RADIOIMMUNOASSAY AND RELATED PROCEDURES IN MEDICINE

VOL. I

PROCEEDINGS SERIES

RADIOIMMUNOASSAY AND RELATED PROCEDURES IN MEDICINE

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CORRIGENDUM

RADIOIMMUNOASSAY AND RELATED PROCEDURES
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(STI/PUB/350)

Paper IAEA-SM-177/87 by Badawi et al.

Page 417, line 7

For MRX 71/222 read MRC 71/222

Page 417, lines 8 - 11

The sentence should be as follows:

It was also found that 1.0 milli-ampoule of the serum standard MRC 71/167 was equivalent to 0.09 ng and 0.9 μU of pituitary standard MRC 71/222 with 95% fiducial limits at 0.06 - 0.14 and 0.6 - 1.4, respectively.

RADIOIMMUNOASSAY AND RELATED PROCEDURES IN MEDICINE
IAEA, VIENNA, 1974
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GLYCOPROTEIN HORMONES (Session III)

PEPTIDE HORMONES (Sessions IV and V)

CALCULATION OF THE RADIOIMMUNOASSAY STANDARD CURVE BY "SPLINE FUNCTION"*

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Abstract

CALCULATION OF THE RADIOIMMUNOASSAY STANDARD CURVE BY "SPLINE FUNCTION".

A project was proposed to fully automate radioimmunoassay. Firstly a computer program was applied to the calculation of the standard curve without distortion of its sigma form. This mathematical process, called "smoothing by spline function", has been applied recently for technical and physical purposes, and calculates trial functions between points on the standard curve, whereby the curve segments on their common end points and their first and second differentials are steady and the total curve has minimal oscillation. The count-rates are fed off-line by paper-tape into a Siemens 404/3 computer. A plotter draws a semilogarithmic graph of the coordinates, sets the calculated standard curve and plots the standard deviation of the points. The smoothing parameter is selectable, and points which are in error can be corrected or eliminated for each case. After examination of the standard curve and eventual correction, the calculation of the hormone concentration of the unknowns is processed by the second part of the program.

1. INTRODUCTION

The number of hormone determinations by radioimmunoassay and competitive protein-binding, both for research and routine clinical purposes, is increasing almost exponentially. Consequently, every effort has been made to replace the widespread but very time-consuming "classical" graphic evaluation of unknowns, where one uses a hand-drawn standard curve, by a mathematical approach. In a project to automate radioimmunoassay completely, we use a computer program which is remarkably different from methods used so far.

2. MATHEMATICAL BASIS OF THE SPLINE APPROXIMATION

Since radioimmunoassay involves the use of experimental data that are subject to accidental and systematic errors, there is a disadvantage in using a single interpolation of the mean values of the response metameter (from here on referred to as points) of a standard dose-response curve, even with functions of a higher degree. It would therefore probably be better to do some smoothing in a mathematically founded and reproducible way. For this purpose, smoothing by spline functions is a very useful method.

Smoothing by spline functions has been used with great success in the calculation of complicated curves for technical and physical purposes and in computer-aided design. The premise of this is that the coordinates either increase or decrease steadily in relation to one another, as is the case in radioimmunoassay, and are not randomly distributed. In short,

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the mathematical basis of this method, invented by Reinsch [1-3], can be expressed as follows: The smoothing function f(x) to be constructed shall minimize the integral of the second derivative of g(x) (expression 1) among all functions g(x), such that the sum of $g(x_i) - y_i$ divided by δy_i, all to the power two, is less than or equal to S (expression 2), where S is defined by expression 3. In our case x_i is the concentration of cold hormone and y_i stands for % bound of x_i, related to maximal binding in the absence of cold hormone (B_0).

$$\int_{x_0}^{x_n} g''(x)^2 \, dx \tag{1}$$

$$\sum_{i=0}^{n} \left(\frac{g(x_i) - y_i}{\delta y_i} \right)^2 \leq S \tag{2}$$

$$N - (2N)^{\frac{1}{2}} \leq S \leq N + (2N)^{\frac{1}{2}} \tag{3}$$

where N = the number of points.

