Microbiological Diffusion Assay II: Design and Applications

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Abstract □ Application of new equipment and new techniques was made to antibiotic diffusion assays. Accumulation of data and computation of potencies were made by an on-line computer. The system was tested by assaying cephalexin with the aid of *Bacillus subtilis* in an FDA single-dose design modified by reducing the number of standards to two. The influence of the thickness of the base layer and the form of the dose–response line were tested. Zone diameter was measured with a resolution of 0.01 mm. The potency of samples was measured with an error usually less than 3%. An error of 0.1 mm in measuring zone size would cause an error of 3% of potency. The usual calibration line was inadequate for extrapolation beyond a twofold range. A dose–response line derived from the Cooper equation was better for standard curves spanning more than a twofold range of concentrations. Precision was better on the plates with the 20-ml base layer. The two-dose method of assaying gave larger errors than the single-dose method. Large variances in measuring zone diameters are a reason for the low precision of diffusion assays and set an inherent limit on precision.

Keyphrases □ Antibiotics—microbiological diffusion assay, design and application □ Microbiology—diffusion assay of antibiotics, design and application □ Diffusion assay—microbiological assay of antibiotics, design and application

Most petri dish diffusion assays done in the United States for quantitation of antibiotics are variants of two Food and Drug Administration (FDA) designs. One is the Knudsen and Randall (1) two-dose assay, and the other is the alternate or single-dose method. The latter is usually thought of as the FDA method (2).

In the single-dose design, the calibration line is assumed to be straight. Sample responses are assumed to fall on a line parallel to the standard curve. Responses of samples and references are assumed to be influenced equally by factors, known and unknown, that affect zone size. If these assumptions are true, then sample responses can be corrected for the several factors, other than concentration of drug, causing plate-to-plate variations in zone size. As shown previously (3), the FDA correction is a good first approximation to complete correction for such variations.

During the last 25 years, the single-dose method has undergone evolution with respect to the spacing of concentrations of the standards but other details have not changed. For example, the 1949 (4) method for penicillin and streptomycin had 10 standards closely spaced on a linear scale covering a 2.5-fold range. The same concentrations were given by Grove and Randall (5). The number of concentrations was later reduced to five, with the middle one used as reference and spaced on a logarithmic scale to facilitate computation of the "best" straight line (6, 7).

Thus the method had assumed its present form by 1957. The number of concentrations of standards and their spacing were selected more for mathematical convenience than for analytical necessity. The full range of the tests was either 2.44- or fourfold. The best straight line was computed through the points by calculating the low point and the high point of the line by means of an unweighted least-squares procedure.

The practice of using many closely spaced points to determine a straight line originated when location of a point on the standard curve was uncertain and shape of the dose–response line had no theoretical basis. A line determined by a number of points was considered more precisely located than one established by only the end-points. Now that the theory of the method has been established and the precision of assays can be much higher than it once was, the intermediate points are redundant. Since the calibration line is straight by definition, any point on it (*e.g.*, either end concentration) could be used as the reference.

Three plates, each holding three zones of each of two concentrations of standards, are sufficient to establish the standard curve, thus reducing the method to the minimum. Such an assay was designed and applied to samples of known concentration. Limits to the precision of the assay were ascertained during the work. To do so required the determination of the precision of measuring zone size and the influence of the form of the dose–response line upon accuracy and precision. Concepts (3) described earlier were applied to these assays to minimize influence of operations upon accuracy and precision. Some data were used to investigate the two-dose method (1).

EXPERIMENTAL

Equipment and Operations—As shown previously (3), variations in agar thickness had only a second-order influence upon zone size attributable to thickness but a first-order influence caused by effective change in the concentration of bacteria of two layer plates. Variations in agar thickness were reduced by employing automatic pipetting equipment and placing the petri dishes containing molten agar on a level surface to cool. A flat slate slab installed on a bench top and carefully leveled with the aid of a machinist's level provided the level surface.

Plastic dishes were placed on the slab, and molten agar was added from an automatic pipetting machine at the rate of 60 or more/min. Both double- and single-layer plates were prepared; when a second layer was added, it was put on the first layer without moving the plates from the slab. Single-layer plates were preferred, but the type of plates that gave the more sharply defined zone edge was used.

