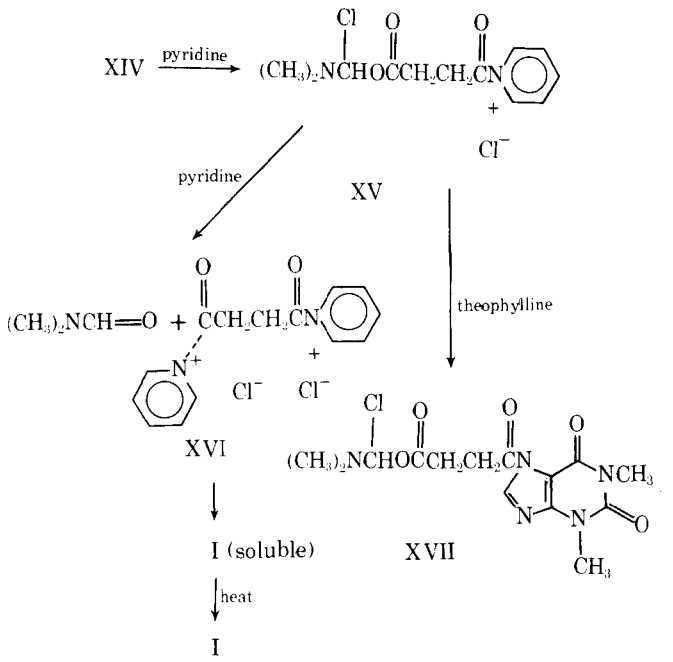

Scheme IV

nium ion is generated from an acid chloride. The crystals of I obtained from this novel synthesis are more stable than those crystals of I obtained when the acid chloride is used in the synthesis of I. In addition, this novel synthesis results in I of consistent physical properties suitable for subsequent formulation.

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Assays and Statistical Analyses for Antibiotic Standards

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Abstract □ Some microbiological assays and statistical analyses of test results used by the National Center for Antibiotics Analysis are described for the establishment of official antibiotic reference standards. Examples are given of both cylinder plate agar diffusion assays and turbidimetric assays. Formulas providing simple and quick analyses of data are shown for calculating potency, determining limits for the potency, and performing validity tests on the results.

Keyphrases □ Microbiological assays—for official antibiotic reference standards, formulas for quick, simple analysis of data □ Antibiotic reference standards, official—microbiological assays, formulas for simple, quick analysis of data

The Food and Drug Administration's National Center for Antibiotics Analysis (NCAA) establishes and maintains official reference standards for all antibiotics subject to certification. When a standard is required for a new antibiotic or when an existing standard must be replaced, NCAA performs a series of assays on representative samples of the proposed batch. The collaboration of other laboratories in assaying samples is requested; the findings are evaluated and certain statistical analyses are performed by NCAA.

In addition, NCAA is frequently called upon to participate and collaborate with other laboratories to establish official standards, such as international standards established by the National Institute for Medical Research in London, England, through the Expert Committee on Biological Standardization of the World Health Organization. Standards are also assayed at the request of the USP and NF.

Kirshbaum *et al.* (1) simplified the bioassay designs described by Bliss (2) and statistical procedures given in USP XV (3). For microbiological assays of antibiotics, they adapted a series of equations to determine potency, error variance, validity, and confidence limits of the assays. This adaptation was published in the USP XVI (4) and is still in use in NCAA laboratories. The calculations are based on a polynomial equation for fitting a line to a parabola. Coefficients and constants are derived from the table of orthogonal polynomials.

METHODS

When evaluating a proposed FDA standard, each participant is supplied with a quantity of the proposed standard and a reference standard and is requested to follow a specific design and to furnish NCAA with the necessary raw data. This design is applied to three-dose assays, where there is a linear response to the log of the dose, where there are parallel

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dose–response lines for the standard and unknown, and where the potency of the unknown is estimated as close to 100% of the standard. The usual instructions are for two weighings daily of the proposed standard to be assayed for 3 days against two weighings of the standard.

Before starting, erlenmeyer flasks used for preparing test solutions are rinsed with distilled water and dried in a hot air oven. The weighed material is dissolved in sufficient solvent to make a convenient stock solution. The same is done with the reference standard. Where possible, only volumetric glassware is used for dilution. All glassware is rinsed with some of the final diluent before use.