In expression (3), S depends on the number of points (determined by different concentrations of cold hormone) of the standard curve. In other words, the definition of optimal smoothing can be expressed as follows: S will be chosen so that the area under the second derivative of the curve, as a measure of the oscillation of the whole function, will be minimized. That means, finally, that the curve segments between the points of a standard curve are described by single trial functions of the common equation:

$$f(x) = a_i + b_i(x - x_i) + c_i(x - x_i)^2 + d_i(x - x_i)^3 \tag{4}$$

The parameters, a_i, b_i, c_i and d_i, beginning with the parameters of Euler-Lagrange, are varied in an iteration slope until the second derivative of the curve segments on their common end points are continuous: from this, by definition, follows the continuity of the whole spline function and its first derivative.

The degree of smoothing of the curve is influenced, firstly, by δy_i (expression 2) which is the estimation of the standard deviation of the single values of one point and, secondly, by the parameter S (expressions 2 and 3).

In our program we can influence the smoothing parameter by an additional factor using the computer terminal. In this way, greater variability is achieved enabling, for example, different parameters to be tested and the best one for radioimmunoassay standard curves to be found by statistical means. It should be stressed that the standard deviation of the single values, relatively weighted as δy_i, influences the smoothing and, consequently, the course of the function. That means that the points with less scattering of their single values will be treated as stronger and the curve will run near their mean value, the distance depending on the other points next to it. On the other hand, values with large scattering (that may be due to errors in pipetting the tracer or in separating bound from free hormone) will be almost ignored because of their relatively less importance (i.e. when δy_i is large). In this way it is unnecessary to correct single false values (rogue readings) because they do not influence the course of the curve.

3. DESCRIPTION OF THE RUN OF THE PROGRAM

3.1. Description of the computer

A Siemens 404/3 computer with a core of 48 K was used. It was provided with a disc with random access. A terminal, a punched tape reader, a punch and a plotter (Hagen-Graphomat, Hagen-Systems, Frankfurt/M., Federal Republic of Germany) with a maximal drawing format of 840 to 600 mm were also used.

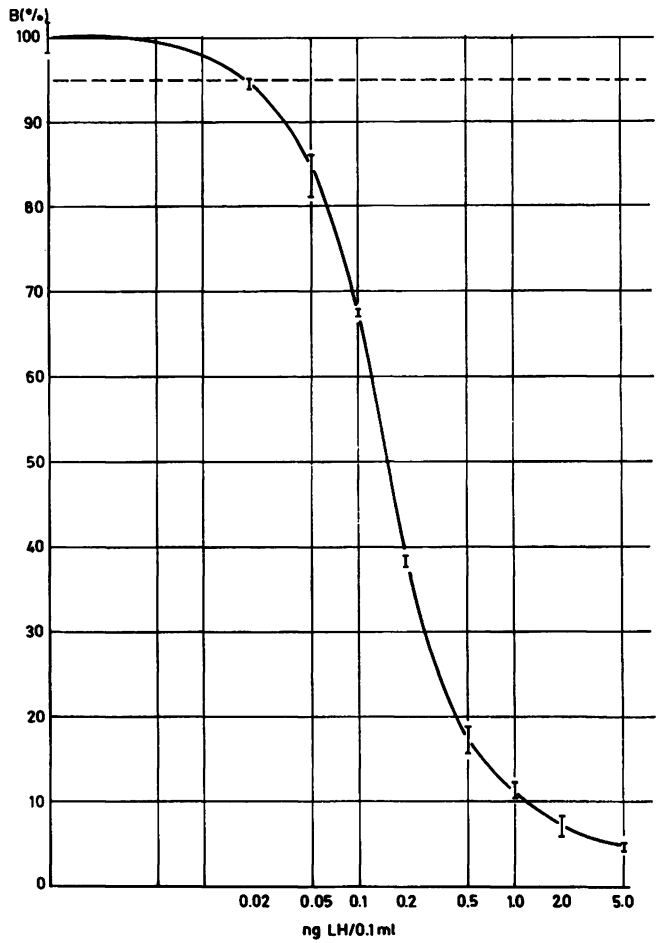

FIG. 1. Standard curve for LH that has been calculated and plotted by using the spline approximation. Ordinate: percentage of bound hormone expressed as B/B_0. Abscissa: concentration of cold hormone expressed on a logarithmic scale. The horizontal line at 95% is the limit of detection, defined by Kaiser as three standard deviations of B_0. In this case the least detectable dose is about 0.02 ng/100 µl.