The poured plates were not refrigerated because using plates of different temperatures can affect zone size (8) and increase variances.

Most cylinders used in the United States are made of stainless steel. The ends of the cylinders may be flat, finished with a radius, or have an inside bevel. Cylinders of the same type of end were used because the effective inside diameters are not the same for flat, rounded, or beveled ends. Cylinders must not leak due to either rough ends or rough handling of plates.

Table I—Duplicate Measurements of Zone Sizes at Four Cephalexin Concentrations

Base Layer	Plate Number	Cephalexin 4 μg/ml Measurement 1	4 μg/ml Measurement 2	8 μg/ml Measurement 1	8 μg/ml Measurement 2	16 μg/ml Measurement 1	16 μg/ml Measurement 2	32 μg/ml Measurement 1	32 μg/ml Measurement 2
		Diameters, mm							
10 ml	1	15.38	15.43	18.24	18.20	20.52	20.43	22.56	22.48
	2	15.28	15.27	18.51	18.45	20.44	20.35	22.47	22.37
	3	15.35	15.27	17.94	17.92	20.35	20.30	22.63	22.62
	Mean	15.34	15.32	18.23	18.19	20.44	20.36	22.55	22.49
	σ	0.051	0.092	0.285	0.265	0.085	0.0656	0.082	0.125
20 ml	1	13.15	13.08	15.84	15.88	18.32	18.42	20.59	20.66
	2	13.32	13.32	16.07	16.11	18.71	18.79	20.68	20.69
	3	13.00	13.01	15.84	16.03	18.54	18.65	20.78	20.86
	Mean	13.16	13.14	15.92	16.01	18.52	18.62	20.68	20.74
	σ	0.16	0.16	0.133	0.12	0.195	0.19	0.095	0.11

The cylinders were added by means of a semiautomatic dropper to a group of plates. Filling was by means of an automatic pipet delivering 0.2 ml of sample. The disposable tips of the pipets were changed between samples. One advantage of the automatic pipet was the impossibility of filling the cylinders to overflowing.

The effect of differences in heating rates upon assays was considered earlier (3). As Cooper and Linton (9) showed, the plates in the center of a stack of five heated much slower than those at the ends. A special incubator was designed to receive plates in a single layer and to incubate them at uniform temperature, thus avoiding the problems caused by incubating plates in a stack. The prototype incubator was constructed from chill-plates through which water circulated from a constant-temperature bath at 20 liters/min through the three shelves connected in series. The walls of the incubator were constructed from 5.1-cm (2-in.) thick, rigid, polystyrene foam insulation. Later, larger electrically heated versions were made and had six shelves measuring 41 cm (16 in.) deep × 86.3 cm (34 in.) long spaced 5.1 cm (2 in.) apart. Each shelf had its own thermoregulator and door and was a closed chamber isolated from all others.

The incubator shelves were so close together that the petri dishes could not be put on them individually. A holder designed for a different system (10) proved ideal. Each holder held three dishes. When the holder was placed on the shelf, each dish had the same air space between its bottom and the shelf as all others. Heat transfer, the same for all, was limited by transfer through the air film.

Zone diameters were measured with a modified antibiotic zone reader (11). Resolution of the measuring system was about 0.01 mm when recorded by computer. Four calibration lines were used. Equation 1 was used as a short-range interpolation formula following FDA practice, as a point-to-point line through an eightfold range of concentrations of standard, and as a best straight line through the same four points:

$$\log C = A + Bd \qquad \text{(Eq. 1)}$$

The fourth line was derived from the Cooper equation (12) as:

$$\log C = E + FX^2 \qquad \text{(Eq. 2)}$$

where X, the distance diffused, was computed from $X = \frac{1}{2}(d - 5.8)$. In this work, d was the zone diameter in millimeters, and 5.8 mm was the inside diameter of the cylinders.

The operational elements of the assay—plate pouring, cylinder dropping, cylinder filling, and incubation—were integrated into one system by using the concept of a work station. The operator sits at the work station, drops the cylinders, fills the cups of three plates in a carrier, covers the plates, inserts the carrier into the incubator, and proceeds to the next carrier. The 18 cylinders of a carrier can be filled in 1 min, a time too short to have much effect by reason of the different prediffusion times. Each shelf has space for seven carriers or a total of 21 plates.

