Automated System for Analytical Microbiology III: Computer Interpolation of Potency

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Abstract □ Highly accurate photometric assays for antibiotic substances, such as those obtainable with a system composed of automated components, require an accurate method of obtaining potencies of samples. The usual manual interpolation of sample potencies from a standard dose–response line may introduce an error of several percent. Errors can come from the use of an inappropriate dose–response line and from the inability of the person interpolating to differentiate potencies with a resolution of less than 1%. A new dose–response line for manual interpolation of antibiotic potencies is described. Interpolations by means of a digital computer from that equation and from the relation log *A versus C* were both shown to reduce errors considerably. In addition, the computer may be put on-line and provide a typed report as soon as the last tube in an assay is processed.

Keyphrases □ Microbiology, analytical—computer interpolation of potency with automated systems □ Dose–response interpolation in automated microbiological methods—computer interpolation of potency □ Automated microbiological methods—use of digital computer to minimize errors introduced by manual interpolation of potencies □ Potency determination, automated microbiological methods—computer interpolation of data □ Computer interpolation of potency data—use with automated microbiological methods

Highly accurate photometric assays for antibiotic substances, such as those that may be obtained with a system composed of automated components[1], require an accurate method of obtaining potencies of samples. Values of sample potencies obtained by interpolation from the standard curve by the usual manual means can be in error by several percent. Such an error is both too large and unnecessary. A new presentation of the dose–response line was developed to reduce errors of manual interpolation for many assay systems. Application of a digital computer to the problem effected further reduction in errors of interpolation.

Unlike certain chemical assays which are absolute, microbiological assays are comparative in that the response of a sample is compared with the responses of standards within the same test. Usually, responses of the standards are plotted on graph paper. Potency of a sample is then obtained by interpolating from the standard response curve to find the concentration of standard corresponding to the response of the sample. The assumptions and pitfalls of such procedures were discussed at length elsewhere (1).

Dose–response lines may vary in shape from straight to strongly curved. Whatever the shape, they are approximated in practice by straight lines drawn between adjacent calibration points. The size of the error in estimation of potency caused by use of such an approximated curve depends upon the curvature of the line and the distance of the entry point from a calibration point. In addition to this error of an arithmetical nature, an error is caused by the inability of the person interpolating to be certain of the location of a point on the line with an error of less than about 1% of potency. Although the turbidity can be measured to the nearest millivolt (1000 mv. = 100% *T*) and absorbance can be calculated with three-digit accuracy, the data cannot be plotted on graph paper with equal accuracy.

Three conventional dose–response lines used in antibiotic assaying and a new one were programmed for a computer and tested to determine which gave the smallest computational errors and the least bias.

Table I—Measured Turbidities of Standards and Samples—Monensin Assayed with *St. faecalis* at pH 6

Substance	Turbidities, mv., Sample Size 0.10 ml.		0.15 ml.	
Standard				
0	378	378	403	403
1	427	426	471	469
2	475	473	529	527
3	512	512	571	569
4	544	544	603	602
5	570	569	636	635
6	591	590	664	663
Sample				
1.5	451	451	501	500
2.5	495	492	551	550
3.5	529	528	590	587
4.5	559	557	621	618
5.5	581	580	651	651

ACCURATE ASSAY SYSTEM

The type of assay system in which computational errors or errors of interpolation as great as 1% may be significant will now be described. A system for performing highly accurate turbidimetric microbiological assays was introduced in 1970[2] by Kuzel and Kavanagh (2, 3). The system consists of three elements: an automatic diluter, an automatic reader, and an incubation bath. The difficult-to-do operations are mechanized so that the critical steps of measuring sample volume, broth volume, and turbidity may be performed with a relative standard deviation of 0.1% or less.

The philosophy of the design was to measure so accurately and to control temperature of incubation and critical operations so carefully that significant errors could occur only external to the system. The answers obtained from this system could then be free

[1] Such as the Autoturb System.

[2] By the Elanco Division of Eli Lilly and Co.

