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PHARMACEUTICAL TECHNOLOGY

Automated System for Analytical Microbiology V: Calibration Lines for Antibiotics

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Abstract □ The accuracy of an automated system for the microbiological assay of antibiotics was increased by improvement attendant to connection to an on-line computer. The system was used to investigate the suitability of four forms of interpolation formulas by assaying for chlortetracycline and erythromycin. The calibration lines were prepared as point-to-point straight-line approximations and as cubic equations. Cubic equations through four calibration points were preferred. Since the automated system was a four-channel instrument, a separate response line was prepared for each channel. Combining the four response lines into one could substantially degrade the accuracy and precision of assays. A new general equation relating the response of the test organism to concentrations of active materials was used to account for factors in addition to the antibiotic upon the dose–response line. Some of these factors were: diluents, growth substances, relative proportions of mixed antibiotics, pH and buffer capacities of the sample solution and assay broth, salts, and organic compounds in samples and not in standard solutions. The equation was used to show under what conditions the dose–response lines of mixtures and single-component antibiotics could be the same. It could also account for the nonspecific nature of turbidimetric assays. The equation showed assay biases to be caused not by differences in composition of antibiotics in standards and samples but by differences in other substances affecting growth of the test organism. A new dose–response line applicable to assays using *Klebsiella pneumoniae* was described.

Keyphrases □ Microbiology, analytical—automated system, calibration lines for antibiotics □ Automated analysis—system for analytical microbiology, calibration lines for antibiotics □ Antibiotics—automated system for analytical microbiology, calibration lines □ Chlortetracycline—automated analysis, evaluation of interpolation formulas, calibration lines □ Erythromycin—automated analysis, evaluation of interpolation formulas, calibration lines

Automation of critical steps in the turbidimetric microbiological assay (1, 2) resulted in a significant increase in the accuracy and precision of assays. A further increase came when interpolation of sample potency was performed by a computer (3). A limitation of the latter was caused by the three-digit resolution of the digital voltmeter used to measure output of the spectrophotometer. Connection of the spectrophotometer to the computer dedicated to analytical services effected a further increase in accuracy and precision (4). The on-line computer had the further advantage of providing a typed report of assay results within 5 min after the last assay tube had been measured. Computerization of reading and recording of turbidity and calculations of potencies completed automation of the operational parts of the assay system.

Operational aspects of automated assays are now of such precision that the form of the calibration line used for interpolating potencies of samples can significantly affect accuracy and precision. Attention will be directed to consequences of using several forms of the lines. One with a theoretical basis and four empirical ones will be considered.

The philosophy guiding the design of the automated system (1, 2) was to minimize variances caused by operation of the electrical and mechanical parts. The same philosophy is applied in this report to treatment of the data to extract potencies with a minimum of computational errors.

EXPERIMENTAL

Preparation of Tests—The five dose–response lines were ap-

plied to assays of erythromycin and chlortetracycline. *Staphylococcus aureus* (ATCC 9144) was the test organism for both tests. The automated turbidimetric system[1] was operated exactly as described previously (2). The turbidity of the suspension of bacteria in the assay tubes was measured photometrically by a spectrophotometer. The output of the spectrophotometer went directly to the computer for storage and subsequent processing. The samples were points of the standard curve and intermediate concentrations of standard for erythromycin assay or chlortetracycline extracted from animal feeds.

The erythromycin solutions were prepared in pH 7 buffer. Chlortetracycline was extracted by, and diluted to assay concentration with, methanol containing 1% concentrated hydrochloric acid solution. The automated diluter measured two 0.1-ml portions of sample into each of two contiguous assay tubes and diluted each to 10 ml with inoculated broth. The diluter also measured 0.15-ml portions of the sample into two assay tubes and diluted each to 10 ml with inoculated broth. The two sets of assay tubes, differing by 50% in quantity of sample and 0.5% in quantity of inoculated broth, constituted an assay of a sample.

Calibration Lines—The calibration line is the means by which responses to the samples are converted into equivalent concentrations of standard. The five lines discussed here are in daily use in pharmaceutical companies. The equations of the lines are given in generalized form. The first is the one derived from the dose–response line and is applicable to most antibiotic assays (5, 6):

$$\log A = E + BC \qquad \text{(Eq. 1)}$$

where A is the absorbance of the bacterial suspension, C is the concentration of the antibiotic in the sample tube, and B and E are constants.

The second is an empirical equation devised for manual interpolation (3) because of the ease by which it can be put into a nearly straight line on graph paper:

$$\log (D - T) = E + BC \qquad \text{(Eq. 2)}$$

where T is the transmittance of the bacterial suspension in the assay tube, and B, D, and E are constants.

The third equation is the dose–response line used for diffusion assays and, by some, for turbidimetric assays:

$$\log C = E + BT \qquad \text{(Eq. 3)}$$

In applying these expressions, four equations are used, one for each channel of the diluter. The average of the four potencies, one from each channel of the diluter, is taken to be the potency of the sample. Interpolation of sample potencies from the responses is done by the computer from the appropriate straight-line segment of the calibration lines drawn from point-to-point.