If an intermediate dilution is required, only one such dilution is made from each stock solution. With appropriate aqueous solvents, dilutions from the stock concentrations are made to three different concentrations. These concentrations are equally spaced at logarithmic intervals and result in the same number of responses so that a constant may be used in the statistical analyses. These concentrations are referred to as S_1, S_2, and S_3 (low, middle, and high concentrations) for the standard and U_1, U_2, and U_3 (low, middle, and high concentrations) for the unknown.

The method may be a cylinder plate procedure or turbidimetric procedure as described in the CFR (5). In the plate assay procedure, each of the six solutions is put in a coded random pattern into six stainless steel cylinders sitting on the surface of a freshly prepared agar plate containing the test organism. Twelve plates are prepared for each weighing. After overnight incubation, the plates are examined visually. All plates with aberrant or missing values are discarded. Nine plates are chosen at random, and the diameters of the zones of inhibition are measured in millimeters with an appropriate instrument. If any of the six zones of inhibition on one plate must be discarded because of aberrance, the entire plate is discarded and one of the remaining plates is used. If more than three plates are discarded, the assay is considered invalid. If only nine plates are prepared, missing or aberrant readings are replaced by methods described in the USP (6).

In the turbidimetric assay, 60 test tubes are placed in one large spool; test solutions are put in the tubes in a coded random pattern, using 1 ml/tube. Then 9 ml of broth containing the test organism is added, and the spool of tubes is incubated in a 37° constant-temperature circulating water bath. After the incubation period, the spool is removed from the water bath and 0.5 ml of 12% formaldehyde solution is added to each tube. The turbidity is measured with a photoelectric colorimeter. The absorbance readings are decoded, and only three absorbance values are used for each concentration. The other values are used only to replace an aberrant reading.

RESULTS AND DISCUSSION

Example 1—The statistical analysis shown in Table I is for a cylinder plate assay. After the zone sizes are decoded and tabulated, the range of the values of each vertical column is obtained by subtracting the lowest value from the highest. The ratio of the largest range to the sum of the ranges should not exceed the appropriate critical value (0.273 for nine plates; $p = 0.05$). If it does, the readings in the column with the largest range are checked to find the aberrant response. The plate with this reading is replaced by a random selection of one remaining plate. If there are no complete plates to substitute, the assay is not considered valid.

This procedure is continued until the table includes data from nine plates with no missing or aberrant values. If a satisfactory assay has been performed, the three extra plates should ensure that the analyst ends up with nine good plates. The columns of values are totaled both horizontally and vertically.

The log potency, M, is determined from:

$$M = \frac{^{*}(U_1 + U_2 + U_3 - S_1 - S_2 - S_3)}{S_3 + U_3 - S_1 - U_1} \qquad \text{(Eq. 1)}$$

and the percent potency, P, is obtained from:

$$P = 100 \times \text{antilog } M \qquad \text{(Eq. 2)}$$

The numerator constant (*) depends on the ratio between doses (see *Formulas for Determining Potency Constant and* L/2 *Constant for All Three-Dose Assays*).

From Example 1:

$$M = \frac{0.12921\,(119.0 + 126.3 + 132.3 - 119.1 - 126.4 - 132.8)}{132.8 + 132.3 - 119.1 - 119.0}$$

$$= -0.00335 \qquad \text{(Eq. 3)}$$

$$P = 100 \times \text{antilog} - 0.00335 = 99.2\% \text{ or } 636\ \mu\text{g/mg} \qquad \text{(Eq. 4)}$$

Table I—Example 1, Assuming a Potency for the Unknown of 641 μg/mg and a Ratio between Doses of 1.25:1

