STATISTICAL SURVEY OF "SATURATION ANALYSIS" CALIBRATION CURVE DATA FOR PREDNISOLONE, PREDNISONE AND DIGOXIN

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ABSTRACT

An extensive survey of radioimmunoassay calibration data for prednisolone, prednisone and digoxin indicated that the common practice of preparing calibration curves with individual subject's pre-dose plasma or serum, and using this to estimate unknown concentrations for the same subject, is not supported by statistical considerations. Preparation of calibration plots from pooled data is better because this introduces less bias in estimated concentrations. Such a method also saves a great deal of time, since it is not necessary to repeat the calibration procedure each time "unknowns" are being assayed. The data suggest that there is no optimum calibration plot for all radioimmunoassays. Rather, each antibody-drug combination should be investigated thoroughly to determine the best calibration plot for the particular combination. We found that the best calibration plots are: the logistic-logarithmic plot for prednisolone; nonlinear least squares fit to a polyexponential equation for prednisone; and a weighted least squares regression of normalized % bound *versus* concentration for digoxin. The error in the radioimmunoassay is usually concentration-dependent, and, in certain regions of the standard curve, is larger than the literature indicates, since, frequently, the error has been gauged from % bound values, but should be gauged from inversely-estimated concentrations.

INTRODUCTION

Plasma concentrations of corticosteroids or digoxin are frequently measured by radioimmunoassay (1,2) or a modification of the competitive protein binding method of Murphy *et al*. (3). In either situation the investigator requires a calibration curve that is often prepared at the same time that "unknown" plasma samples are analyzed. A calibration curve is prepared by adding known amounts of unlabeled drug to the subjects' own pre-dose plasma. "Unknown" plasma concentrations of drug are then calculated in one of the following ways: (a) from least squares parameters obtained from logistic-logarithmic plots (4); (b) the % bound or counts per minute (cpm) values *versus* concentration data are fitted to a polyexponential equation and "unknown" plasma concentrations are calculated by an iterative process (5); or (c) the data are linearized

by dividing % bound values in the absence of drug, B(0), by the % bound values in the presence of drug, B(X). This ratio is plotted against concentration, and "unknown" concentrations are estimated from least squares parameters obtained from such plots (6). Often, the choice of the method utilized in presenting "saturation analysis" data depends on the investigator who may choose any of the methods discussed without giving any scientific reason why one method was chosen over the others. In the process of performing large numbers of radioimmunoassays for prednisolone (11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione), prednisone (17α,21-dihydroxy-1,4-pregnadiene-3,11,20-trione) and digoxin, it was necessary to find the optimum methods of presenting calibration data. This article summarizes analyses of extensive radioimmunoassay calibration data for these three compounds. The extension of such statistical analyses to data obtained by the competitive protein binding method is obvious. The analyses of variance reported in this paper were performed on a digital computer using the BMD Biomedical Computer Programs described by Dixon (7).

RADIOIMMUNOASSAY PROCEDURES

Plasma concentrations of prednisolone were assayed by a modification of the radioimmunoassay method of Colburn and Buller (1) reported by Sullivan et al. (5). Plasma concentrations of prednisone were measured by the method of Sullivan et al. (8). Plasma digoxin levels were measured by the method of Stoll et al. (2) using commercial antiserum.

RESULTS

Analysis of Radioimmunoassay Data for Prednisolone, Prednisone and Digoxin.

The prednisolone data (Tables 1-3 and 6 and Figures 7 and 8) were based on average duplicate B(0) and B(X) values, corresponding to concentrations of 0.4, 0.8, 2, 4, 6 and 10 ng of prednisolone/ml of plasma, for 12 subjects' pre-dose plasma (after dexamethasone suppression of endogenous cortisol) taken on the same day--a total of 168 individual values and 84 averages. The prednisone data (Tables 1, 3 and 6 and Figures 1-4) were based on average duplicate B(0) and B(X) values, corresponding to concentrations of 0.0505, 0.253, 0.505, 1.01 and 2.01 ng of prednisone/ml of plasma, for 12 subjects' pre-dose plasma (after dexamethasone suppression of endogenous cortisol) taken on four different days

at one week intervals--a total of 576 individual values and 288 averages. The digoxin data (Tables 1, 3 and 5 and Figures 5 and 6) were based on average duplicate B(0) and B(X) values, corresponding to concentrations of 0.08, 0.4, 1.2 and 2.0 ng of digoxin/ml of plasma, for 8 subjects' pre-dose plasma taken twice with an intervening period of two weeks--a total of 160 individual values and 80 averages.