In a variant of Eq. 3, responses of the two 0.10-ml samples are averaged and the two responses from the 0.15-ml samples are averaged for each concentration, C, of standard. The averages are then used in constructing the standard curve. The concentrations of standard in the sample tubes are in the ratio of 1:2:4. To construct the standard curve, the 0.1-ml sample of one is assigned a value of 1, the 0.15-ml sample of one is assigned a value of 1.5, the 0.1 ml of two is assigned a value of 2, the 0.15 ml of two is assigned a value of 3, *etc.* Thus, one standard curve—not two—is made from the mean responses. Interpolation of sample potency is done from the individual responses. The two responses from the 0.15-ml amounts are divided by 1.5 before the mean of the four potencies is taken to represent the potency of the sample.

Cubic Equations—A graph of Eq. 3 usually has one inflection; therefore, it can be approximated by a cubic equation such as:

$$\log C = F + BT + DT^2 + ET^3 \qquad \text{(Eq. 4)}$$

A line used by the Food and Drug Administration (7):

$$A = K + L \log C \qquad \text{(Eq. 5)}$$

can be fitted by a cubic equation similar to Eq. 4.

[1] AUTOTURB System, Elanco Division of Eli Lilly and Co.

Table I—Responses to Standards and Samples of Erythromycin

Sample Number	Concentration, μg/ml	Responses, T, for Sample Volumes of 0.10 ml		0.15 ml	
1	0	54.65	54.70	54.86	54.86
2	1	60.40	60.49	64.66	64.86
3	2	67.49	67.44	72.86	73.11
4	3	72.56	72.83	77.96	78.01
5	4	76.04	75.97	81.08	81.03
6	5	78.83	78.94	83.46	83.49
7	3	72.67	72.80	78.01	78.09
8	2.5	70.50	70.70	75.79	75.82
9	3	72.60	72.62	77.72	77.82
10	3.5	74.45	74.62	79.82	79.79
11	4	75.96	75.93	81.07	80.95
12	2	67.41	67.65	72.92	72.91

In applying Eqs. 4 and 5, a separate equation was derived for each channel of the analyzer, as was done for point-to-point interpolations. These computations were done off-line, using data in Table I; therefore, unlike results for Eqs. 1 and 3, they would contain the rounding-off errors of data in the table. A cubic expression will pass through all four points of a four-point standard curve. When the number of standards is more than four, a line of best fit (least squares) is found. Such a line may not pass through all calibration points.

Two forms of a cubic equation were used to obtain potencies given in Table II. They are labeled 4(4) for Eq. 4 fitted to four calibration points and 4(5) for Eq. 4 fitted to all five points. A similar numbering system identified the four-point versions of Eqs. 1 and 5.

Computer Interpolation—The computer was programmed to compare sample responses with those of standards to decide what segment of the dose–response line to use for the interpolations. When sample responses were identical with those of a standard, the value of that standard was selected as the potency of the sample without further computation. When sample response was different from a standard, interpolation was from a line drawn between responses of the two standards bracketing the sample. The line was straight for the point-to-point lines and was a smooth curve for the cubic equations. Although the results in Table II are reported to four significant digits, the significance of the fourth is questionable. It was retained to avoid rounding-off errors.

Errors made in preparing the standards can cause a measurable error in response. The errors in concentrations of standards should

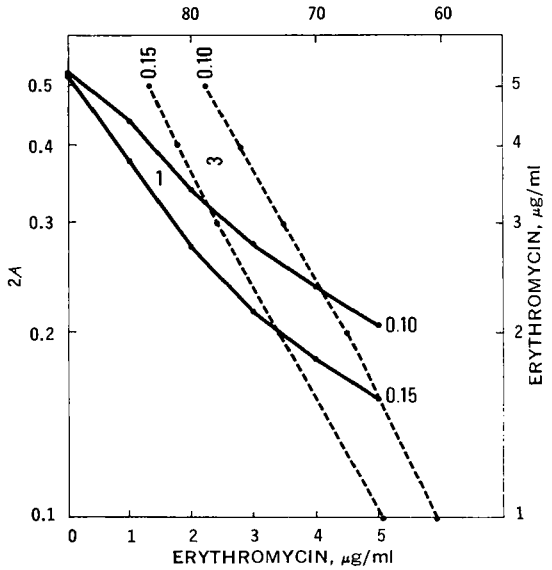

Figure 1—*Erythromycin dose–response lines for Eqs. 1 and 3. Only one of each pair of responses is shown. For convenience in plotting, 2A is used in place of A in Eq. 1.*

Table II—Assay of Erythromycin Standard Solutions (Potencies Obtained from the Indicated Interpolation Equations)