If the modified FDA single-dose design is used, one carrier suffices for standards and the remaining six carriers on a shelf are for six samples.

Design of Assays—As already described, the assay is a slight modification of the single-dose method of FDA (7). One carrier of three plates holds the standards, which have the same number of responses per concentration as in the FDA design. The mean of the high standard and the mean of the low standard determine the calibration line. The higher concentration is the reference standard. There are three plates (one carrier) for each sample. One-half of the zones are reference and one-half are sample. The sample is diluted to fall between the two standards.

The reference concentration in these procedures as in that of FDA is one of the standards. This need not be so. The only restriction placed upon the reference is that it be the same on all plates. The reference concentration could be unknown. It is made one of the standards solely in the interest of efficiency. The reference is used to correct for certain variations in operations; it cannot correct for blunders, bad diluting, or misreading of zone sizes.

On the sample plates, the mean of the nine sample zones is corrected for deviation of the mean of the nine reference zones on the sample plates from the mean reference (high standard) on the standard plates. The corrected sample zone is used to interpolate its potency from the standard curve. Interpolation may be done

Table II—Replicate Measurements of Zone Diameters of Standard Curves

Base Layer	Plate Number	Cephalexin 4 μg/ml Measurement 1	4 μg/ml Measurement 2	8 μg/ml Measurement 1	8 μg/ml Measurement 2
		Diameters, mm			
10 ml	1	15.84	15.93	18.32	18.27
		15.93	16.00	18.57	18.47
		15.85	15.87	18.22	18.15
	2	15.68	15.70	18.43	18.47
		15.93	15.87	18.09	17.98
		15.63	15.67	18.25	18.23
	3	15.55	15.55	18.33	18.41
		15.96	15.97	18.42	18.37
		15.44	15.54	17.67	17.66
	Mean	15.757	15.789	18.255	18.223
	σ	0.188	0.177	0.259	0.265
20 ml	1	13.63	13.57	16.19	16.20
		13.53	13.57	16.18	16.09
		13.42	13.39	16.19	16.22
	2	13.37	13.41	16.20	16.21
		13.21	13.22	16.08	16.02
		13.48	13.43	16.04	16.17
	3	13.45	13.38	16.15	16.16
		13.47	13.54	16.07	16.08
		13.36	13.35	15.97	15.91
	Mean	13.436	13.429	16.119	16.118
	σ	0.118	0.115	0.082	0.103

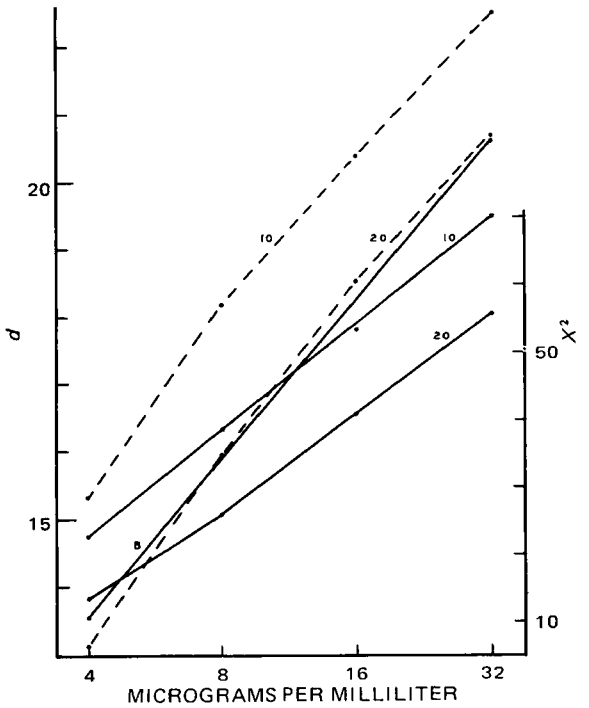

Figure 1—*Two forms of dose–response lines of a cephalexin*–B. subtilis *assay. The dashed lines are for* d versus *log* C, *and the continuous lines are for* X^2 versus *log* C, *where* d *is the zone diameter in millimeters and* X^2 *is the square of the distance diffused computed from* $X^2 = \frac{1}{4}(d - 5.8)^2$. *The numbers on the lines refer to volumes of the base layers. The straight line labeled B is the best straight line through the points for the 20-ml base layer as in the FDA method.*

manually or by computer. Only mean values are used in the calculations, and only one answer is obtained from the nine responses of a sample.