Formerly, the authors had fitted % bound values to a triexponential or biexponential equation, then estimated the concentration of "unknowns" for each subject by an iterative method using an electronic calculator. Hence, the data for each subject were treated separately. Although the mean squares for "Between Subject's Plasma Samples" are significant (see Table 1) analyses of the partitioned corrected sums of squares of the % bound, B(X), values indicated that intersubject variation contributed only 0.7, 2.28 and 0.8% to the total variability for prednisolone, prednisone and digoxin, respectively (Table 1). On the other hand, different concentrations accounted for 98.6, 93.6 and 97.8% of the total variability for prednisolone, prednisone and digoxin, respectively (Table 1). Thus, single or duplicate B(X) values for an individual subject are just one or two members of a distribution of such values corresponding to a given concentration of compound. Preparation of a calibration curve from such data obtained at several different concentrations most probably introduces considerable bias into concentrations estimated for unknowns.

Usually B(X)/B(0) ratios are more homogenous than the B(X) values themselves, where B(X) is the % bound at a known concentration of compound and B(0) is the % bound in the absence of the compound. Table 2 gives $\frac{B(X)}{B(0)} \times 100$ values for prednisolone after averaging duplicate B(X) and B(0) values. Since the success of the radioimmunoassay depends so much on the accuracy with which B(0) values are determined, currently it is the policy of our laboratory to obtain at least four B(0) values for each subject using pre-dose plasma and measuring binding on the same day that the "unknowns" for the same subjects are assayed. The normalization process is then carried out with the average of the four B(0) values. Analyses of variance of B(X)/B(0) values given in Table 3, shows that the sums of squares attributed to "Between Subjects' Plasma

Table 1

Results of Analyses of Variance of B(X) Values for Prednisolone, Prednisone and Digoxin Radioimmunoassays and Percent of Total Sum of Squares Accountable for by Various Variables

Drug	Source of Variation	Degrees of freedom	Sum of Squares	% Total Sum of Squares	Mean Squares	F	Significance Level
Prednisolone	Between Subjects' Plasma Samples	11	124	0.700	11.3	7.08	$p<.0001$
	Concentrations	6	17,624	98.6	2937.	1843.	$p<.0001$
Prednisone	Between Subjects' Plasma Samples	11	3,017	2.28	275	21.3	$p<.0001$
	Concentrations	5	123,374	93.6	24,675	1917.	$p<.0001$
Digoxin	Between Subjects' Plasma Samples	7	166	0.800	23.7	11.3	$p<.0001$
	Concentrations	4	20,262	97.9	5066.	2421.	$p<.0001$

Samples" contribute very little to the total sum of squares; the bulk of the variability is contributed by concentrations. Therefore, the pooling of data for the calibration curves of prednisolone, prednisone and digoxin appears to be justified.

Table 2

Normalized % Bound Values, $\frac{B(X)}{B(0)}$ x 100, Calculated From Average Calibration Data for Prednisolone Radioimmunoassay of Plasma Samples of Prednisolone Study No. 2 Using Ottawa Antiserum

Plasma		Assay	$\frac{B(X)}{B(0)}$ x 100 at Prednisolone Plasma Concentration (ng/ml) of					
Subject	Phase	Operator	0.4	0.8	2	4	6	10
1	1	T	60.2	43.5	22.8	13.1	9.88	5.62
2	1	H	54.5	44.9	24.8	14.6	11.1	8.60
3	1	S	61.0	45.6	28.2	18.1	13.9	9.06
4	1	T	58.1	47.0	24.3	14.2	11.7	7.34
5	1	S	63.1	45.9	26.2	16.3	12.6	8.87
6	1	H	57.3	45.4	24.3	13.9	12.1	8.81
7	1	H	55.0	44.0	23.6	14.7	11.7	8.13
8	1	S	68.4	53.4	30.8	19.3	14.8	10.7
9	1	H	61.0	44.0	26.0	16.8	12.4	8.23
10	1	H	60.2	45.0	25.0	14.7	10.8	8.01
11	1	S	61.8	45.7	26.5	15.8	11.8	7.55
12	1	S	55.2	41.5	23.0	14.5	10.7	7.50
		Ave.	59.7	45.5	25.5	15.5	12.0	8.20
		S.D.	3.97	2.86	2.29	1.83	1.37	1.22
		C.V.(%)	6.65	6.29	9.01	11.8	11.4	14.9

Estimation of Prednisone Concentration from Three Different Types of Calibration Plots

The results of statistical analyses of data obtained by the prednisone radioimmunoassay are shown in Table 4. Three different calibration methods were studied. It is clear that the best method (i.e., the one that is associated with the lowest coefficients of variation in the back-calculated values of prednisone over the entire range of concentrations and the lowest bias) is the nonlinear least squares fit of the calibration data to a triexponential equation (Fig. 1). Although the parabola (Fig. 2) actually gave the lowest coefficients of variation it is much less desirable because of the lack of information at lower concentrations of drug and the higher bias. The coefficients of variation of the estimated concentrations obtained for prednisone by the triexponential equation were 46.0, 15.3, 8.42, 14.2 and 27.6% at 0.0505, 0.253, 0.505, 1.01 and 2.02 ng/ml, respectively.

