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DATA-PROCESSING OF ENZYME IMMUNOASSAY MEASURMENTS USING POGRAMMABLE DESK-TOP CALCULATOR

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INTRODUCTION

Various types of enzyme immunoassay are now used routinely for quantitative determination of antigens and antibodies. As the label is enzyme, addition of an appropriate substrate yields a chromogenic product measurable by spectrophotometer.

Concentration of unknown samples is determined on the basis of calibration curve. Manual graphing and handling of data is time consuming and can be a source of erroneus results.

This is an obvious situation for employment of computing methods.

Two simple programs have been designed for measuring alpha-fetoprotein (AFP) by enzyme-linked immunosorbent assay (ELISA)

and progesterone by enzyme immunoassay (EIA) using TI-59 desk-top calculator which is inexpensive, universally avaible and easy to use.

MATERIALS AND METHODS

1. Determination of AFP using double sandwich ELISA method

1.1. The immunoassay was performed in 96-well flat-bottom microtiter plate (Dynatech Lab. Inc.) coated with anti-AFP (0.1 ml).
1.2. The plate was incubated overnight at 4 °C and washed three times with PBS-Tween 20 buffer.
1.3. AFP-standards were then added (0.1 ml) and the plate was incubated again for an hour at 30 °C. After the incubation step the plate was washed.
1.4. Then anti-AFP-horseradish peroxidase (HRP) conjugate (0.1 ml) was added to each well and incubated for an hour.
1.5. After washing the plate was incubated with hidrogen peroxide-o-phenilenediamine (OPD) substrate solution (0.15 ml).
1.6. The enzyme reaction was terminated after 15 minutes by adding of 4N sulphuric acid (0.05 ml).
The absorbance was measured at 492 nm.

2. Determination of progesterone by competitive double antibody EIA

2.1. Progesterone standards, anti-progesterone antiserum and progesterone-HRP conjugate solutions (0.1-0.1 ml) - diluted adequately - were incubated together in glass tubes for an hour at room temperature (RT).
2.2. Then 1 ml of second antiserum-PEG solution was added and incubated for 30 minutes (RT).
2.3. The tubes were centrifugated and the supernatant discarded.
2.4. 1 ml freshly prepared substrate solution was added to each tube and incubated in dark for an hour (RT).
2.5. The enzyme reaction was stopped by adding of 0.2 ml 4N sulphuric acid. The absorbance was measured at 492 nm.

A home-made "TI-59" programmable calculator was mounted on a KA-100 (HTSZ) printer. It contains 800 steps storage for program and 20 storage register for data. These can be distributed depending on the length of the program.
For storaging of the programs, magnetic cards are used. The programs were written in machine language.

RESULTS

The general form of the logistic equation which can be used as a model for the relationship between the concentration and the optical density (OD) is as follows:

$$y = \frac{a-d}{1 + \left(\frac{x}{c}\right)^b} + d \qquad (1.)$$

Where y represents the measured OD, x is the known concentration of progesterone or AFP, a is the OD when x=0, d is the OD for infinite concentration, c is the concentration resulting from an OD halfway between a and d, b is a slope factor.

The linearization of eq. 1. can be done by the following way:

$$\ln\left(\frac{a - y}{y - d}\right) = b \ln(x) - b \ln(c) \qquad (2.)$$

$$Y = A + BX \qquad (3.)$$

where B=b, X=ln(x), A = -b ln(c)

The concentration of an unknown sample can be calculated using A, B, $\sigma(A)$, $\sigma(B)$ and $\sigma(A,B)$ parameters which were determined on the basis of standard points:

$$X = \frac{\bar{Y} - A}{B} \qquad (4.)$$

$$\sigma^2(X) = \frac{1}{B^2}\left(\sigma^2(\bar{Y}) + \sigma^2(A)\right) + \frac{(\bar{Y} - A)^2}{B^4}\sigma^2(B) + 2\,\frac{(\bar{Y} - A)^2}{B^3}\,\sigma^2(A,B) \qquad (5.)$$

$$\sigma^2(\bar{Y}) = \frac{\sigma^2(a)}{(a - \bar{y})^2} + \frac{\sigma^2(d)}{(d - \bar{y})^2} + \frac{(a - d)^2}{(a - \bar{y})^2(\bar{y} - d)^2}\,\sigma^2(\bar{y}) \qquad (6.)$$

$$x = \exp(X) \qquad (7.)$$

$$\sigma^2(x) = x^2\,\sigma^2(X) \qquad (8.)$$

After the logit transformation (eq. 2.) a straight line was obtained by the method of least squares. (It would have been more correct to use the weighted least squares method but the restricted storage capacity of the calculator limited it.)