3.2. Data input

The transfer of data between the gamma-counter (Gamma-Guard, Fa. Tracer-Lab, Mechelen, Belgium) and the computer is done off-line by paper-tape. Therefore every gamma-counter is equipped with a Teletype (ASR 33) with paper-tape output. All the count-rates of the assay, beginning with the standard curve, control-samples and unknowns, are read from paper-tape and stored on the disc. False data may be corrected, if necessary, by a subroutine. Necessary information about the concentrations of cold hormone used in the standard curve and the number of tubes containing no cold hormone (B_0), cold hormone (duplicates, triplicates, etc.),

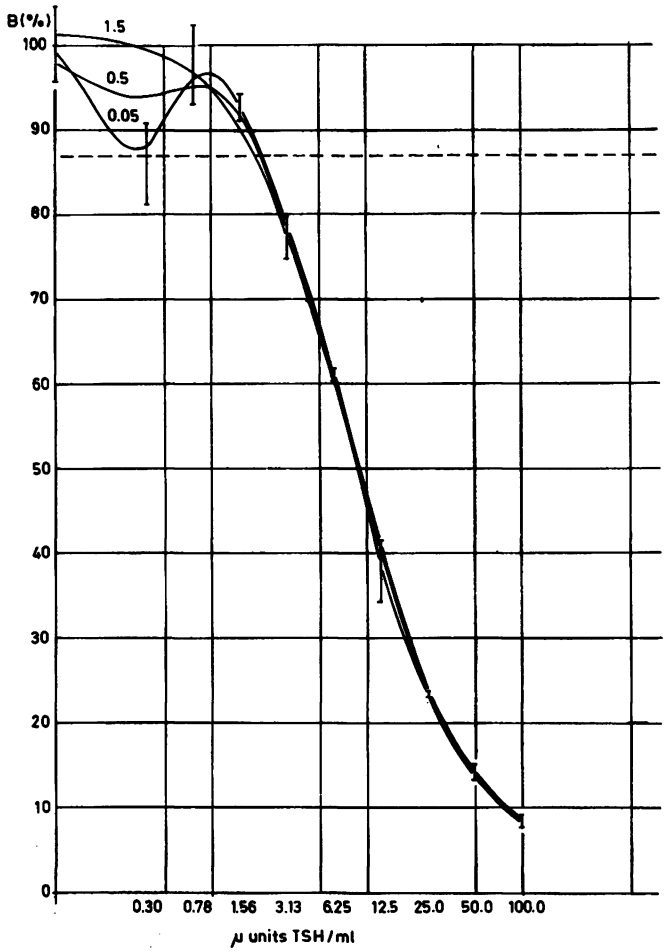

FIG.2. Example of the effect of various smoothing factors.
The lowest measure-point (0.39 μU TSH/ml) of this TSH dose-response curve is a pipetting error. The true concentration is higher and the precision is poor. With increasing smoothing factors (0.05, 0.5 and 1.5) this point is increasingly ignored. Despite this, the usable part of the curve remains almost unaffected.

and no first antibody (N) are read in the paper-tape reader from a short second paper-tape, called the specification-strip. With this information, the computer is able to find the corresponding data on the disc and calculate the standard curve.

3.3. Calculation and plotting of the standard curve

The standard curve takes about two seconds to calculate. The program allows us to decide whether or not we want the plotter to draw a 40 cm×50 cm semilogarithmic graph of the coordinates before plotting the standard curve (Fig. 1). Alternatively, results may be calculated without use of the plotter. In each case one must decide on a smoothing factor. The plotting of the standard curve with various smoothing factors can be repeated as often as required (Fig. 2). Different standard curves can also be plotted in the same graph of the coordinates to show, for example, the between-assay variation of standard curves. During plotting, Table I is printed out.

After this part of the program has been stopped (i.e. after the best-fitting smoothing factor has been found: for routine purposes we only use 0.2), the matrix containing the parameters a_i, b_i, c_i and d_i for every trial function is stored in the disc. Provided that the recovery curve (i.e. a standard curve in serum with a low or immeasurable hormone level) is contained in the same assay it may be similarly processed. In this way we can calculate the regression coefficient between two selectable segments of the standard curve. With this regression coefficient or recovery factor the results of the unknowns can be adjusted [4].