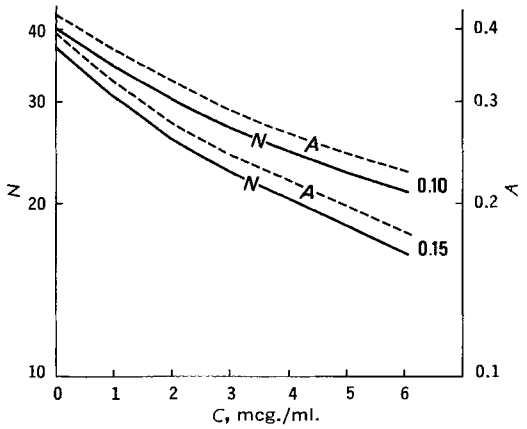

Figure 1—*Dose–response lines for monensin*–St. faecalis *assay for the two volumes of sample of 0.10 and 0.15 ml. Curves labeled* N *are for Eq. 1; curves labeled* A *are for Eq. 2. Only one of each pair of curves is shown.*

from operational bias and have a small relative error. In some assays, interpolation of potency from a standard curve seemed to be the main source of error. To reduce this last source of error to insignificance and to increase ease and speed of analysis, potencies are interpolated by a digital computer from the standard curve.

The automatic diluter prepares two sets of dilutions from each sample by means of four separate diluting channels arranged in pairs. Therefore, four responses are obtained for each sample or standard (no distinction is made in treatment of standards and samples). In the data reported here, one pair of samples received 1.5 times as much antibiotic as the other pair. Since four tubes are prepared for each sample, the data occur in sets of four (Table I). Each of the four entries for a sample represents a separate diluting channel. All standards and all samples pass once through each channel. The standards and samples pipeted by a channel are the assay unit. Potencies of samples in an assay unit are interpolated from the standard curve prepared from the standards in that unit. The advantage of this procedure is that the absolute volume pipeted by a channel need not be known because it is the same for standards and samples. What is important is that the volume be exactly ($\leq$0.1% error) the same for standards and samples. Apparently the automatic diluter achieves such accuracies.

When manual interpolation is used to obtain sample potencies in this system, the two responses from one sample size are averaged before interpolating from the standard curve. Thus, there are two standard curves, one for each size of sample. The curves usually are different because of a 0.5% difference in concentration of inoculum (1).

DOSE–RESPONSE LINES

At least 10 relations between concentration of antibiotic and the response obtained from the test have been used in antibiotic assaying. Of these, the one with a theoretical basis (4), a variant of it, and the new one just mentioned were investigated because usually these three are nearer to straight lines than the other seven. In addition, a relationship much used in official methods was included.

A theoretical relation between concentration, C, of the antibiotic and the response, N, obtained as concentration of bacteria

Table II—Potencies of the Five Samples of Table I Interpolated from Eq. 4

Putative Concentration	Concentration, mcg./ml., Interpolated from Eq. 4 — 0.10		0.15		Mean	*RSD*
1.50	1.483	1.515	1.493	1.510	1.500	1.00
2.50	2.526	2.471	2.503	2.527	2.506	1.04
3.50	3.516	3.485	3.575	3.526	3.526	1.06
4.50	4.564	4.507	4.523	4.462	4.514	0.93
5.50	5.512	5.512	5.512	5.548	5.521	0.33

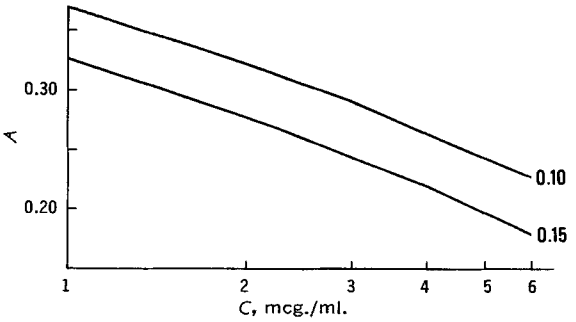

Figure 2—*Data of Fig. 1 plotted to relate absorbance and logarithm of the concentration.*

(1, 4) was shown to be of the form:

$$\log N = A - BC \qquad \text{(Eq. 1)}$$

where A and B are constants.