Table 3

Results of Analyses of Variance of B(X)/B(0) Values for Prednisolone, Prednisone and Digoxin Radioimmunoassays and Percent of Total Sum of Squares Accountable for by Various Variables

Drug	Source of Variation	Degrees of Freedom	Sum of Squares	% Total Sum of Squares	Mean Squares	F	Significance Level
Prednisolone	Between Subjects' Plasma Samples	11	270	1.05	24.5	10.8	$p<.001$
	Concentrations	5	25,430	98.5	5086.	2242.	$p<.0001$
Prednisone	Between Subjects' Plasma Samples	11	341	0.180	31.0	2.44	$.01<p<.025$
	Concentrations	4	188,017	98.4	47,004	3701.	$p<.0001$
Digoxin	Between Subjects' Plasma Samples	7	0.073	0.110	0.01	1.48	NS($.1<p<.25$)
	Concentrations	3	65.50	98.8	21.8	3110.	$p<.0001$

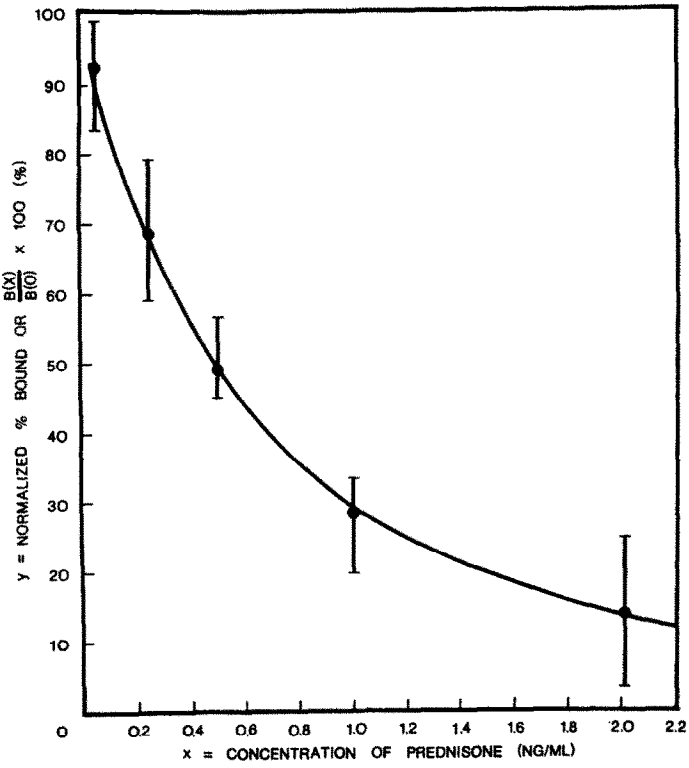

Fig. 1 - Calibration curve for the prednisone radioimmunoassay based on the nonlinear least squares fitting of 240 points to a triexponential equation. Data were obtained from the pre-dose plasmas of 12 subjects for each of four phases of a crossover study. The circles are the mean $\frac{B(X)}{B(0)}$ x 100 values. The bars mark off the ranges of the same values. The equation of the fitted line is: $\hat{y} = 52.627e^{-0.6724x} + 51.669e^{-2.889x} - 3.652e^{-4.668x}$, where $\hat{y}$ is the estimated value of $\frac{B(X)}{B(0)}$ x 100 and x is the concentration of prednisone in ng/ml.

The logistic-logarithmic plot (Fig. 3) gave coefficients of variation (Table 4) that are comparable to those given for the triexponential equation, but in this case the mean estimated concentrations show excessive bias at both the lowest concentrations and the highest concentra-

Averages, Standard Deviations, Coefficients of Variation and Bias of Inversely Estimated Concentrations from Three Different Types of Calibration Plots For Prednisone

Equations for Calibration Plot		For y at Actual Concentration (ng/ml) 0.0515	0.253	0.505	1.01	2.02
$\hat{y} = 52.627e^{-0.6724x} + 51.669e^{-2.889x} - 3.6520e^{-4.668x}$	Mean[a]	0.0526	0.250	0.507	1.07	2.10
	S.D.	0.0242	0.0383	0.0427	0.152	0.580
	C.V.(%)	46.0	15.3	8.42	14.2	27.6
	Bias ng/ml	+0.021	-0.003	+0.002	+0.06	+0.08
	Bias %	+4.16	-1.19	+0.4	+5.94	+3.96
where $\hat{y} = \frac{B(X)}{B(0)} \times 100$						
x = concentration of prednisolone (ng/ml)						