Plate Number	S1	S2	S3	U1	U2	U3	Plate Sum (T_r)
1	13.3	14.1	14.8	13.2	13.9	15.0	84.3
2	12.7	13.7	14.7	12.9	13.8	14.6	82.4
3	12.9	14.1	14.6	13.1	13.9	14.7	83.3
4	12.9	13.8	14.8	13.2	13.5	14.1	82.3
5	13.6	14.6	14.9	13.8	14.4	14.9	86.2
6	12.8	13.6	14.7	12.9	14.3	14.9	83.2
7	13.6	14.0	14.8	13.2	14.3	14.4	84.3
8	13.7	14.2	14.8	13.0	14.0	14.8	84.5
9	13.6	14.3	14.7	13.7	14.2	14.9	85.4
Sum (T_t)	119.1	126.4	132.8	119.0	126.3	132.3	755.9 (T)
Range	1.0	1.0	0.3	0.9	0.9	0.9	

observed $R = \frac{1.0}{5.0} = 0.2000$ critical $R = 0.273$

The error variance, s^2, from cylinder to cylinder and from plate to plate is determined using:

$$s^2 = \frac{\sum y^2 - \frac{\sum T_r^2}{6} - \frac{\sum T_t^2}{9} + \frac{T^2}{54}}{40} \qquad \text{(Eq. 5)}$$

where:

y = individual response, zone measurement, or single observation

$\sum y^2$ = sum of the squares of all 54 zone sizes

$\frac{\sum T_r^2}{6}$ = sum of the squares of the totals of the horizontal rows divided by the number of treatments (6)

$\frac{\sum T_t^2}{9}$ = sum of the squares of the totals of the vertical columns divided by the number of plates (9)

$\frac{T^2}{54}$ = squares of the sum of the totals of the horizontal rows or the square of the sum of the total of the vertical columns (they should be equal) divided by the number of observations (54)

40 = degrees of freedom

From Example 1:

$$\sum y^2 = 10{,}605.87 \qquad \text{(Eq. 6a)}$$

$$\sum T_r^2 = 63{,}501.00 \qquad \text{(Eq. 6b)}$$

$$\frac{\sum T_r^2}{6} = 10{,}583.5017 \qquad \text{(Eq. 6c)}$$

and:

$$\sum T_t^2 = 95{,}413.59 \qquad \text{(Eq. 7a)}$$

$$\frac{\sum T_t^2}{9} = 10{,}601.51 \qquad \text{(Eq. 7b)}$$

and:

$$T^2 = 571{,}384.81 \qquad \text{(Eq. 8a)}$$

$$\frac{T^2}{54} = 10{,}581.2002 \qquad \text{(Eq. 8b)}$$

Therefore:

$$s^2 = \frac{10{,}605.87 - 10{,}583.3017 - 10{,}601.51 + 10{,}581.2002}{40} \qquad \text{(Eq. 9a)}$$

$$s^2 = \frac{2.0585}{40} = 0.0515 \qquad \text{(Eq. 9b)}$$

(The values obtained for $\sum y^2$, $\sum T_r^2/6$, $\sum T_t^2/9$, and $\sum T^2/54$ should all be quite close numerically.)

To test the assay validity, three equations are used. The value ab is used to test whether the dose–response lines are parallel. The value q is used to test combined curvature in the same direction. The value aq is used to test separate curvatures in opposite directions. The three values obtained from these equations should not exceed the value obtained for three times the error variance, $3s^2$. If any value is greater than three times the error variance, the significant F-test is used to determine validity.

The three values are added and their sum is divided by $3s^2$. This value should not exceed the critical F (2.84). If it does, the assay is not valid and is discarded. Therefore:

$$ab = \frac{[S_1 + U_3 - (S_3 + U_1)]^2}{36} \quad \text{(Eq. 10)}$$

$$q = \frac{[S_1 + U_1 + S_3 + U_3 - 2(S_2 - U_2)]^2}{108} \quad \text{(Eq. 11)}$$

$$aq = \frac{[(U_1 + U_3 + 2S_2) - (S_1 + S_3 + 2U_2)]^2}{108} \quad \text{(Eq. 12)}$$

$$F = \frac{ab + q + aq}{3s^2} \quad \text{(Eq. 13)}$$

From Example 1:

$$ab = \frac{[119.1 + 132.3 - (132.8 + 119.0)]^2}{36} = 0.0044 \quad \text{(Eq. 14)}$$

$$q = \frac{[119.1 + 119.0 + 132.8 + 132.3 - 2(126.4 + 126.3)]^2}{108} = 0.0448 \quad \text{(Eq. 15)}$$

$$aq = \frac{[119.0 + 132.3 + 252.8 - (119.1 + 132.8 + 252.6)]^2}{108} = 0.0015 \quad \text{(Eq. 16)}$$

$$F = \frac{0.0044 + 0.0448 + 0.0015}{0.1545} = 0.3282 \quad \text{(Eq. 17)}$$

Critical $F = 2.84$. The assay is valid.