Sample	Equation[a]	Individual Potencies, μg/ml				Mean Potency, μg/ml	σ
		Sample Volume, ml					
		0.10		0.15			
8	1	2.578	2.588	2.553	2.532	2.563	0.025
	1(4)	2.546	2.536	2.506	2.483	2.518	0.029
	3	2.544	2.556	2.524	2.503	2.532	0.023
	3 (1 line)	2.500	2.537	2.613	2.620	2.567	0.059
	4(4)	2.549	2.546	2.510	2.487	2.525	0.032
	4(5)	2.559	2.586	2.524	2.504	2.543	0.036
	5(4)	2.532	2.477	2.486	2.455	2.487	0.032
9	1	3.011	2.958	2.949	2.958	2.969	0.028
	1(4)	3.008	2.950	2.936	2.948	2.960	0.032
	3	3.010	2.953	2.943	2.953	2.965	0.030
	3 (1 line)	2.916	2.921	3.060	3.085	2.996	0.090
	4(4)	3.009	2.950	2.939	2.950	2.962	0.032
	4(5)	2.883	3.004	2.959	2.974	2.955	0.052
	5(4)	3.008	2.944	2.933	2.944	2.957	0.034
10	1	3.529	3.558	3.580	3.574	3.560	0.025
	1(4)	3.501	3.514	3.542	3.541	3.525	0.020
	3	3.507	3.535	3.561	3.555	3.539	0.024
	3 (1 line)	3.449	3.506	3.623	3.614	3.548	0.095
	4(4)	3.496	3.503	3.541	3.536	3.519	0.023
1 standard	4(5)	1.000	1.001	1.000	1.000	1.000	0.000
2 standard	4(5)	1.997	1.986	1.995	1.995	1.993	0.005
3 standard	4(5)	3.014	3.053	3.020	3.024	3.028	0.017
4 standard	4(5)	3.980	3.921	3.969	3.964	3.959	0.026
5 standard	4(5)	5.036	5.066	4.982	5.005	5.022	0.037

[a] Results from four lines drawn point-to-point for both Eqs. 1 and 3. Calibration points for Eq. 3 (1 line) was obtained from responses to standards 1, 2, and 4 μg/ml. Results for Eqs. 4 and 5 were obtained from four cubic equations, one for each channel of the system. The equations were fitted exactly to four calibration points for 1(4), 4(4), and 5(4). A best cubic equation was fitted to five points for 4(5).

be less than 0.2% to cause an error in response not larger than the resolution (1 in 3400) of the analog-to-digital converter of the spectrophotometer. Class A volumetric pipets (≥2 ml) and volumetric flasks (≥100 ml) must be carefully used to avoid significant errors of concentrations.

RESULTS

Erythromycin—Concentrations of standards and samples and the corresponding responses are given in Table I for the erythromycin assay. The first six entries are standards and the last six are samples. The 2-, 3-, and 4-μg/ml samples are the same solutions as the corresponding standards. Standard curves are depicted in Fig. 1. Only one set of responses is given for each sample volume. The calibration lines are approximated by straight-line segments because the computer was so programmed. In this example, the log *C versus T* lines were less curved than the log *A versus C* lines. The latter could have been straightened in the region of significant curvature by modifying the expression to be log *(A − D) versus C*. When this straightening was done, the difference in answers was very small and the difference in precision was insignificant.

A comparison of the results of interpolating from four different dose–response lines is given in Table II. The differences between potencies interpolated from Eqs. 1 and 3 were small, and the standard deviations were the same. The single-line version of Eq. 3 was poorest, as indicated by the large standard deviations. Potencies computed from 1(4), 4(4), and 5(4), the four-point cubic equations, were nearly the same as those interpolated from straight-line versions of Eqs. 1 and 3. Equation 4(5) could give a computational bias of about 1%, as shown by results on the standard curve given in the lower part of Table II. These values were obtained by using responses of the standards to compute equivalent potencies. Similar computations using 1(4), 4(4), and 5(4) gave potencies of 3.000 for the 3-μg/ml standard whereas 3.028 was obtained from 4(5).

Chlortetracycline—The assay for chlortetracycline differs from most antibiotic assays in the large difference in turbidities between the assay tubes without antibiotic. Figure 2 shows the calibration lines for Eqs. 1 and 3 and for the single-curve version of Eq. 3. Both Eqs. 1 and 3 are more strongly curved than the lines for erythromycin. Therefore, the straight-line approximations are not quite as good as for erythromycin. The line for the single-curve version of Eq. 3 is rather unusual for a calibration line. The corresponding line for erythromycin and other antibiotics has the same general shape but is much less extreme.

Results for three samples are given in Table III. The single-curve version of Eq. 3 produced potencies lower than those obtained from Eqs. 1 and 3 and with significantly higher standard deviations. Computational biases are evident and are a principal cause of the large standard deviations. The errors are most pronounced at the lower concentrations, as the line in Fig. 2 indicates they should be. The last line in Table III shows the size of computational errors inherent in the procedure. These numbers were obtained by entering the responses for the 0.2-μg/ml standard in the section of the protocol devoted to samples and then calculating to obtain the indicated potencies.

The 0.120-μg/ml values were obtained from the segment of the calibration line lying between 0.1 and 0.15 μg/ml. The 0.1 point represented responses to 0.10 ml of the 0.1-μg/ml standard. The other end of the segment represented responses obtained from 0.15 ml of the 0.1-μg/ml standard. Thus, the values of 0.120 μg/ml were obtained by using responses to 0.10 ml of sample to interpolate from a calibration line quite different from that for 0.10-ml samples. In contrast to the 0.10-ml samples, the results for the 0.15-ml portions were interpolated from a region of the calibration line (very near a calibration point) where the slope had little influence. Results would be different away from one of the calibration points. Generally, the single-line version will bias the 0.10-ml samples low and the 0.15-ml samples high. The mean of such biased answers is not as near to the correct answer as the automated system is capable of producing.