		For y at Actual Concentration (ng/ml) 0.0515	0.253	0.505	1.01	2.02
$y = 1.2366x + 0.9032$	Mean[a]	0.0636	0.257	0.499	1.04	2.40
where	S.D.	0.0262	0.0360	0.0410	0.154	1.24
$\hat{y} = \left[\ln \frac{100 - \frac{B(X)}{B(0)} \times 100}{\frac{B(X)}{B(0)} \times 100}\right]$	C.V.(%)	41.2	14.0	8.21	14.9	51.9
	Bias ng/ml	+0.0121	+0.004	-0.006	+0.03	+0.38
x = ln (concentration of prednisone, ng/ml)	Bias %	+23.5	+1.6	-1.2	+3.0	+18.8

		For y at Actual Concentration (ng/ml) 0.0515	0.253	0.505	1.01	2.02
$\hat{y} = 3.3200 - 0.9275x - 0.1762x^2$	Mean[a]	b	0.2735	0.491	0.988	2.13
	S.D.		0.0317	0.0375	0.141	0.897
	C.V.(%)		11.6	7.64	14.3	42.1
	Bias ng/ml		+0.0205	-0.014	-0.022	+0.11
	Bias %		+8.1	-2.8	-2.2	+5.4
where $y = \ln \frac{B(X)}{B(0)} \times 100$						
x = ln (concentration of prednisone, ng/ml)						

[a] Each mean is based on 48 observations.

[b] Values not calculated since, on inverse estimation, high $\frac{B(X)}{B(0)} \times 100$ values gave negative numbers under the square root sign for the roots of the quadratic equation.

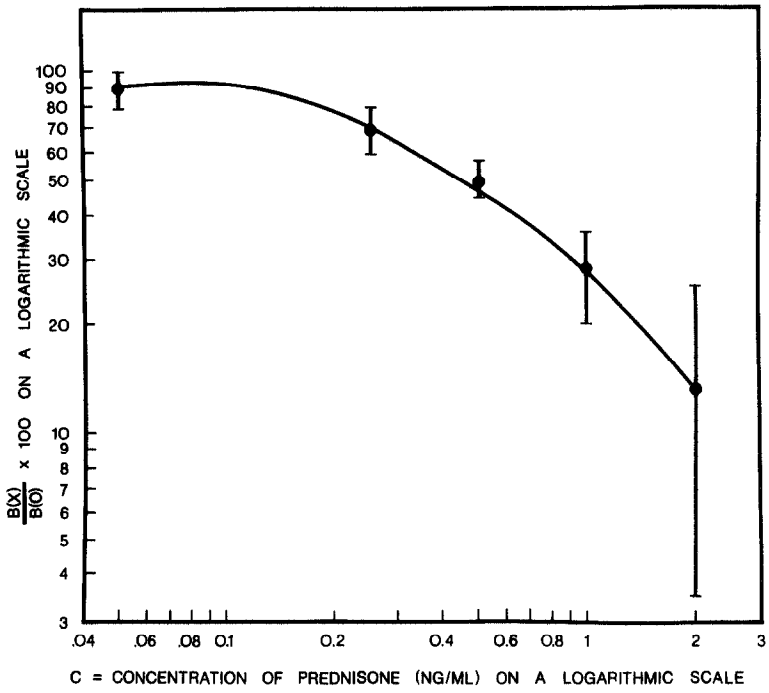

Fig. 2 - Calibration curve for the prednisone radioimmunoassay based on fitting to the equation of a parabola. Same data as used for preparation of Fig. 1. The equation of the fitted line is: $\ln\left[\frac{B(X)}{B(0)}\right] \times 100 = 3.3200 - 0.9275 \ln c - 0.1762(\ln c)^2$.

tions (Fig. 2). This plot failed the linearity test over the entire range of concentrations. Fig. 4 shows the marked heteroscedasticity for the logarithmic-logistic transformation applied to prednisone. Hence, the logistic-logarithmic transformation is optimum for prednisone only in the very narrow range of drug concentrations where this plot (Fig. 4) shows a minimum.