If the assay is valid, the precision of the assay, C, and the confidence interval, L, are determined next:

$$C = \frac{(S_3 + U_3 - S_1 - U_1)^2}{(S_3 + U_3 - S_1 - U_1)^2 - 147.06s^2} \quad \text{(Eq. 18)}$$

and:

$$\frac{L}{2} = \sqrt{(C - 1)(CM^2 + *)} \quad \text{(Eq. 19)}$$

The confidence interval depends on the precision with which the slope of the dose–response curve has been determined.

From Example 1:

$$C = \frac{(132.8 + 132.3 - 119.1 - 119.0)^2}{(132.8 + 132.3 - 119.1 - 119.0)^2 - 147.06 \times 0.0515} = 1.0105 \quad \text{(Eq. 20)}$$

and:

$$\frac{L}{2} = \sqrt{(1.0105 - 1)[1.0105\,(-0.00335)^2 + 0.02504]} = 0.0162 \quad \text{(Eq. 21)}$$

Finally, the upper and lower 95% confidence limits are computed. There is 95% assurance that the true potency lies between the upper and lower limits, with the best estimate of potency being that derived by the appropriate calculation for potency:

$$X_u = 100 \times \text{antilog}\left(CM + \frac{L}{2}\right) \quad \text{(Eq. 22)}$$

$$X_1 = 100 \times \text{antilog}\left(CM - \frac{L}{2}\right) \quad \text{(Eq. 23)}$$

X_u = percent potency, upper limit of 95% confidence interval (Eq. 24)

X_1 = percent potency, lower limit of 95% confidence interval (Eq. 25)

From Example 1:

$$X_u = 100 \times \text{antilog}\,[1.0105 \times (-0.00335) + 0.0162] = 103.0\% \text{ or } 660\ \mu g/mg \quad \text{(Eq. 26)}$$

$$X_1 = 100 \times \text{antilog}\,[1.0105 \times (-0.00335) - 0.0162] = 95.6\% \text{ or } 613\ \mu g/mg \quad \text{(Eq. 27)}$$

Example 2—The statistical analysis of turbidimetric assays (Table II) is performed in a similar manner. After the absorbance values are decoded, the first three readings are recorded for testing. The range of the values in each vertical column is obtained by subtracting the lowest value from the highest. The ratio of the largest range to the sum of the ranges should not exceed the appropriate critical value (0.389 for three tubes). If it does, the readings in the column with the largest range are checked to find the aberrant response. A reading from another tube of the same dose chosen at random is substituted.

Table II—Example 2, Assuming a Potency for the Unknown of 1000 μg/mg and a Ratio between Doses of 1.25:1[a]

Tube Number	S1	S2	S3	U1	U2	U3
1	32.3	26.8	21.6	33.0	26.1	21.5
2	31.1	25.5	21.0	31.8	26.4	21.0
3	31.9	25.3	21.3	32.2	26.0	21.8
Sum (T_t)	95.3	77.6	63.9	97.0	78.5	64.3
Range	1.2	1.5	0.6	1.2	0.4	0.8

observed $R = \frac{1.5}{5.7} = 0.2632$ critical $R = 0.389$

[a] To simplify calculations, the absorbance readings are recorded as whole numbers instead of thousandths.

This procedure is continued until the table has 18 values with no missing or aberrant readings. If there are no values left to be substituted, the assay is considered invalid.