The zone sizes of the standards are not subjected to an FDA correction because none is required. The low concentration of the standard could be corrected for the plate difference between the plate high standard and the mean reference (high standard). The result would be the same as the mean of the nine responses of the low standard.

Application of the concepts and designs discussed in this and the previous paper (3) was made to an assay for cephalexin by a cylinder-plate method, employing *Bacillus subtilis* (ATCC 6633) as the test organism. This assay was selected because the antibiotic was stable and the zone edges were sharp. Zone sizes were accumulated and processed by an on-line computer system. Diameters are reported to 0.01 mm in the tables.

Two base layers (10 and 20 ml) were used with 5 ml of top layer. The plates were poured from the same bottle of molten agar, had the top layers poured from the same bottle of inoculated agar, and received the same solutions. All work was done by one person. Two extended standard curves were prepared at the same time from the same bottles of agar.

The precision of the measurement of zone sizes was obtained by having the same operator measure the same set of plates a second time. One person produced the data in Table I, and another produced the data in Table II.

RESULTS AND DISCUSSION

Single-Dose Assays—Results of the measurement of zone diameters are given in Tables I and II. Table I contains data for an eightfold standard line obtained on plates with either a 10- or 20-ml base layer. Data for the replicate measurements of the standard curve for the single-dose method of assay are given in Table II for the two base layers.

Several conclusions can be drawn from the data. Means of the two sets of measurements of zone diameters differed by 0.1 mm or less; one set of mean diameters is as good as the other. One-half of the comparisons differed by 0.06 mm or less. Only three differences, or one-twentieth of the total, were as great as 0.1 mm, which is the resolution of the manual zone reader when it is fitted with a millimeter scale. These results, as well as many others, indicate that measuring zone diameters with a resolution of 0.01 mm can give useful information. Precision of measurement was somewhat greater for zones formed on a 20-ml base layer.

The zone edge of *B. subtilis* inhibited by cephalexin was sharp enough for most measurements of diameter to be replicated within 0.08 mm or within 2% of potency. Most assay systems are less precise.

The standard deviations in Tables I and II include variances in actual zone diameters as well as measuring errors. One zone of 8-μg/ml concentration on Plate 3 of the 10-ml base layer was responsible for the rather large standard deviation. Zone diameters were more variable on plates with the 20-ml base layers.

Standard lines representing the data in Table I are plotted in Fig. 1 in three ways:

1. As zone diameter (*d*) *versus* logarithm of concentration (usual way for FDA type of assays).
2. As $\frac{1}{4}(d - 5.8)^2$ *versus* logarithm of concentration.
3. As the best straight line through the points obtained on the plates with the 20-ml base layers.

The second relationship was derived from the Cooper equation (12), assuming the inside diameter of the cup (5.8 mm) to be equivalent to a hole of that size. If the Cooper equation is a valid representation of the facts, the first set of lines should curve toward the concentration axis and the second set should be straight. Figure 1 shows that the Cooper expression is followed by the cephalexin–*B. subtilis* assay from 4 to 32 μg/ml. A low concentration of 6–7 μg/ml would give better zone edges than 4 μg/ml gave on the plates with 20 ml of base layer and would straighten the line.

The lines in Fig. 1 show quite clearly that for high accuracy assays, the usual dose–response relationship (Eq. 1) would add a small error for a narrow (twofold) range assay and a larger error to a wider range assay because the line is curved and not straight as assumed.

An assay for potency of three samples is given in Table III. Standards are at the top of the table. The six zones for one plate are on one line. The relative standard deviation (*RSD*) of zone sizes is reported as percentage. The values of *RSD* are not used in any computation and are given solely as a measure of quality of the work. A large *RSD* should alert the analyst to a problem. The computer program interpolated potencies of the unknowns from a line of the form *d versus* log *C*. The samples were the 8-μg/ml standard and two concentrations approximately 10% above and below it. These latter samples bracket the concentrations of most samples analyzed by a quality control laboratory.