Estimation of Digoxin Concentrations from Different Types of Calibration Plots

The best calibration plot for digoxin radioimmunoassay is the

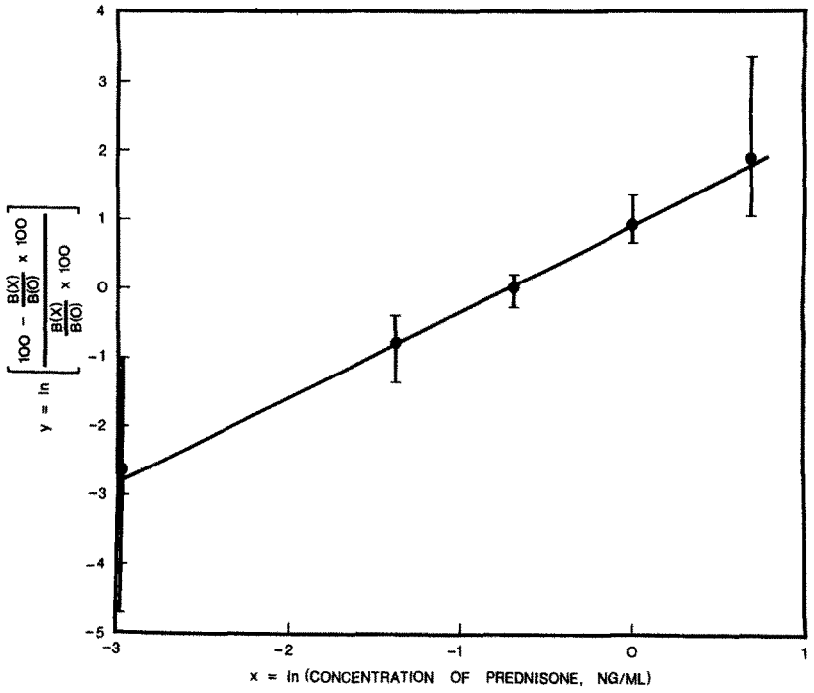

Fig. 3 - Calibration curve for the prednisone radioimmunoassay based on the logarithmic-logistic function. Same data as used for preparation of Fig. 1. The equation of the weighted least squares is: $\hat{y} = 1.2366x + 0.9032$, where $\hat{y}$ = the estimated value of

$$\ln\left[\frac{100 - \left\{\frac{B(X)}{B(0)}\right\} x\ 100}{\frac{B(X)}{B(0)}\ x\ 100}\right]$$

and x = the natural logarithm of the concentration of prednisone in ng/ml.

weighted least squares regression of B(0)/B(X) versus concentration (Table 5 and Fig. 5). The equation of the line is of the form:

$$\frac{B(0)}{B(X)} = a + b\ C \qquad \text{Eq. (1)}$$

where "a" and "b" are constants and "C" is the concentration of digoxin. For this type of calibration plot for digoxin, the calculated coefficients of variation of concentrations and the bias values are given in Table 5. Fig. 6 shows the non-uniformity of variance of the B(0)/B(X) values over the range of concentrations utilized. In the case of digoxin the variance of the B(0)/B(X) parameter appears to be a linear

Averages, Standard Deviations, Coefficients of Variation and Bias of Inversely Estimated Concentrations for Digoxin

			Actual Concentration (ng/ml)			
			0.08	0.4	1.2	2.0
Equation B[a]		Ave.	0.0802	0.399	1.20	2.00
		S.D.	0.0261	0.0429	0.0901	0.113
		C.V.(%)	32.5	10.7	7.48	5.65
	Bias	ng/ml	+0.002	-0.001	0	0
		%	+0.25	-0.25	0	0

[a] Equation B is: $\frac{B(0)}{B(X)} = 0.974 + 1.361\ C$

Inverse estimation was performed with the equation:

$$C = \left[\frac{B(0)}{B(X)} - 0.974\right] \Big/ 1.361$$

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Table 6

Averages, Standard Deviations, Coefficients of Variation and Bias of Concentrations Estimated Inversely Using the B(0) and B(X) and the Least Squares Regression Equation[a] Which Fits Each Set of Data

			Actual Concentration (ng/ml)					
			0.4	0.8	2	4	6	10
Equation A[a]		Ave.	0.405	0.770	2.12	4.29	6.02	9.79
		S.D.	0.0725	0.0906	0.266	0.625	0.843	1.99
		C.V.(%)	17.9	11.8	12.6	14.6	14.0	20.3
	Bias	ng/ml	+0.005	-0.003	+0.120	+0.29	+0.02	-0.21
		%	+1.25	-3.75	+6.0	+7.25	+0.33	-2.1

[a] Equation A is: $\ln\left[\frac{100 - \frac{B(X)}{B(0)} \times 100}{\frac{B(X)}{B(0)} \times 100}\right] = S \cdot \ln C + \ln Q$ where $S = 0.8853$ and $\ln Q = 0.4209$

Inverse estimation was performed with the rearranged equation:

$$C = e^{\left[\ln\left\{\frac{100 - \frac{B(X)}{B(0)} \times 100}{\frac{B(X)}{B(0)} \times 100}\right\} - \ln Q\right] / S}$$

function of the mean value of the parameter over the concentration range tested (Fig. 6).

Estimation of Prednisolone Concentration from Different Types of Calibration Plots

A summary of the results of analyses of calibration data for the prednisolone radioimmunoassay are given in Table 6 and Figs. 7 and 8.