The "a" and "d" values are needed for the transformation (eq. 1.). In case of progesterone-EIA "a" was obtained without progesterone, and "d" without anti-progesterone antiserum. The standard points were choosen as not to come close to "a" and "d" within twofold standard deviation (SD) range.

To simplify the program the average of transformed values were taken directly. Fig. 1. shows an EIA calibration curve; a typical logit versus log plot of data from Fig. 1. is shown on Fig. 2.

In case of AFP ELISA the value of "d" is unknown, so it should be determined. Eq. 1. is linearized with a postulated "d" value and the $\chi^2(A,B,d)$ function is calculated, then the program searches the minimum of this function by an iterative method, choosing the proper "d" value. At the minimum of this function the A, B, $\sigma(a)$, $\sigma(b)$ and $\sigma(A,B)$ values are calculated, while $\sigma(d)$, $\sigma(d,A)$ and $\sigma(d,B)$ parameters are not. $\sigma(d)$ is supposed as 5 per cent.

The iteration ends when the deviation of "d" from the minimum is less than 0.1 (OD) or its value is larger than 9.

Figures 3. and 4. show the calibration curve of an AFP ELISA and the straight line after transformation.

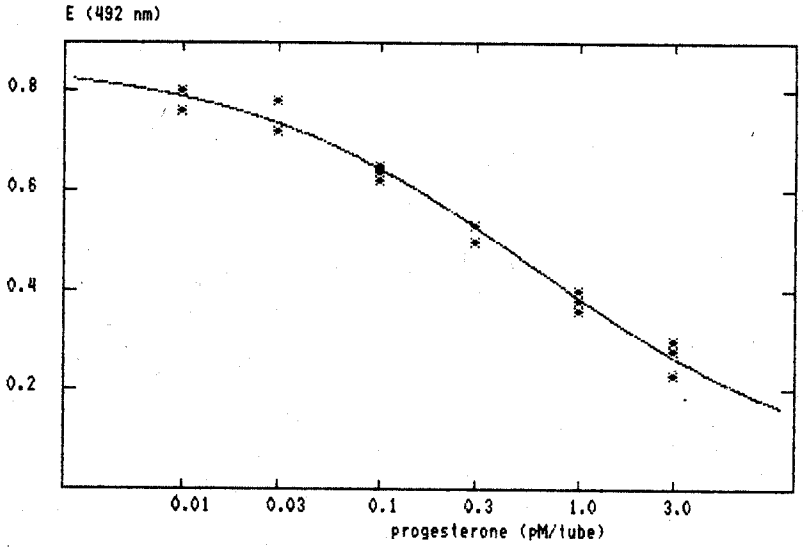

Fig. 1. EIA of progesterone, manually graphed calibration curve

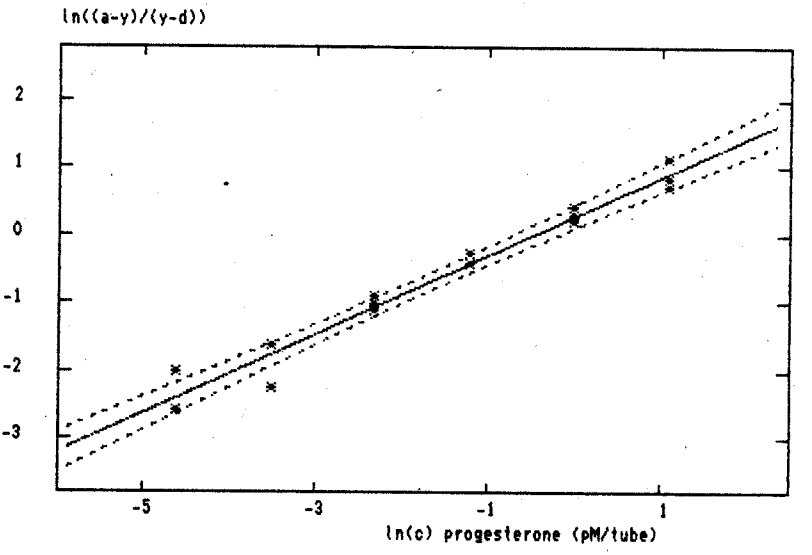

Fig. 2. Straight line after transformation with the 95 % confidence limit

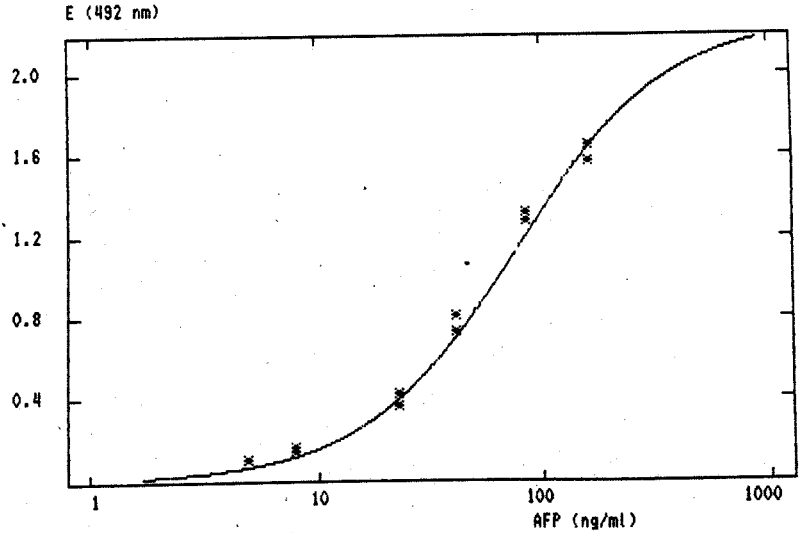

Fig. 3. ELISA for AFP, graphed calibration curve

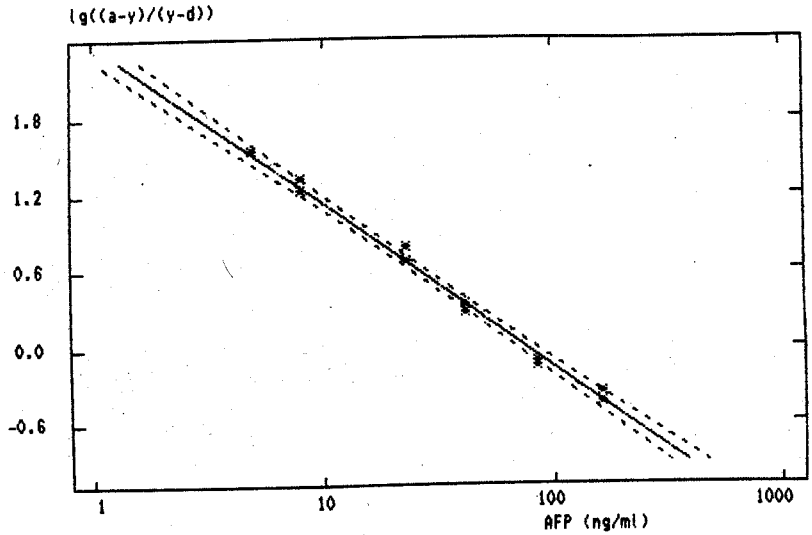

Fig. 4. ELISA for AFP, logit transformation with the 95 % confidence limit

The programs and sample runs are avaible on request.

DISCUSSION

Application of different enzyme immunoassay methods needs handling lots of data. Desk-top calculators (with even little storage capacity) can be used for storage of data, calculation of concentrations of unknown samples and determination of errors and "r" i.e. the linear-correlation coefficient (1., 3., and 4.).

The simple approach for EIA and ELISA measurments to linearize the standard curve is the four parametric logit-log transformation with two, respectively three unknown parameters.