TABLE I. TYPICAL PRINT-OUT OF INFORMATION YIELDED BY THE COMPUTER AFTER THE "SMOOTHING" CALCULATION

This table, which is related to the curve in Fig. 1, is printed out at the same time as the curve is plotted.
Column 1: Hormone concentration of standards.
Column 2: Ordinate, calculated by spline approximation.
Column 3: Mean value of the single points.
Column 4: Estimation of the standard deviation of the single values (y_i).
Column 5: Differences between values in columns 2 and 3.
SDIF: Sum of values in column 5. SDIF increases with increasing smoothing factor.

ABSZISSE	ORDINATE	MITTELWERT	ABW	DIFF
Ø.ØØ	1ØØ.Ø9	1ØØ.ØØ	1.7Ø	Ø.Ø9
Ø.Ø2	94.55	94.5Ø	Ø.5Ø	Ø.Ø5
Ø.Ø5	84.9Ø	83.72	2.51	1.18
Ø.1Ø	67.3Ø	67.56	Ø.36	Ø.27
Ø.2Ø	39.Ø9	38.31	Ø.64	Ø.78
Ø.5Ø	17.33	17.26	1.52	Ø.Ø7
1.ØØ	11.Ø6	11.35	Ø.88	Ø.3Ø
2.ØØ	7.21	7.Ø5	1.21	Ø.16
5.ØØ	4.73	4.74	Ø.45	Ø.Ø1

SDIF = 2.9Ø

TABLE II. EXAMPLE OF PRINTING THE RESULTS AS DESCRIBED IN SECTION 3.4(a)

Column 1: Sample number.
Column 2: Count rate.
Column 3: Bound as % of B_0.
Column 4: Hormone concentration.

PLATZNR.	CPM	PROZ.	HORM.KONZ.
100	5381	34.10	1.93
101	3605	16.05	4.45
102	4075	20.83	3.45
103	4007	20.13	3.58
104	3868	18.72	3.86
105	4075	20.83	3.45
106	4049	20.56	3.49
107	3885	18.89	3.82
108	3493	14.91	4.71
109	3789	17.92	4.03
110	4134	21.42	3.34

TABLE III. EXAMPLE OF HOW THE RESULTS ARE PRINTED AS DESCRIBED IN SECTION 3.4(b)

The first line contains the serum number, time of venipuncture, name of patient, date of birth and diagnosis. The results of a TRH-stimulation test, seen as two lots of triplicates, have been taken from a hypothyroid patient with endemic goitre. The upper triplicate group are the TSH values measured before TRH administration (mean in the upper right-hand corner) and the lower group, the TSH levels, 30 min after an intravenous injection of 200 µg TRH (mean lower right-hand side).

805	0	ZANKL, RUDOLF		17.11.32	STRUMA	6.77
	82	8201	78.8	6.64		
	83	8146	78.2	6.78		
	84	8099	77.7	6.90		
805	30					40.59
	85	2608	18.6	41.64		
	86	2658	19.1	40.33		
	87	2679	19.3	39.81		

3.4. Calculation of the unknowns and printing of the results

To calculate the hormone concentrations of an unknown sample we use a second program which again reads the parameters a_i, b_i, c_i and d_i from the disc. For data input we can choose between disc (which is the normal case), paper-tape reader, or terminal. After reading one single count-rate, the appropriate curve segment is ascertained. Then the hormone concentration is calculated by linear iteration, which is continued until the value is approximated to four decimal places.

The results can be printed in two ways:

(a) Line by line after stipulating the first and the last desired sample number (Table II),

(b) By reading a paper-tape which contains the serum number, the position number of the samples with the same serum (duplicates, triplicates, etc.), the time of venipuncture (e.g. before or after application of a releasing hormone), the patient's name and any additional information like the diagnosis. Compared with the first way, Table III shows an additional heading with the specifications and the mean hormone concentration of single values belonging to it. With this method, probe identification is facilitated.