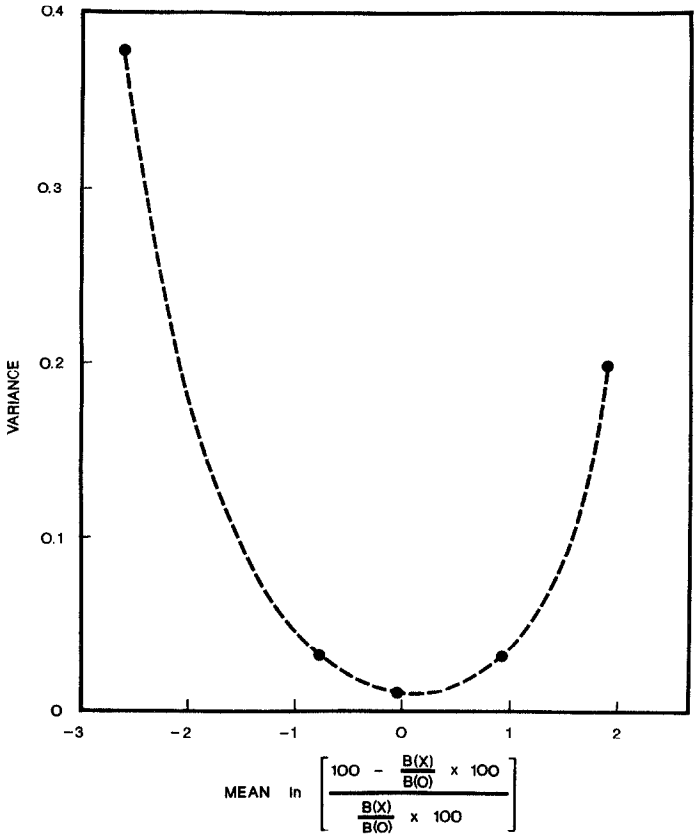

Fig. 4 - Showing the marked heteroscedasticity of the logit function derived from prednisone radioimmunoassay data.

(Fig. 8 is drawn to the same scale as Fig. 4, for the purposes of comparison). The logistic-logarithmic plot (Fig. 7) is the best transformation to use for estimating plasma concentrations of prednisolone. Fig. 8 shows that the variances are essentially homogenous over the entire concentration range of the logistic-logarithmic plot. The coefficients of variation associated with this plot average 15.2%, with a range of 11.8 to 20.8% (Table 6). It is interesting that these coefficients of variation for the prednisolone radioimmunoassay are much more concentration independent than those obtained in either the digoxin or prednisone radioimmunoassays.

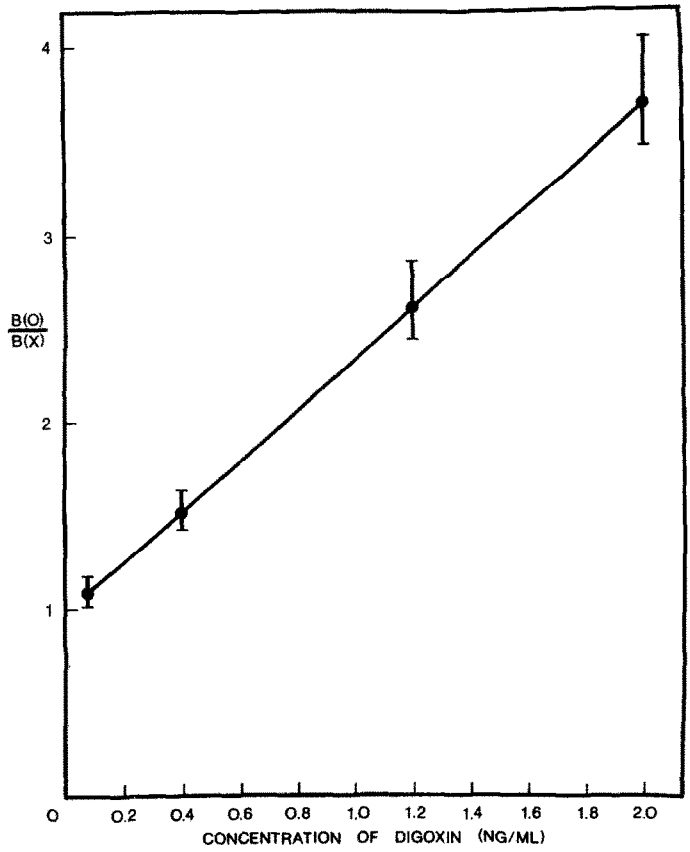

Fig. 5 - Calibration curve for the digoxin radioimmunoassay based on 16 points at each concentration or a total of 64 points. The circles are the mean B(0)/B(X) values and the bars mark off the ranges of the values. The weighted least squares regression line has the equation: $\hat{y} = 0.974 + 1.361\ x$, where $\hat{y}$ is the estimated value of B(0)/B(X) and x is the concentration of digoxin in ng/ml.

The authors believe that if anyone running radioimmunoassays collects as much data as exists in this laboratory, comparable coefficients of variation would be found in concentrations calculated by inverse estimation. Also, supportive evidence for the "pooling concept" was obtained when parameters for the logistic-logarithmic equation for prednisolone, based on six subjects, were found to be essentially the same as those obtained for twelve subjects.