DISCUSSION

Selection of Interpolation Formulas—Agreement between an answer and the expected result is not sufficient to permit selection of the best interpolation formula. Even procedures that minimize standard deviation may not be the best. Anyone who follows these guides may be trapped into selecting incorrect methods. The principle applied here is that "correct" answers are not obtained by procedures with inherent biases. In deciding which one to use, the assumption is made that responses of the standard curve are without error.

When sample responses are compared one-to-one with responses of the standard, the sample response indicates whether the sample

Table III—Assay for Chlortetracycline in Animal Feeds

Sample	Equation	Individual Potencies, μg/ml: Sample Volume, ml 0.10		0.15		Mean Potency, μg/ml	σ
2	1	0.467	0.471	0.469	0.464	0.468	0.003
	2	0.467	0.471	0.468	0.464	0.467	0.003
	3 (1 line)	0.452	0.459	0.467	0.470	0.462	0.008
9	1	0.356	0.346	0.335	0.325	0.341	0.013
	2	0.353	0.343	0.330	0.319	0.336	0.014
	3 (1 line)	0.284	0.279	0.350	0.346	0.315	0.038
11	1	0.186	0.159	0.150	0.150	0.163	0.016
	3 (1 line)	0.121	0.113	0.176	0.179	0.147	0.035
0.2 standard	3 (1 line)	0.125	0.124	0.201	0.200	0.162	0.043

measures more or less than the standard. Only those interpolation procedures are valid that always give potencies that deviate from the standard in the same direction as the responses indicate they should. This criterion, when applied to the four kinds of calibration lines, shows that all give acceptable results when a separate line is used for each channel of the diluter.

Cubic equations fitted to more than four points degrade accuracy and precision, as shown by lines 4(5) in Table II. The single-line version of Eq. 3 gives grossly erroneous results, as shown by those in Table III and confirmed by those in Table II. In Table II, Sample 9 is a standard solution assayed as a sample, the response of which can be compared with the responses of the 3-μg/ml standard. Potencies interpolated from Eq. 3 (one line) have only one result, 2.921, that deviates from Eq. 3 in the direction indicated by the responses; the three other results are biased. Inspection of the table reveals the large standard deviations of results obtained from the one-curve version of Eq. 3 to be caused by systematic errors.

Regardless of the form of the interpolation equation, the one that governs responses is Eq. A1 (see *Appendix*). Therefore, anything that affects coefficients of Eq. A1 also affects results interpolated from any other equation. Equations 1–3 and 5 represent convenient interpolation formulas—not dose–response lines.

A conclusion of interest is that in any assay corresponding to the assumptions (6) made in deriving Eq. A1, one antibiotic can be assayed in terms of another and the potency of the sample will be independent of dose. Lack of identity of standard and sample will not cause an obvious bias in the assay, even though potency of the sample may be grossly erroneous.

"Best" Calibration Lines—"Best" straight lines and curves of best fit are popular calibration lines without any evidence that the lines should be straight or that the best fit is such in other than a statistical sense. Use of an equation of best fit in place of one that goes through the actual calibration points or a point-to-point line implies that deviations of the calibration points from the best fit are caused by variances in responses.

Experience with the automated assay system indicates small variances when it is correctly operated. Each response may be assumed to be at or very near to the true response and to deviate, if at all, by a small unknown amount. Since deviation from true is unknown and unknowable for each point of a standard curve because each is determined by only one response, the simplest assumption is that of no error in the standard. This procedure attributes all error to the sample but does not, of course, necessarily increase the size of the error.

Equation 1, derived from the dose–response line (Eq. A1), should not be straight because of several approximations made in deriving it. To obtain Eq. 1, absorbance was substituted for N in Eq. A2 whereas, in reality, A and N were related by a quadratic expression (6). Light-scattering properties of the bacteria may not be independent of antibiotic concentration, thereby adding an additional nonlinearity to A. For these reasons, Eq. 1 is unlikely to be straight over an extended range of concentrations. Portions of the line may approximate a straight line as plotted, especially if specifications of accuracy are relaxed (Fig. 3).

Although Eq. 1 was derived for the simplest system, it was a satisfactory interpolation formula for more complex ones. Published data were used to show its applicability to antibiotics that bind to ingredients of the medium (8), to antibiotics enzymatically degraded in the biophase of the test organism (9), and to systems showing two phases in the drug-affected growth curve (10).

Equations 2, 3, and 5 exhibit degrees of curvature varying from assay to assay. Usually Eq. 2 can be put into a nearly straight line. There are two reasons not to use a single calibration line with the automated system: the two sample sizes always produce different dose–response lines because of the difference in initial concentrations of test organisms, and the menstrum effect (Eq. A1) may cause distortion (Fig. 2). Another defect in the single-line version of Eq. 3 is that the geometric spacing of concentrations of standards is too great. Concentrations of standards for turbidimetric assays should be uniformly spaced as in the erythromycin assay (Table I), regardless of the form of the calibration line used for obtaining potencies because the real dose–response line is Eq. A1 (or A2). The other forms (Eqs. 2–5) are simply convenient interpolation formulas with no theoretical foundations.

The best practice for more than four calibration points seems to be the point-to-point line whether the equation be 1, 2, 3, or 5. Whether 1, 3, or 5 is used for computer interpolations depends upon which gives lines with less curvature over the region of interest. When interpolation is done from a graph, the same criterion applies to Eqs. 1–3 and 5. Sometimes Eq. 5 is a little less curved than Eq. 3. If a cubic equation is used, it should be restricted to four points of the standard curve selected to cover the least curved portion of the line if such restriction meets the analytical needs. The cubic equation, when appropriate, probably gives the smallest error of interpolation.