A notable feature of the assays reported in Table III is the plate-to-plate uniformity of adjusted mean responses. The worst was the second plate of Sample 5. Its adjusted mean was 18.11 mm, which indicated a potency of 7.640 instead of the expected 8.00. Thus, the worst plate gave an error of −4.5%. Most mean plate deviations from the true values were less than 2% of potency.

Results of the assays are given in Table IV. The maximum error was +3%. Such an error would be caused by a 0.1-mm error in mean zone size. The difference between the 8-μg/ml standard used as samples and the values found could be caused by an error of 0.025 mm in the mean of the zone diameters. Better agreement is not to be expected because 0.02–0.03 mm is about the size of the difference in means between two sets of measurements of the same set of zones.

The resolution of the zone measurements was 0.01 mm, which was equivalent to 0.022 μg/ml at 8 μg/ml, or 1 part in 400 of concentration, or 1 part in 1800 of diameter, or 1 part in 600 of distance diffused. Therefore, rounding the potencies to three digits (Table IV) introduced little error even in the high accuracy assays reported here. Such rounding should be done in assays to avoid the appearance of unreal and unrealizable accuracy.

The results in Table IV indicate accuracy to be substantially independent of thickness of the base layer in the range from 10 to 20 ml. Extrapolation by less than 10% did not degrade accuracy greatly. However, the lines in Fig. 1 show that the usual extrapolation (Eq. 1) beyond the 8-μg/ml point with the slope determined by the 4–8-μg/ml region will cause error in potency. These errors come

Table III—Assay for Cephalexin Standards in Micrograms per Milliliter, 10-ml Base Layers

	Standards					
	4 μg/ml			8 μg/ml		
	15.84	15.93	15.85	18.32	18.57	18.22
	15.68	15.93	15.63	18.43	18.09	18.25
	15.55	15.96	15.44	18.33	18.42	17.67
Mean		15.76			18.26	
RSD, %		1.19			1.42	

Samples

Number	Sample Zones, mm			Reference Zones, mm			Adjusted Mean	Result
1	18.18	18.39	18.65	18.23	18.71	18.38	18.23	7.944
	18.01	18.34	18.10	18.33	18.61	17.81		
	18.18	18.37	18.45	18.11	18.46	18.26		
Mean		18.30			18.32			
RSD, %		1.08			1.46			
2	18.09	18.50	18.26	18.40	18.23	18.37	18.16	7.788
	17.89	18.27	18.00	18.29	18.42	18.06		
	18.02	18.48	18.31	18.27	18.57	18.08		
Mean		18.20			18.30			
RSD, %		1.18			0.89			
3	18.52	18.72	18.12	18.33	18.50	17.82	18.51	8.590
	18.25	18.75	18.27	18.45	18.15	18.02		
	18.46	19.03	18.09	18.62	18.13	17.88		
Mean		18.47			18.21			
RSD, %		1.73			1.54			
4	17.81	18.32	18.20	18.12	18.46	18.09	18.07	7.603
	18.08	18.28	18.23	18.33	18.39	18.46		
	17.72	18.46	17.96	18.21	18.59	18.06		
Mean		18.12			18.30			
RSD, %		1.36			1.03			
5	18.36	18.17	18.36	18.09	18.11	18.47	18.23	7.946
	17.82	18.02	18.30	18.03	18.45	18.12		
	18.02	18.39	18.33	18.04	18.44	18.24		
Mean		18.20			18.22			
RSD, %		1.11			1.01			
6	18.97	18.86	18.87	18.75	18.52	18.46	18.53	8.641
	18.47	18.46	19.04	18.02	18.56	18.39		
	18.14	18.69	18.26	18.03	18.66	17.87		
Mean		18.64			18.36			
RSD, %		1.73			1.70			

from use of inappropriate standard lines and are not inherent in the assay.

Potencies of the 8.70-μg/ml samples were also obtained from lines drawn point to point to include the 4–16-μg/ml region of the standard curve.