Values of N are not measured directly but are derived from the turbidity of the suspension by means of a calibration curve relating N to a corresponding absorbance. Accurate values of N can be obtained only by calculations which, however, are too extensive to do manually for a test involving 80 responses as in this automated system.

As a first approximation, N is proportional to absorbance, A. Substituting A for N in Eq. 1 gives Eq. 2:

$$\log A = E - FC \qquad \text{(Eq. 2)}$$

Accurate values of absorbance are easily computed from percent transmittance. Equations 1 and 2 are only approximately straight in actual practice. Therefore, a straight-line approximation of the equations will be slightly erroneous between the break points. This is illustrated by the curves in Fig. 1. Such straight-line approximations are employed in both manual and digital computer treatments. The commonly used relation, Eq. 3:

$$A \textit{ versus } \log C \qquad \text{(Eq. 3)}$$

is illustrated by Fig. 2.

A method of plotting and of computation possessing minimum inherent errors was needed. The method should use the response of the photometer (millivolts) to avoid the effort and errors of conversion. A new equation was devised to give a straight or nearly straight dose–response line when photometer response in millivolts was plotted against the concentration of antibiotic eliciting the response. The response, described by Eq. 4, is called an inverted logarithmic response (1) and is illustrated by Fig. 3. The equation of the line is of the form:

$$\log (D - V) = G - HC \qquad \text{(Eq. 4)}$$

where V is the photometer output in millivolts, C is the concentration of antibiotic, and G and H are constants for a particular line.

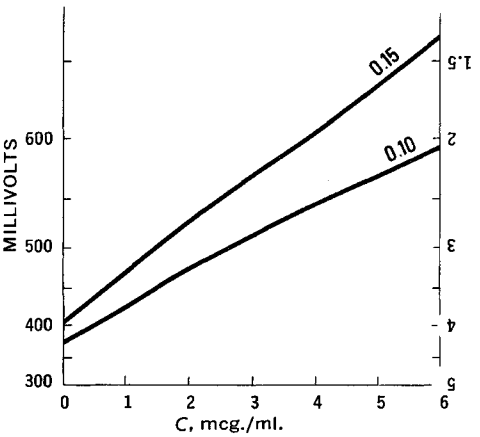

Figure 3—*Inverted logarithmic plot (Eq. 4) of the same data used in Fig. 1,* D = *800.*

Table III—Potencies and Relative Standard Deviations of the Five Samples in Table I Interpolated from Four Equations

Putative Concentration	Eq. 1		Eq. 2		Eq. 3		Eq. 4	
	Potency	*RSD*	Potency	*RSD*	Potency	*RSD*	Potency	*RSD*
1.5	1.536	1.04	1.516	1.01	1.430	1.06	1.500	1.00
2.5	2.536	1.05	2.519	1.05	2.474	1.09	2.506	1.03
3.5	3.550	1.08	3.537	1.08	3.507	1.12	3.526	1.06
4.5	4.538	0.85	4.526	0.88	4.504	0.87	4.514	0.93
5.5	5.544	0.41	5.533	0.38	5.516	0.41	5.521	0.33
ARE[a], %	1.17		0.72		0.64		0.38	
Bias	+		+		−		+	

[a] *ARE* is the average relative error as a percentage of the average potency. The errors were summed without regard to sign.

By shifting the starting point (changing value of D) of the curves up or down on the graph, the lines may go from concave to convex. At some intermediate starting point, the lines are straight or slightly sigmoid. Any starting point not at a major division of the semilogarithmic paper is very inconvenient except when a computer is employed. Therefore, as a practical matter, the starting point is selected to fall on the major division that gives a dose–response line nearest to a straight line over the region of principal interest. In many assays, the line is as straight as it can be plotted and drawn by hand. Interpolation of potencies from such a line is still subject to small errors because of the difficulty of estimating the value of C corresponding to a measured value of turbidity. Computer interpolation eliminates this last error.