Enough evidence has been given to establish the importance of considering consequences of Eq. A1 when selecting computational

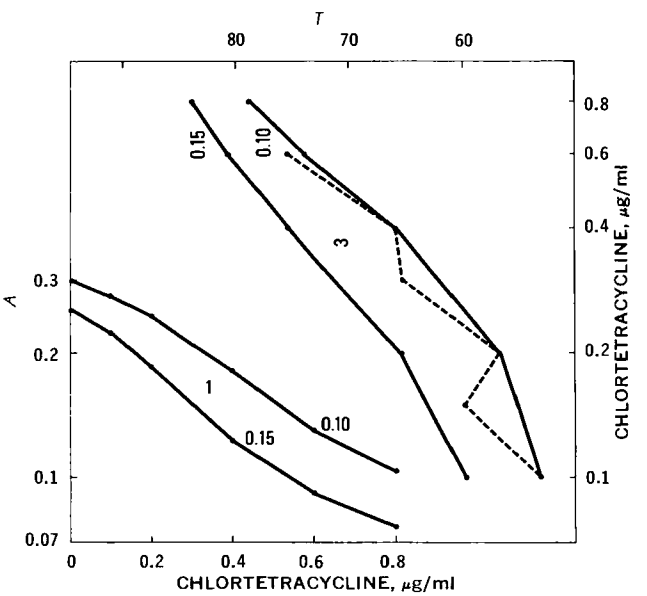

Figure 2—*Chlortetracycline dose–response lines for Eqs. 1 and 3. Only one of each pair of responses is shown. The dashed line is the single-line version of Eq. 3.*

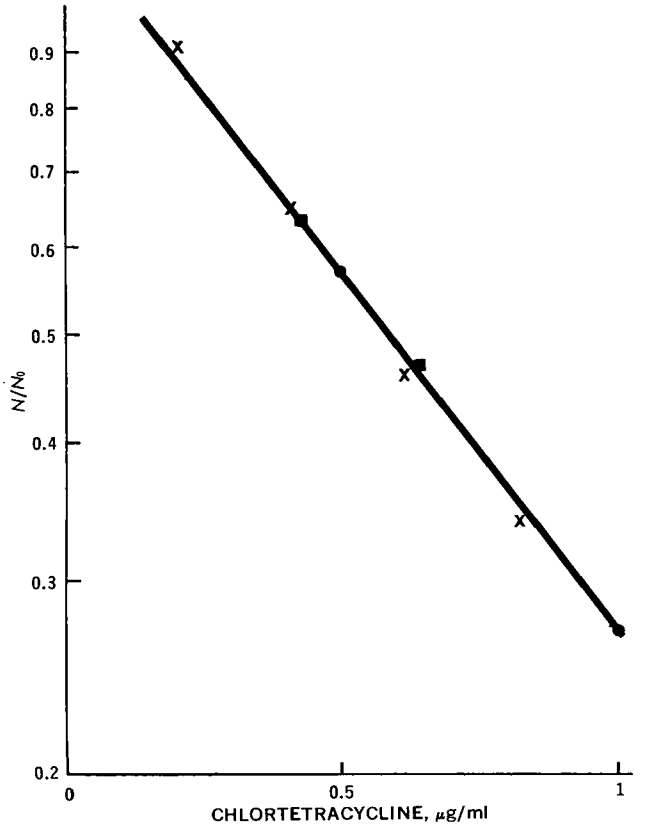

Figure 3—*Transformation of doses (Eq. A4) of erythromycin (■) and tylosin (×) to correspond to the chlortetracycline line. The erythromycin and tylosin lines were made to coincide with the chlortetracycline line at about 0.4 μg/ml. The multiplier of dose was 0.22 for erythromycin and 0.102 for tylosin.* N *was the concentration of bacteria in tubes with antibiotic, and* N_0 *was the concentration in tubes without antibiotic.*

procedures for the automated system. If only low accuracy and precision assays are wanted, the form of the dose–response line and computational procedures are not important. But the influence of $f(v)k_m$ and N_0 of Eq. A1 cannot be ignored if full accuracy and precision of the automated system are to be achieved. A separate interpolation equation for each channel of the diluter must be used to obtain potencies with minimum interpolation error. Thus, each assay requires construction of four interpolation lines or equations, an operation most conveniently done by a computer. If calculations must be done by hand, straight-line interpolation using Eq. 3 requires the fewest operations of a calculator[2].

In a turbidimetric assay, the test organism is exposed to all components of a mixture in the same proportion as they occur in the sample. This is in contrast to diffusion methods in which the several components may reach affected bacteria in ratios quite different from those in the sample. Such fundamental differences in behavior are part of the explanation for different potencies being obtained from the two methods applied to mixtures of antibiotics.