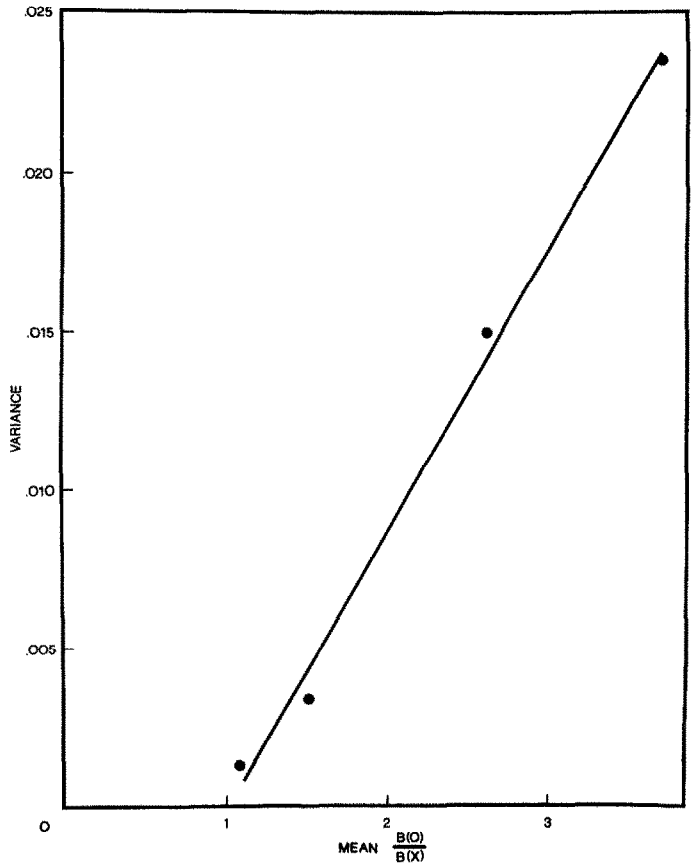

Fig. 6 - Showing the heteroscedasticity of the parameter B(0)/B(X) derived from digoxin radioimmunoassay data. Here the variance is a linear function of the mean.

DISCUSSION

Various measures of precision of radioimmunoassays have been discussed in the literature (9-14). Among these are: (a) the standard deviations calculated from duplicate % bound values; (b) the standard deviations and coefficients of variation of $\frac{B(X)}{B(0)} \times 100$ values; (c) the standard deviations and coefficients of variation of the transformation used on the ordinate to linearize the data; and (d) lastly, the standard deviations and coefficients of variation of the inversely estimated concentrations. The standard deviations and the coefficients of variation

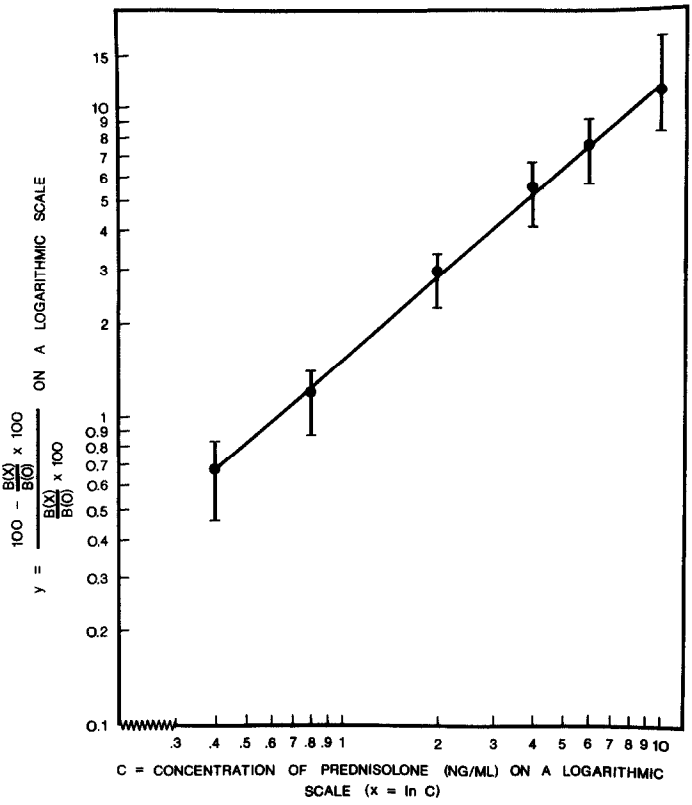

Fig. 7 - Calibration curve for the prednisolone radioimmunoassay based on the logarithmic-logistic function. Plot is based on total of 12 different subjects' pre-dose plasma at each of the 6 concentrations. The circles are the mean parameter values and the bars mark off the ranges.

of the inversely estimated concentrations are the most valid criteria of precision. This method also yields estimates of bias (Tables 4, 5 and 6).