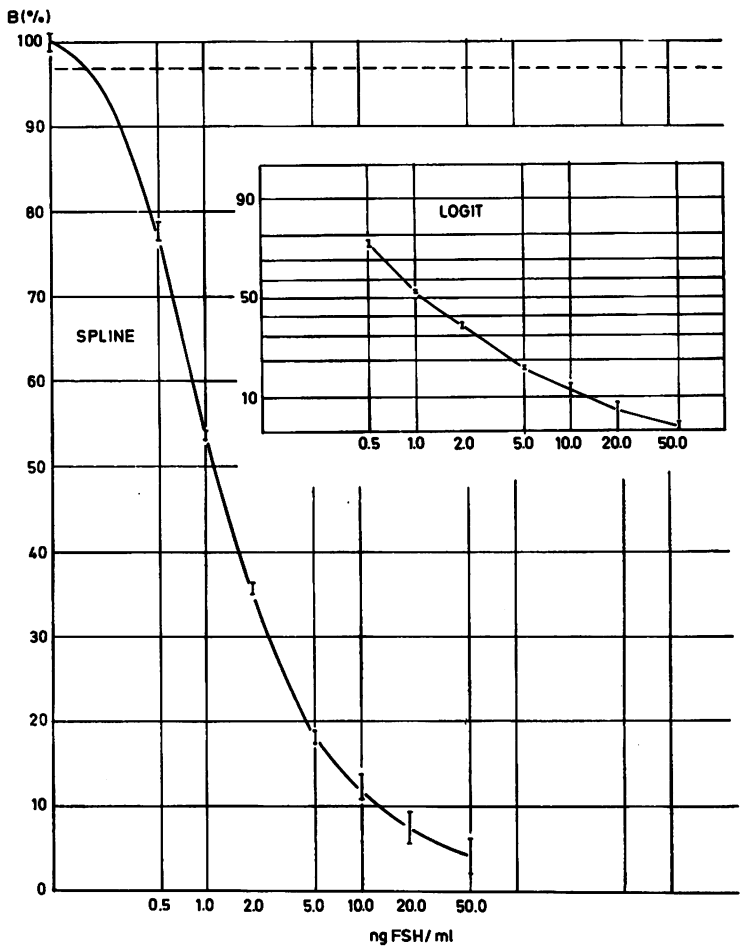

FIG.3. Comparison of logit transformation and spline approximation on a FSH dose-response curve. Logit transformation gives a satisfactory result only when there is an approximately linear gradient in the middle range of the standard curve.

4. DISCUSSION

The approximation of radioimmunoassay standard curves by spline functions is a considerable improvement in the automatic calculation of assay data and unknown hormone concentrations. It is very important that (a) the sigma form of this standard curve does not show any distortion and (b) the whole curve is calculated from 100% B/B_0 to any required concentration of cold hormone. A systematic error is, of course, possible, but this remains at the same low level throughout the whole range and does not increase at either end of the curve, as is the case in logit transformation [5]. There is no pre-established function to which the standard curve has to conform (e.g. parabola, arcus-sinus), but rather it is fitted in a previously defined "optimal" way to the points. It should be emphasized that spline approximation is not a chance procedure, as are logit transformation [6-8], arcus-sinus transformation [9], and hyperbolic or parabolic regression [10].

In fact, we have compared two types of commonly occurring standard curves by using both logit transformation and spline approximation. The usual type of standard curve with an approximately linear gradient in the middle range gives a satisfactory logit transformation. However, in some cases, as with the standard curve for FSH shown in Fig. 3, the spline function method is clearly superior to the logit transformation. The advantage of spline function is that it can be applied to all types of curves found in radioimmunoassay. Furthermore, the mathematical derivation of spline-approximation has nothing to do with the theoretical fundamentals of radioimmunoassay [5, 11, 12, 13]. Rather, it is a method by which one is able to calculate from points (at least duplicates, because of the relative weight) the best-fitting curve and therefore it is not dependent on the characteristics of the antibody. The precision of approximation increases with the number of points on the standard curve. This too is an important advantage over the other methods mentioned previously because we do not necessarily get a better fitting with these other methods if we have more points on the curve.

The accuracy of the spline-approximation is easy to demonstrate by the following example (Fig. 4). By using a simple program, the computer plotted a sinus curve. The abscissa from $\pi/2$ to $3\pi/2$ was divided into 100 equal divisions. From every tenth division an imaginary perpendicular was drawn and the value of the ordinate of its point of intersection with the curve was calculated. For each value an arbitrary range was assigned by calculating the values for perpendiculars one division apart from either side of that corresponding to a given tenth division. By using spline functions the computer drew a curve through these ranges. As can be seen from the figure these curves are almost identical.