Influence of pH on Activity—The general equation for dose–response lines, Eq. A1 (see *Appendix*), may be used to study the effect of the pH of the assay broth upon activity. The equation is applicable to mixtures as well as to single-component antibiotics. Although the pH of the medium affects the concentration of the active form of the drug, attributing the changes in activity solely to changes in the size of the specific inhibitory coefficient (k_a) simplifies the discussion. The changes in k_a change the slope of the dose–response line, which shows as a change in activity. If the standard and sample contain the same single species of drug, the relative activity of the sample and standard is independent of pH.

Mixtures may give a different response. The k_a of one component of a mixture may change more with the pH of the assay broth than the k_a of another. The effect is to change the size of B, slope, in Eq. A4 more for certain samples than others, resulting in a change of relative potencies. A sample could be less active than the standard at one pH and more active at a different pH of broth. Consequently, potency of mixtures should be measured under carefully specified and attained conditions; otherwise, different assays may find significantly different answers.

Mixtures, unlike single-component antibiotics, do not have a potency directly translatable into weight except in the rare instances where the standard and sample have identical ratios of components. The potency of a mixture is equivalent to the activity in a particular assay of a certain concentration of standard and not to the concentration of standard. Samples of many acidic and basic antibiotics are mixtures not necessarily of the same relative composition as the standards. Assays of these antibiotics may show high interlaboratory variances in potency.

Indications of Invalidity—The second sample volume of 0.15 ml was introduced into the automated system solely to test the invalidity of the assay of a sample. It was not required for purely analytical purposes. If the response of the standard and sample falls on the same dose–response line, the potency of the sample is independent of the concentration at which it is assayed. In other words, if the standard and sample are indistinguishable, there is no evidence of invalidity. When the two sets of potencies (from 0.10 and 0.15 ml of sample) for a sample differ by more than experience shows is normal for the substance and type of sample assayed, potencies are of questionable validity.

In practice, especially with the assay of extracts of animal feeds, potencies obtained from the 0.1- and 0.15-ml volumes of samples may not be the same, thereby indicating bias. Such bias casts doubt upon the accuracy of the potency. The bias may be caused by inhibiting or stimulating substances in the extract, the $f(v)k_m$ effect (Eq. A1). Equation A1 indicates that antibiotics in the sample but not in the standard may not cause assay bias.

Once the nonantibiotic source of bias is recognized, steps can be taken to minimize it. For example, the inhibitory substance (acid methanol) in the chlortetracycline extract was not removed by evaporation because of the difficulty of being certain that standards and samples were treated in an identical manner. Instead, samples and standards were diluted in the same solution used to extract the antibiotic from the feeds. This procedure could be followed because the automated system measured acid methanolic solutions as accurately as aqueous ones. Acid methanol inhibited growth of *S. aureus*, as shown by the large difference in turbidity of the zero tubes in Fig. 2. Tubes of the lower line (Eq. 1) received 0.05 ml more acid methanol than those of the upper line.

Apparent bias can be caused by use of the wrong calibration line. For example, all results, except for Sample 2 in Table III, obtained from the one-curve version of Eq. 3 would be considered invalid or suspect. These invalidities were caused solely by erroneous computational procedures. Thus, biases caused by the single-line standard curve throw valid assays into doubt. Any single dose–response line constructed from responses obtained from more than one concentration of inoculum gives biased answers. Furthermore, such lines have no advantage when the data are processed either by computer or by hand.

Sources of Errors—Experience shows the absolute necessity of treating the sample and standard in an indistinguishable manner. The automated equipment will do its part to produce high accuracy assays by so processing standards and samples. The analyst must make certain that events external to the equipment do not degrade overall performance. The standard and sample must be in solutions of identical composition with respect to inorganic ions, organic solvents and other organic compounds, pH, and buffer capacity before high accuracy assays are possible. These conditions can be approximated for many samples but are not. Results of the theoretical treatment (Eq. A1) and extensive experience with diverse assay systems emphasize the importance of diluting standards and samples in *exactly* the same solvents. Not doing so has caused analysts to suffer much grief. Even using water for one and buffer for the other may affect assays not so accurate as those done with the automated equipment.

The mechanical operations are done so precisely by the automated system that external influences, not noticed in low accuracy assay, become apparent. Quite often, obvious deviations, first at-

[2] Hewlett-Packard model H-P 45 advanced scientific pocket calculator.

tributed to malfunction of equipment, are found to be caused by poorly controlled operations external to the equipment. The style of operations, considered adequate for manual assays, is soon found to be the source of error and variance. Some sources of error are: use of a water bath inadequate with respect to temperature uniformity, heating of the assay tubes in a steam bath instead of a water bath to stop growth at the end of the test, change in temperature of the inoculated broth during the diluting stage, dilution of standards and samples to assay concentrations by inaccurate means, too wide spacing of concentrations of standards, use of inappropriate calibration lines, measuring samples against a standard curve incubated in a different assay-tube carrier, use of different solvents for standards and samples, and use of degraded standards (high bias) or standards that increased in concentration (low bias of samples) because solvent evaporated during storage of the solution. These errors of operations affect results by influencing the size of one or more elements of Eq. A1.

APPENDIX

General Equation—The fallacy of a single calibration line when applied to the particular automated system or to any other in which different assay tubes receive different concentrations of inoculum is easily explained. Antibiotic assays of concern here are really growth rate assays. The simplified explanation is that the antibiotic reduces growth rate for a time of incubation which is the same for all tubes in an assay. The effect of lag period and other variants was discussed elsewhere (6) and will be ignored here. The assumptions stated there also apply to this treatment. Turbidity at the end of the incubation period is the integrated effect of growth rate over time.