The log potency, M, and the percent potency, P, are obtained from Eqs. 1 and 2, respectively. From Example 2:

$$M = \frac{0.12921\,(97.0 + 78.5 + 64.3 - 95.3 - 77.6 - 63.9)}{63.9 + 64.3 - 95.3 - 97.0} = -0.0061 \quad \text{(Eq. 28)}$$

$$P = 100 \times \text{antilog} - 0.0061 = 98.6\% \text{ or } 986\ \mu g/mg \quad \text{(Eq. 29)}$$

The error variance, s^2, is determined using:

$$s^2 = \frac{\sum y^2 - \frac{\sum T_t^2}{3}}{12} \quad \text{(Eq. 30)}$$

From Example 2:

$$\sum y^2 = 12{,}967.68 \quad \text{(Eq. 31a)}$$

$$\sum T_t^2 = 38{,}892.80 \quad \text{(Eq. 31b)}$$

$$\frac{\sum T_t^2}{3} = \frac{38{,}892.80}{3} \quad \text{(Eq. 31c)}$$

Therefore:

$$s^2 = \frac{12{,}967.68 - \frac{38{,}892.80}{3}}{12} \quad \text{(Eq. 32a)}$$

$$= \frac{12{,}967.68 - 12{,}964.2667}{12} \quad \text{(Eq. 32b)}$$

$$s^2 = 0.2844$$

(The values obtained for $\sum y^2$ and for $\sum T_t^2/3$ should be very close to each other numerically.)

To test the assay validity as in Example 1, the following equations are used:

$$ab = \frac{[S_1 + U_3 - (S_3 + U_1)]^2}{12} \quad \text{(Eq. 33)}$$

$$q = \frac{[S_1 + U_1 + S_3 + U_3 - 2(S_2 + U_2)]^2}{36} \quad \text{(Eq. 34)}$$

$$aq = \frac{[U_1 + U_3 + 2S_2 - (S_1 + S_3 + 2U_2)]^2}{36} \quad \text{(Eq. 35)}$$

$$F = \frac{ab + q + aq}{3s^2} \quad \text{(Eq. 36)}$$

From Example 2:

$$ab = \frac{[95.3 + 64.3 - (63.9 + 97.0)]^2}{12} = 0.1408 \quad \text{(Eq. 37)}$$

$$q = \frac{[95.3 + 97.0 + 63.9 + 64.3 - 2(77.6 + 78.5)]^2}{36} = 1.9136 \quad \text{(Eq. 38)}$$

$$aq = \frac{[97.0 + 64.3 + 155.2 - (95.3 + 63.9 + 157.0)]^2}{36} = 0.0025 \quad \text{(Eq. 39)}$$

Table III—Potency and $L/2$ Constants for Three-Dose Assay

•	1:1.12	1:1.2	1:1.25	1:1.33	1:1.5	1:1.56	1:2	1:4
P	0.06563	0.10557	0.12921	0.16513	0.23479	0.25749	0.40137	0.80275
$L/2$	0.00646	0.01672	0.02504	0.04090	0.08268	0.09945	0.24165	0.96660

$$F = \frac{0.1408 + 1.9136 + 0.0025}{0.8532} = 2.4108 \quad \text{(Eq. 40)}$$

Critical $F = 3.49$. The assay is valid.

If the assay is valid, the precision, C, is determined:

$$C = \frac{(S_3 + U_3 - S_1 - U_1)^2}{(S_3 + U_3 - S_1 - U_1)^2 - 56.964\, s^2} \quad \text{(Eq. 41)}$$

The confidence interval, L, is determined using Eq. 19.

From Example 2:

$$C = \frac{(63.9 + 64.3 - 95.3 - 97.0)^2}{(63.9 + 64.3 - 95.3 - 97.0)^2 - 56.964 \times 0.2844} = 1.0040 \quad \text{(Eq. 42)}$$

and:

$$\frac{L}{2} = \sqrt{(1.0040 - 1)\,[1.0040\,(-0.0061)^2 + 0.02504]} = 0.0100 \quad \text{(Eq. 43)}$$

Finally, the upper and lower 95% confidence limits are computed using Eqs. 22 and 23. From Example 2:

$$X_u = 100 \times \text{antilog}\,[1.0040 \times (-0.0061) + 0.0100] = 100.9\% \text{ or } 1009\ \mu\text{g/mg} \quad \text{(Eq. 44)}$$

$$X_l = 100 \times \text{antilog}\,[1.0040 \times (-0.0061) - 0.0100] = 96.4\% \text{ or } 964\ \mu\text{g/mg} \quad \text{(Eq. 45)}$$

Formulas for Determining Potency Constant and $L/2$ Constant for All Three-Dose Assays (Plate and Turbidimetric)—Determine the log of the interval of the ratio:

ratio	logarithm of interval between successive log doses (i)
1:1.12	0.04922
1:1.2	0.07918
1:1.25	0.09691
1:1.33	0.12385
1:1.5	0.17609
1:1.56	0.19312
1:2	0.30103
1:4	0.60206

Thus, $P = i(4/3)$ and $(L/2) = i^2(8/3)$ (Table III).