The plotting of the coordinates as well as the calculation of the standard curve in a semilogarithmic graph corresponds to the classical procedures of radioimmunology [11, 14]. Because of the described weighting of the measure-points by the estimation of the standard deviation, correction of errors is only necessary when there is good precision and poor accuracy (e.g. false concentration of the standard and good pipetting precision). The possibility of calculating the regression between standard curves in buffer and in serum (recovery curves) allows a systematic adjustment of the unknowns.

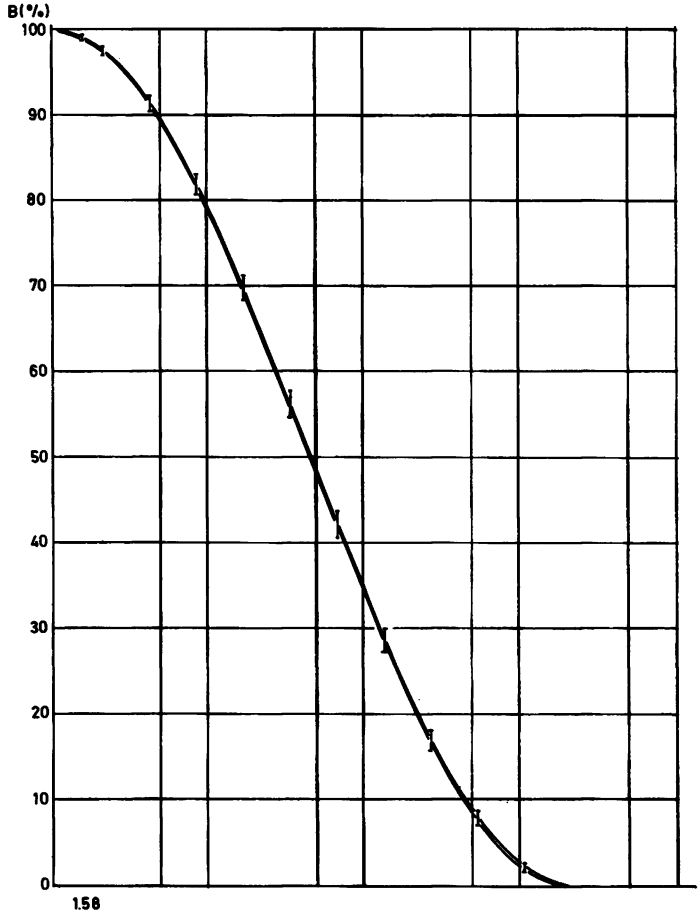

FIG.4. Original sinus curve and its approximation by spline functions. (See Section 4 for a description.)

A technician would require approximately one day to evaluate an assay with 800 unknowns. The computer does the calculation within 5 seconds and prints the results in 20 minutes.

Since the introduction of this program, radioimmunoassays and competitive protein-binding assays are routinely carried out in our Endocrinology Department on the following substances: thyroid-stimulating hormone, insulin, luteinizing hormone, growth hormone, thyroxine, tri-iodo-thyronine, follicle-stimulating hormone, antidiuretic hormone and angiotensin I.

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DISCUSSION

D. RODBARD: I think you have developed a very fine method of curve fitting for RIA dose-response curves. However, in weighing its advantages with respect to the logit-log method, it is important to bear in mind the pros and cons of both methods. Since your discussion has tended to be somewhat unilateral, I would like to comment on the pros of the logit-log method.

First of all, the logit-log model is essentially an application of linear regression, i.e.:

$$Y' = a + bX'$$

Although this model involves transformations of both the X and Y variables, it is an extremely simple mathematical model, which is readily adaptable to graphical analysis or to small desk-top calculators. Use of a linear model vastly simplifies the statistical analyses, e.g. calculation of the standard error of the slope.

Second, the logit-log model can be justified on the basis of the first-order mass action law, although certain approximations are involved.

Third, the logit-log method is successful, i.e. satisfactory linearity is obtained, in more than 90% of all RIA systems examined to date. It is a rare exception to the rule if the logit-log plot is not linear over the entire observable range.

Fourth, the logit-log approach vastly facilitates calculation of potency estimates when both the standard and unknown have been measured at

several dose levels. This corresponds to the calculation of parallel-line bioassay statistics by the methods of Finney and Bliss. Thus, the logit-log method permits a combined test of parallelism and potency estimate and of the confidence limits. I believe this is very difficult, if not impossible, when using the "spline function" approach.