The general equation can be written:

$$N_t = N_0 e^{(k_0 + f(v)k_m - k_a C)t} \qquad \text{(Eq. A1)}$$

where N_t is the bacterial concentration after incubation time, t, for an initial concentration of bacteria of N_0, at a concentration, C, of antibiotic which has an inhibitory coefficient, k_a, for the test organism; k_0 is the generation rate constant in the absence of antibiotic (11); k_m is the coefficient for menstruum effect; and $f(v)$ is a function of the volume of sample added to the assay tube.

The $f(v)k_m$ was introduced to recognize the influence of substances other than the antibiotic upon growth of the test organism. The term can account for the effect of such inhibitory agents as acid methanol in chlortetracycline assays as well as the effect of growth-promoting substances in extracts of animal feeds and of excipients in pharmaceutical dosage forms. Equation A1 reduces to the form of Eq. A2, as does the equation (5, 6) derived for the special case of $f(v)k_m = 0$.

Strictly, Eq. A1 holds only so long as growth is in the log phase, a good first approximation in practice. Since N_t is proportional to N_0, anything that affects N_0 changes N_t by the same proportion. As stated earlier, the diluter of the automated system dilutes each sample to 10 ml, causing one-half of the assay tubes to receive 9.9 ml of inoculated broth and the other half 9.85 ml of broth. Therefore, one-half of the assay tubes has a concentration of inoculum of N_0 and the other half has an inoculum concentration of 0.995 N_0. That difference of 0.5% in inoculum concentration is important when turbidity is as accurately measured as it is in the system. The difference in inoculum concentration is the principal cause of the differences of turbidities in the tubes without erythromycin in Table I. The extreme sensitivity to inoculum concentration explains failure of antibiotic assays inoculated, as in vitamin methods, by adding a drop of inoculum to each tube.

Other substances, in addition to antibiotic, can affect growth rate. If any inhibitory substance is added with the antibiotic, then one-half of the assay tubes in the automated system receives 50% more of it than the other half. The additional 0.05 ml of solution added by the 0.15-ml channels can have a profound influence upon growth rate. Examples are the acetone used as solvent in the monensin assay (12), the methanol–pH 8 buffer (40:60) in the tylosin assay (13), and the acid methanol used in the chlortetracycline assay (Fig. 2).

In Eq. A1, the algebraic sign of k_m is positive for growth-promoting substances such as amino acids, vitamins, purines, pyrimidines, other organic compounds, essential inorganic ions, and potentiating agents. The sign is negative when other inhibitory substances are added with the antibiotic. The k_m may be factored into at least three parts: one for growth, one for effect of diluent, and one for interaction between ions in the sample and the antibiotic. In a particular assay, from none to all three kinds of k_m may be involved. The numerical value of k_m (growth) is a function of nutritional requirements of the test organism, nutritional completeness of the assay medium, and composition of the sample solution.

For assays for penicillins, cephalexin, and erythromycin using *S. aureus* growing in antibiotic assay broth, k_m (growth) is zero or nearly zero for samples dissolved in pH 7 phosphate buffer. This is not true for the monensin assay, which is done in nutritionally limiting assay broth (12). The value of k_m (diluent) may be zero, as in the example above, a small number when acetone is the solvent, as in the monensin assay, or a much larger number, as in the chlortetracycline assay. If salts in the sample solution interfere with the activity of the antibiotic, then k_m (interaction) is not zero. Examples are reduction of activity of neomycin by aluminum, ferrous, and magnesium ions (14), potassium chloride, and potassium phosphate (15) and reduction of activity of novobiocin by magnesium ion (16). Thus, there are at least three k_m affecting an assay.

The $f(v)$ has at least three forms, all unknown: one form for growth-promoting substances, one for such inhibitory products as acetone and acid methanol, and one for substances that reduce activity of the antibiotic such as certain inorganic ions interacting with aminoglycoside antibiotics.

The apparent influence of the several $f(v)k_m$ depends upon the design of the assay. It is not apparent when one volume of standard solution (or sample) is added to a constant volume of inoculated assay broth. The effect of $f(v)k_m$ upon dose–response lines may be significant when more than one volume of sample is diluted to a constant volume with inoculated assay broth, as is done by the automated system used in this work. The lines for Eq. 1 in Fig. 2 illustrate this point (compare with equivalent lines in Fig. 1).

Although an exact Eq. A1 cannot be written, enough is known of the general form to use it to explain observations and to make predictions. Application of Eq. A1 will be made to tylosin and monensin assays. In the tylosin assay, k_m (growth) and k_m (diluent) may not be zero. The sign of k_m (growth) may be negative for some samples because of the presence of inhibiting concentrations of heavy metals. It was to remove such interferences that treatment with calcium phosphate was instituted (13). The methanol–pH 8 buffer (40:60) gave k_m (diluent) a negative sign. There was no evidence for k_m (interaction). The dose–response line for tylosin in feed extracts will always be affected by k_m (diluent) but usually not by k_m (growth).