An important advantage of Eq. 4 is the use of raw data; no transformation of photometer response is made as in Eqs. 1–3. This is an advantage in manual but not in computer interpolation. A value of D must be assigned before the equation can be used. A restriction is that D must always be greater than the largest response from the photometer. Usually, values of D between 800 and 1200 are used; it is 800 in Fig. 3. Had the 400-mv. point been put at 6 on the log scale, D would have been 1000. A change of 100 in the value of D has a trivial effect upon calculated potency for those assays to which the equation is applicable; *i.e.*, gives a nearly straight line. The value of D may be found by means of the computer program mentioned later or by plotting on semilog paper. If the latter procedure is followed, D is changed in value by steps of 100, and the points of the standard curve are plotted. The value of D that gives the lines nearest to straight in the region of interest is entered into the computer program.

Although Eq. 4 has no apparent theoretical basis, it does have the advantage of being more nearly straight than the others for many antibiotic assays.

If a line is mathematically straight, any deviation of the actual points from the line is presumed to be the result of an error. Upon this assumption, the "best" straight line through the points of a standard line may be found by means of a least-squares solution. A computer program was written to do this for Eq. 4. Values were assigned to D in steps of 10, the best straight line was found, the values of turbidity corresponding to each C were computed from the equation for the line, and the difference between computed and measured turbidity was found, squared, and summed. The value of D giving a minimum sum was then used in calculating the dose–response line from which potencies of samples were computed. This iterative computation of the best straight line could occupy the computer for as long as 75 sec.

When experience showed that most dose–response lines were not straight but slightly sigmoid, this approach to computation of dose–response lines was abandoned in favor of point-to-point interpolation. The computer programs to do this were written in Dartmouth Basic and run on a computer[3]. Basic was used because of the ease of programming. The programs in Basic were discarded at the end of this experiment. New programs were written in FORTRAN and assembly language to be used for routine data acquisition and for calculations by an on-line computer.

TEST OF EQUATIONS

The equations were tested with data from assays known to be capable of the precision required. These were: penicillin G–*Staphylococcus aureus*, erythromycin–*S. aureus*, and monensin–*Streptococcus faecalis* assays (5). Data from monensin and penicillin assays were selected to illustrate the point-to-point interpolation by computer from the four equations. Responses in the form of the millivolt output of the photometer, concentrations at the points of the standard curve, values of D, and the coefficients of the calibration equation needed to compute N were entered into the computer. A set of four potencies and their mean were obtained for each sample. The relative standard deviation (coefficient of variation) for each set of four potencies was printed with their mean.

The samples were of two kinds: standards prepared to have concentrations between points on the standard curve and concentrations used as points of the standard curve. Since the samples were prepared from the same stock solution as the standards, the assumption was made that the sample concentrations were as accurately known as the standards. Errors in preparing the samples and standards should be less than 1%.

DISCUSSION

The data for a standard curve and five samples are given in Table I. The readings of turbidities of the pairs of assay channels differ because the channels are not identical, as was shown by diluting dye solutions (3), and because of small errors.

Potencies for the samples of Table I are given in Table II. Potencies were interpolated by means of Eq. 4. Each of the first four entries on a line represents the potency obtained from the corresponding channel of the diluter. Potency was independent of sample size. Relative standard deviation (*RSD*, coefficient of variance) was used as a statistical measure of agreement within the set of four responses constituting an assay of a sample. It was a measure of the ability of the assay system (antibiotic, organism, and equipment) to perform reliably, but it was not a measure of the accuracy of the assay. The *RSD* of about 1% is quite satisfactory for such an assay system and is about the size of error caused by an uncertainty of 1 mv. in measuring turbidity. The digital voltmeter of the reader had a last place uncertainty of reading of 1 digit or 1 mv. The agreement among the four potencies obtained for a sample is typical of assays performed with this automated system when the assay system of organism and antibiotic is capable of high precision (1).

The data of Table I were processed with the aid of the four interpolation equations, and the results are shown in Table III. Each potency is the mean of the four responses obtained in assaying a sample. The potencies are not strongly dependent upon the equation used in the interpolation, because none of the dose–response lines is strongly curved (Figs. 1–3) and all may be approximated by straight-line segments. The values of *RSD* are about the same for all equations, because they are principally a measure of agreement among the four turbidities obtained for a sample. The equation used for interpolation had only a second-order effect upon *RSD*.

