable (or at least no correlation between errors in the dependent and independent variables); (b) uniformity of variance exists for the dependent variable, and; (c) the error in the dependent variable follows a normal distribution. When these assumptions are not justified, as is often the case, inaccurate estimates of K_D and B_{max} are likely to be obtained.

In addition to the increased accuracy of the direct linear plot as described herein, the simplicity of this method of analysis is re-emphasized, in that the plot is very easy to construct, requiring no transformation of data, and the binding parameters can be read directly from the graph. It is cautioned, however, that the direct linear plot is unsuitable for analysis of data which for some reason (e.g., two orders of binding sites, cooperativity, non-achievement of equilibrium) is not linear.

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