The monensin assay (12) is more complex. Acetone in the extract reduced growth rate, fats in the extract increased growth rate, and potassium ion reduced activity of monensin. Activity of the fats in the feed extracts was compensated so that, in effect, k_m (growth) = 0. Problems with postassium ions were avoided by using acetone to extract monensin but not appreciable potassium ions. Thus, only k_m (diluent) remained to affect the assay.

Multiple-Factor Antibiotics—The logarithmic form of Eq. A1 may be written as:

$$\ln N_t = A - BC \qquad \text{(Eq. A2)}$$

where $A = k_0 t + f(v)k_m t + \ln N_0$ and $B = tk_a$; A is the logarithm of the concentration of test organism obtained in the absence of antibiotic. The value of A is affected by the initial concentration of bacteria in the assay tube, growth rate of the organism, incubation time, and the assorted influences represented by $f(v)k_m$. Temperature of incubation affects growth rate and, consequently, the value of A. In a test (one carrier of the automated system), t and k_0 are the same for all tubes and N_0 and $f(v)k_m$ should be the same for all tubes receiving the same volume of sample. The size of A is not independent of sample volume because N_0 is not and $f(v)k_m$ may not be.

Slope, B, of the line depends upon t and k_a. If the antibiotic has a single active component, k_a is the same in all tubes of an assay. If the antibiotic is a mixture, $k_a C$ has several components such as $k_{a1}C_1 + k_{a2}C_2 + k_{a3}C_3 \ldots$. Furthermore, the standard and sample may be different mixtures. Usually one factor, say 1, will be present in the greatest concentration and the concentrations of the other components can be expressed as a fraction of factor 1 such

as: $C_2 = bC_1$, $C_3 = cC_3$, ... In the same way, the several specific inhibitory coefficients can be related to that of component 1 by $k_{a2} = ek_{a1}$, $k_{a3} = gk_{a1}$, ... Therefore, the components of k_aC can be written as:

$$k_{a1}C_1 + ek_{a1}bC_1 + gk_{a1}cC_1 = (1 + eb + gc)k_{a1}C_1 = hk_{a1}C_1 \quad \text{(Eq. A3)}$$

Substitution in Eq. A2 leads to:

$$\ln N_t = A - hBC_1 \quad \text{(Eq. A4)}$$

Equation A4 differs from Eq. A2 written for a standard composed solely of factor 1 by the constant h. Therefore, Eq. A4 can be made identical with Eq. A2 by means of a change in the dose scale. This conclusion was tested by results from an assay containing responses of chlortetracycline (0–1 μg/ml), erythromycin (2–3 μg/ml), and tylosin (2–8 μg/ml). The agreement of the transformed erythromycin and tylosin doses with those of chlortetracycline (Fig. 3) giving the same responses are as good as can be expected considering the graphical conversions required to obtain values of N (6). Chlortetracycline was about five times as active as erythromycin and about 10 times as active as tylosin in the assay. The values of A in Eq. A2 were 2.08 for chlortetracycline, 2.05 for erythromycin, and 2.11 for tylosin. A consequence of the slopes of the standard and sample being related by a constant multiplier is that any mathematical transformation keeps the relationship unchanged. No form of interpolation line causes bias attributable to form. Of course, a line that does not fit the calibration points causes bias.

Differences in A (Eq. A2) between the standard and sample cause bias. If the differences are large enough, the validity of the assay is questioned. Differences in A are most likely to be caused by differences in $f(v)k_m$ because N_0, t, and k_0 should be the same for the standard and sample. If they and $f(v)k_m$ are not, the assay should be considered invalid. Within a test, if A is the same for the standard and sample, responses of the sample even at several doses cannot establish the identity of the sample with that of the standard.

Nothing written here should be considered a recommendation for assaying one antibiotic in terms of another. That always is a bad practice.

The assumption made in deriving Eq. A1, that inhibition of the growth rate of the test organism was proportional to the concentration of antibiotic, may not always be true. Certain combinations of test bacteria and antibiotics seem to show inhibitions proportional to a higher power of concentration of antibiotic such as the square. When this is true, Eq. A2 should be written:

$$\log N_t = M - PC^2 \quad \text{(Eq. A5)}$$

Assays following Eq. A5 are exemplified by those employing *Klebsiella pneumoniae* (ATCC 10031). The response with chlortetracycline, oxytetraycline, hygromycin A, dihydrostreptomycin, and streptomycin was studied. Examples of published data are those on the latter two antibiotics (17) and chlortetracycline alone and mixed with a constant proportion of neomycin (18). For these systems, graphs in the form of Eqs. 1–3 and 5 are highly curved. Interpolation from a point-to-point straight-line version of Eq. 1 causes a negative error in potency because of poor approximation to the highly curved calibration line. The size of the error depends upon the closeness of spacing of concentrations of antibiotic in the standard curve. The closer the spacing and the higher the concentration, the smaller is the error. Calculations from a model showed errors as large as 5% at the lower concentrations.

Although use of an incorrect calibration line (Eq. 1) can cause significant error, the relative size of the errors change so slowly with concentration that invalidity probably will not be indicated by the test applied to the automated system. Even though use of an inappropriate calibration line might not cause substantial error, such systems should be identified and a more nearly straight line (Eq. A5) should be used. When *K. pneumoniae* is the test organism in assays providing data required for calculating relative inhibitory coefficients (19) of families of antibiotics, calculations should be based upon Eq. A5—not Eq. A1.

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