The errors caused by the approximations inherent in the interpolation expressions were different for the four equations. Equation 1 gave the largest error because the relation used to compute N from turbidity (millivolts) was itself only an approximation. Equation 2 was next in error. Equation 3 gave the third largest errors; the low bias at the 1.5 and 2.5 parts of the dose–response line was a principal cause of the large errors. At the higher part of the dose–response line, Eq. 3 was the best. It was rejected in favor of Eq. 4 because it was not as applicable over the whole range of the dose–response line. Equation 4 gave the least error over the entire range

[3] Hewlett-Packard model 2115.

Table IV—Assay of Nine Samples of Penicillin G with *S. aureus*[a]

Known Potency of Sample	Eq. 1		Eq. 2		Eq. 3		Eq. 4	
	Mean	*RSD*	Mean	*RSD*	Mean	*RSD*	Mean	*RSD*
2.50	2.574	2.1	2.534	2.1	2.484	2.1	2.504	2.0
3.00	3.099	1.3	3.090	1.2	3.079	1.0	3.083	1.1
3.33	3.366	2.5	3.341	2.5	3.310	2.4	3.322	2.4
3.33	3.383	1.9	3.358	1.9	3.326	1.8	3.338	1.9
3.00	2.979	0.5	2.976	0.6	2.971	0.7	2.973	0.6
3.00	3.127	1.3	3.115	1.2	3.101	1.1	3.106	1.1
2.50	2.602	1.3	2.562	1.3	2.512	1.4	2.532	1.3
2.00	2.000	0.7	2.000	0.7	1.998	0.8	1.998	0.7
1.50	1.505	1.5	1.505	1.4	1.504	1.2	1.504	1.3
ARE[b], %	2.00		1.61		1.09		1.05	

[a] Samples are listed in the order in which they occurred in the test. [b] *ARE* is the average relative error as a percentage of the average potency. The errors were summed without regard to sign.

of the assay, as shown by the size of the average relative error (*ARE*). A quite different assay, penicillin G by an *S. aureus* method, supported these conclusions (Table IV). The indication of bias given in the last line of Table III shows the direction of the error caused by the straight-line approximation. The errors were small even for the worst condition. Occasionally, Eq. 3 or the response *V versus C* will give the best straight line in an antibiotic assay. The response to use for growth-promoting substances is *N* or *A versus C*.

The best relation is the one giving a dose–response line nearest to straight over the region of interest. Although the inverted log response has been the most generally applicable for antibiotic assaying, other relations may be better under certain circumstances. The analyst who wishes to minimize computational errors must be prepared to use the form of dose–response line best for a particular assay.

When only the relative standard deviations of the four responses obtained for each sample are considered, there is little reason to choose one of the four equations relating response and concentration over the others. Those who believe the inherent error of a microbiological assay to be at least $\pm 10\%$ would consider the equations to be equivalent. Those analysts who judge the quality of an assay by the statistics of the responses constituting a potency would draw the same conclusion. However, a pharmaceutical preparation is sold on the basis of the quantity of active drug, not upon the statistics of the responses. Therefore, the equations to be used in computing potencies should be the ones giving minimum error.

The emphasis placed here upon achieving minimum error should not be taken as denigration of the statistics of response for a sample. Such statistics of this automated system provide the analyst a quick check of the operations of the equipment and of identity of standard and sample. The potencies obtained from each element of a pair of channels should be nearly identical when the equipment is operating correctly. The two potencies obtained from the two sets of channels will be nearly identical when sample and standard contain the same active drug. Any substantial difference indicates a difference in composition of standard and sample and a questionable assay.

In addition to reducing errors and increasing resolution of an assay, the computer can calculate all parts of an assay and be put on-line to provide a typed report as soon as the turbidity of the last tube in a test is measured.

To achieve the inherent accuracy of this automated system, a computer must be used to make the interpolations from a dose–response line selected to minimize computational errors.

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