Fifth, some of the deficiencies of the original logit-log method, which you correctly pointed out, have already been remedied. In the original version of the method, the zero dose response, in terms of B/T or counts, was kept fixed. However, the methods of Burger, et al.[1], of Arrigucci, et al.[2] and of Healy[3] permit the computer to make an optimal adjustment of the value for $(B/T)_0$. Likewise, the value for non-specific counts (the lower asymptote) was kept constant in the original logit-log method. However, the approach adopted by Leclercq, Täljedal and Wold[4] and by Healy[3] permit the computer to estimate this value as well. The approach which I will present later today embodies the advantages of all of these modifications, together with the often crucial ability to provide proper weighting to each point on the dose-response curve.

Finally, when all else fails, one can fit a parabola in the logit-log co-ordinate system. We have adopted this approach quite successfully in those few assays which show a non-linear logit-log plot. This method is still far simpler, from a mathematical and statistical point of view, than the use of "spline function" curve fitting.

One additional technical comment: your method of weighting apparently weights points in a manner inversely related to the observed standard deviation of replicates of the response variable for a given dose. This method can be very unstable and unsatisfactory on account of the large sampling error in the sample standard deviation based on only a few degrees of freedom. Only with ten or more replicates of y for each dose does this method become "well-behaved". This was shown by Jaquez et al. a few years ago in the Journal of Biometrics. Accordingly, I would suggest that you plot δ_y^2 versus y, fit a smooth function to this relationship, and then use it as a basis for your weighting function. This should improve the efficiency of the curve fitting procedure.

I. MARSCHNER: Many thanks for your comments, Dr. Rodbard. I agree with you that the logit-log transformation is a procedure that is simple and relatively easy to understand from the mathematical point of view, and one which has been shown to be of value in the statistical analysis of RIA standard curves.

However, since it is difficult to gain insight into such various factors involved in RIA as non-homogeneous antibody populations, cross-reaction, influences of the tracer due to varying specific activity and damage, and non-specific protein interference, we are reluctant to believe that the use of a mathematical method related to the principles of the first-order mass action law is justified. Indeed, the criteria for applying such a principle will be met only in rare cases. In our experience, only half of all RIA standard curves can be made linear with the logit-log transformation. An

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[1] Ref.[9] in paper SM-177/208.
[2] Ref.[10] in paper SM-177/208.
[3] Ref.[11] in paper SM-177/208.
[4] Ref.[8] in paper SM-177/208.

attempt to fit a parabola in a logit-log co-ordinate system is neither theoretically sound nor often applicable.

For purposes of quality control we make regular determinations of control sera, which we think is more important than the quality control of standard curves. This is one of the reasons why we do not need some of the parameters calculated by your program, which, in any case, are only designed for the latter.

As I mentioned in my paper, we calculate, by a sub-routine, the regression between the standard curve and recovery curve. This method is free from the difficulties inherent in the calculation of the regression between different standard curves, or between standard curves and serum dilution curves.

As regards your final point, I think I should stress that weighting in spline approximation has a completely different mathematical meaning from the same term when used for the calculation of a weighted regression in statistical analysis – a feature which unfortunately is liable to cause misunderstandings. Hence, the number of the degrees of freedom plays no role. Details of the weighting in spline approximation are described in the original paper on the subject by Reinsch[5]. The greatest advantage of spline approximation is that we do not need to analyse, by means of statistical methods, what kind of procedure (i.e. logit, parabola, four-component logit, etc.) will fit for the individual curve, and that we can analyse all standard curves deriving from RIA, CPBA, IRMA, or two-site IRMA with this procedure.

E. KELLER: By how much do calculated values differ in samples with very low and very high amounts of hormone, when using (a) the logit-log transformation, and (b) the spline function?

I. MARSCHNER: Clearly, the differences between the hormone values which are read from logit-log or from spline approximated standard curves depend on the kind of assay involved, and on the slope of the standard curve. If, as in an ideal case, one gets a linear curve by logit-log transformation, the differences do not exceed 5%. If one does not obtain linearity with the logit-log transformation, the method should not be used at all, or else one should restrict oneself to the linear area in the middle of the standard curve, since the deviation at both ends increases with distance from the middle; such is not the case, however, with the spline approximation.

[5] Ref. [1] of the paper.