From all of the valid assays, the mean of the potency and 95% confidence limits of the mean are calculated:

$$\text{C.L.} = \overline{X} \pm t\left[\frac{\sqrt{\dfrac{\sum X^2 - \dfrac{(\sum X)^2}{N}}{N-1}}}{\sqrt{N}}\right] \quad \text{(Eq. 46)}$$

where:

C.L. = confidence limits
X = individual result or observation
$\overline{X}$ = mean, or average, of results
t = constant obtained from t table for $N - 1$ degrees of freedom (use a probability = 0.05)
N = number of results

Furthermore:

$$s = \sqrt{\frac{\sum X^2 - \dfrac{(\sum X)^2}{N}}{N-1}} = SD \quad \text{(Eq. 47)}$$

$$s_x = \frac{\sqrt{\dfrac{\sum X^2 - \dfrac{(\sum X)^2}{N}}{N-1}}}{\sqrt{N}} = SDM\ (SEM \text{ or } SE) \quad \text{(Eq. 48)}$$

For example, assume an estimated potency of 800 μg/mg:

assay number	potency
1	812
2	814
3	798
4	830
5	816
6	814

and:

$$\sum X = 4884.0 \quad \text{(Eq. 49)}$$

$$X = 814 \quad \text{(Eq. 50)}$$

$$t = 2.571 \quad \text{(Eq. 51)}$$

$$N = 6 \quad \text{(Eq. 52)}$$

$$\sum X^2 = 3{,}976{,}096.0 \quad \text{(Eq. 53)}$$

$$(\sum X)^2 = (4884.0)^2 = 23{,}853{,}456.0 \quad \text{(Eq. 54)}$$

$$\frac{(\sum X)^2}{N} = 3{,}975{,}576.0 \quad \text{(Eq. 55)}$$

$$\sqrt{N} = 2.4495 \quad \text{(Eq. 56)}$$

The confidence limits are:

$$\text{C.L.} = 814 \pm 2.571\,\frac{\sqrt{\left(\dfrac{3{,}976{,}096 - 3{,}975{,}576}{5}\right)}}{2.4495} \quad \text{(Eq. 57a)}$$

$$\text{C.L.} = 814 \pm 2.571\ (10.198/2.4495) \quad \text{(Eq. 57b)}$$

$$\text{C.L.} = 814 \pm 2.571\ (4.1633) \quad \text{(Eq. 57c)}$$

$$\text{C.L.} = 814 \pm 10.7038 \quad \text{(Eq. 57d)}$$

$$\text{C.L.} = 803\text{–}825 \quad \text{(Eq. 57e)}$$

The six assays, with a mean of 814 μg/mg, have 95% confidence limits of 803–825 μg/mg; *i.e.*, 95% of the time, the mean of six such assays will fall between 803 and 825 μg/mg.

An estimate of the variation of the individual results (X) used in determining the 95% confidence limits is made by computing the coefficient of variation:

$$\%\,CV = \frac{SD}{\text{mean}} \times 100 \quad \text{(Eq. 58a)}$$

$$\%\,CV = \frac{10.198}{814} \times 100 \quad \text{(Eq. 58b)}$$

$$\%\,CV = 1.25 \quad \text{(Eq. 58c)}$$

After the data analysis is completed and a potency is determined, the results and statistical analyses are sent to the participants with a request for their comments. Calculated potency is agreed upon, and the standard then becomes official. Before the advent of these equations, analyses of this kind were performed by statisticians. The use of these formulas has enabled NCAA to analyze the results of this type of studies quickly and simply.

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