Many laboratories prepare individual subject calibration curves at the same time that "unknown" plasma samples are being analyzed for a particular drug by a radioimmunoassay method. Data presented in this paper show that intersubject variation contributes very little to the total sum of squares. Also, the normalized % bound values were compared with the B(X) values themselves. Normalizing the data with respect to B(0) reduced the coefficients of variation in the case of prednisone and

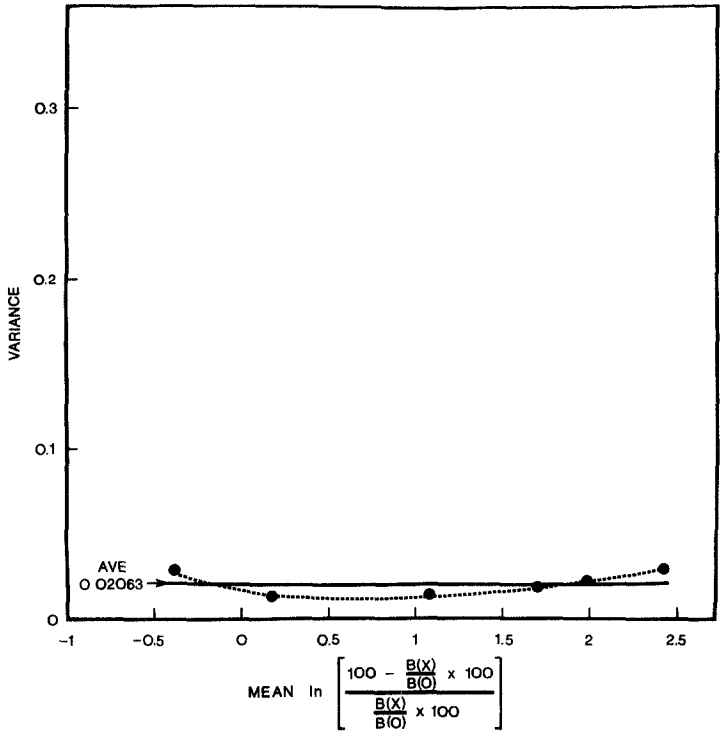

Fig. 8 - Showing the homoscedasticity of the logit function derived from prednisolone radioimmunoassay data. (Drawn to the same scale as Fig.4 for comparison purposes).

digoxin, but not for prednisolone. Normalizing also reduced the fraction of the total sum of squares accounted for by the "between plasma samples" sum of squares in the case of prednisone and digoxin, but not in the case of prednisolone. The statistical survey of calibration curve data also indicated that: (a) even a slight degree of curvature on a supposedly linear calibration plot introduces considerable bias in estimated concentrations, particularly at the lower and upper concentration range (e.g., Fig. 3). (b) The coefficients of variation calculated from duplicate % bound values, or % bound values observed on different days, are much smaller than coefficients of variation obtained from inversely estimated concentrations. Particular attention should be paid to the relationship between the variance and the mean for the parameter to be used on the

ordinate of the calibration plot (e.g., Figs. 4, 6 and 8). Finally, the error in the radioimmunoassay is much larger than the literature indicates.

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REFERENCES

1. Colburn, W. A. and Buller, R. H., Steroids 21, 833-846 (1973).
2. Stoll, R. G., Christensen, M. S., Sakmar, E. and Wagner, J. G., Res. Comm. Chem. Path. Pharm. 4, 503-510 (1972).
3. Murphy, B. P., Engleberg, W. and Pattee, C. J., Clin. Endocr. 23, 293-300 (1963).
4. Ruder, H. J., Guy, R. L. and Lipsett, M. B., J. Clin. Endocr. 35, 219-224 (1972).
5. Sullivan, T. J., Stoll, R. G., Sakmar, E., Blair, D. C. and Wagner, J. G., J. Pharmacok. Biopharm. 2, 29-41 (1974).
6. Larbig, D., Kochsiek, K. and Schrader, Chr., Deutsch. Med. Wschr. 97, 139-145 (1972).
7. Dixon, W. J., BMD Biomedical Computer Programs, Health Science Computing Facility, Department of Biomathematics, School of Medicine, University of California Press, Berkeley, CA, 1974.
8. Sullivan, T. J., Hallmark, M. R., Sakmar, E., Weidler, D. J., Earhart, R. H. and Wagner, J. G., J. Pharmacok. Biopharm. (1976) in press.
9. Rodbard, D., Lenox, R. H., Wray, L. and Ramseth, D., Clin. Chem. 22, 350-358 (1976).
10. Park, H. M., Chen, I., Manitasas, G. T., Lowey, A. and Saenger, E. L., J. Nucl. Med. 14, 531-533 (1973).
11. Phillips, A. P., Clin. Chim. Acta 44, 333-340 (1973).
12. Zeegers, J. J. W., Maas, A. H. J., Willebrands, A. F., Kruyswijk, H. H. and Jambroes, G., Clin. Chim. Acta 44: 109-117 (1973).
13. Abraham, G. E., Path. Biol. 23, 885-888 (1975).
14. Ekins, R. P., Brit. Med. Bull. 30, 3-11 (1974).