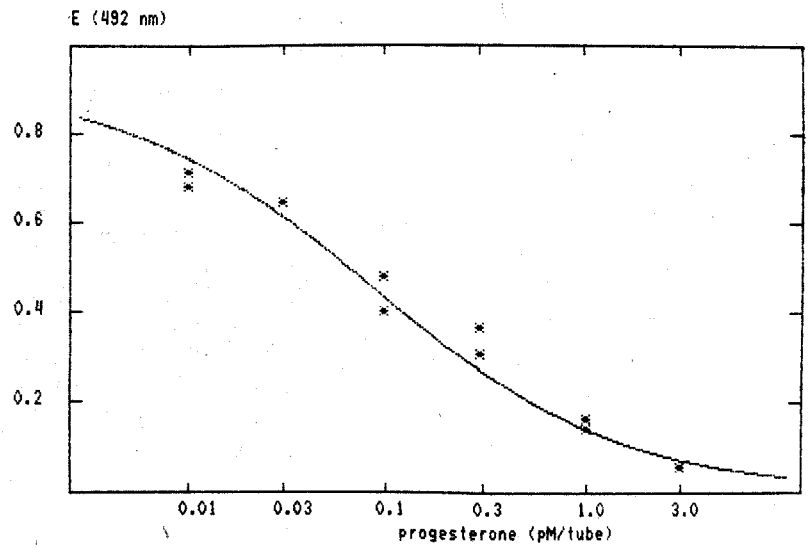

Fig. 5. Progesterone EIA including wrong data points, graphed calibration curve

As it is displayed in Fig. 1. the probability of errors in manually graphed curves can be decreased.

At the low and high concentrations the scatter of errors is broaden indicating that the determination of concentration is not quite adequately interpetable here.

Interpolation between the standarad points is suggested by Fey (2.). The disadvantage of this procedure is demonstrated on the Fig. 5 and 6. It shows a calibration curve with a "deviating" standard point. The method of interpolation would give erroneus results in surrounding of this point and it has not any parameter which would indicate the "deviating" trend of the curve.

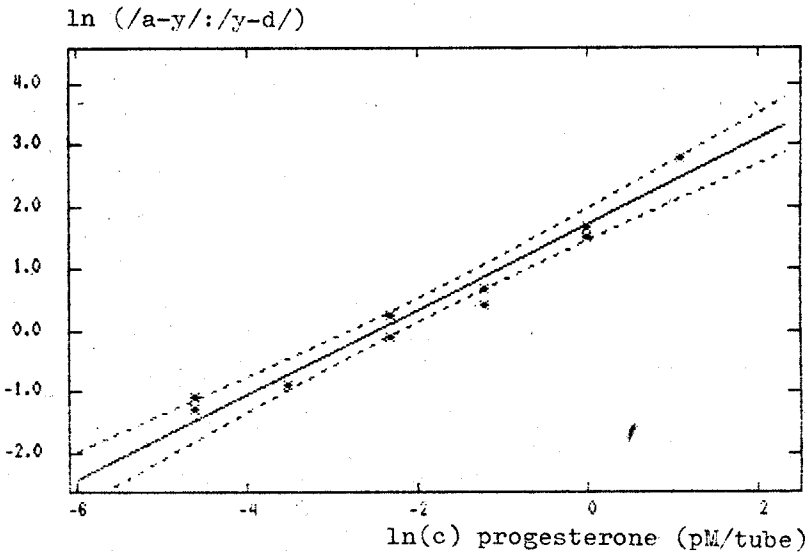

Fig. 6. Straight line after transformation with a broadening confidence limit

Employing the method of the least square fit to a straight line, the uncertainties are indicated by the significant deviation of linear-correlation coefficient (r) from 1, and by the

SD value of the fitted parameters.

The program designed for measuring AFP by ELISA uses iteration method to search the best fitting curve for a matching slope from calibration data.

(There were six standard points in our case, so the storage capacity was enough for six paralell measurements.)

It was necessary to use some simplification to compute data. Data-points close to the maximum OD or background can have an effect on parameters used for transformation, so the results can be distorted. The limited storage capacity of this calculator does not allow to use the weighted least squares method but restrictions concerning calibration data eliminate this source of error.

Programs described here give correct estimation of SD of final results considering the uncertainty of the measurment of unknown samples and calibration curve.

Data processing with fitting method makes the interpretation of results more safe, errors coming from methods of interpolation and drawing can be eliminated and the reproducibility of measurements can be improved.

ABBREVATIONS

ELISA: enzyme-linked immunosorbent assay,
AFP: alpha-fetoprotein,
EIA: enzyme immunoassay,
PBS: phosphate buffered saline,
HRP: horseradish peroxidase,
OPD: o-phenilenediamine,
PEG: polyethylene glycol,
OD: optical density

SUMMARY

Two programs for analysis of data from progesterone EIA and AFP ELISA were written for TI-59 calculator. It can be concluded that these programs are useful, practicable, the errors of manual handling of data are decreased and some information can be obtained about the validity of results. The advantages ot method of least squares were demonstrated comparison to method of interpolation.

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