The rationale for correcting the FDA and Cooper types of lines for plate-to-plate variations is fundamentally different. The FDA correction assumes slope of lines of standards and sample to be identical (intercept changes). This is the same as saying that the value of A in Eq. 1 may not be the same for all plates whereas B is the same.

According to the Cooper equation, factors (not concentration of antibiotic) causing first-order changes in zone size do so by affecting the slope of the line (intercept constant). Thus, in Eq. 2, F is affected by operational and environmental variations. In reality, B of Eq. 1 is also affected by the same factors. When using Eq. 2 to correct for operational variances (Table IV), the value of E is computed from the responses of the standard curve. The value of F is computed from the mean of the responses of the reference on the set of three plates in question and then the equation is used to interpolate values of sample concentrations. A different value of F is computed for each set of three plates. Results of these interpolations are given in Table IV for the 8.7-μg/ml sample.

If the extrapolated portion of the calibration line is to be used without degrading accuracy, the slopes of the lines above or below the adjacent standard must be the same. This restriction, when ap-

Table IV—Assay of Samples of Known Concentrations (Data from Table III)

Base Layer	Concentration of Samples, μg/ml	Form of Equations			
		d versus log *C*		$1/4(d - 5.8)^2$ *versus* log *C*	
		Potencies, μg/ml	Error, %	Potencies, μg/ml	Error, %
10 ml	7.55	7.79, 7.60	3.2, 0.66	7.76, 7.55	2.78, 0.00
	8.00	7.94, 7.95	−0.75, −0.63	7.93, 7.93	−0.88, −0.88
	8.70	8.59, 8.64	−1.26, −0.69	8.68[b], 8.73[b]	−0.21, 0.34
	8.70	8.65[a], 8.71[a]	−0.57, 0.11	8.64[c], 8.68[c]	−0.69, −0.23
20 ml	7.55	7.62, 7.58	0.93, 0.40	7.57, 7.52	0.26, −0.40
	8.00	8.08, 7.94	1.00, −0.75	8.10, 7.93	1.25, −0.88
	8.70	8.74, 8.74	0.46, 0.46	8.86[b], 8.88[b]	1.88, 2.09
	8.70	8.77[a], 8.77[a]	0.80, 0.80	8.68[c], 8.70[c]	−0.23, 0.00

[a] Interpolated from a three-point calibration line drawn point to point; same data as on line above. [b] Obtained from extrapolated standard curve for the 4–8-μg/ml region. [c] Line computed for the 8–16-μg/ml region; same data as for line above.

Table V—Two-Dose Assays of 8-μg/ml Standard

Agar Layer	Sample Number	Potency, μg/ml	*SD*	Error	Error, %
10 ml	1	8.274	0.34	+0.274	+3.43
	2	7.615	0.37	−0.385	−4.81
20 ml	1	7.851	0.11	−0.149	−1.86
	2	7.819	0.12	−0.181	−2.26

plied to the examples, requires that the lower standard be 4 μg/ml for plates with 10 ml of base layer and 8 μg/ml for 20-ml base layer plates and that Eq. 2 be used. If Eq. 1 is the interpolation formula, then the standard curve should be drawn point to point. The worst possible line is the best straight line through four or five widely spaced points (B in Fig. 1).

In diffusion assaying where at least four types of calibration lines are used, compensation of errors may occur through the use of an incorrect line. Therefore, agreement with an expected answer is not proof of correctness. The answer must also be obtained by procedures free from obvious technical errors.

The results in Table IV show that extrapolation can lead to significant errors because of changes in slope of the calibration line. Equation 2 probably is better than Eq. 1 as an interpolation formula and requires no more work when a computer is available. For assays done without special precautions and a computer, the FDA procedure is adequate. The data also show that potency of the sample should fall between the ends of the standard line and that the span of the line should, as a general rule, be restricted to twofold when the FDA method is used.

Previous theoretical treatment (3) showed that the unique feature of the FDA single-dose design, the method for correcting for plate-to-plate variations, gives a good first approximation to reality and is essential to high accuracy assays (Table IV).

Although errors of several percentages caused by manual interpolation of potencies from graphed calibration lines were avoided by having the computer make the interpolations mathematically, a further advantage was the practical one of removing restrictions upon the form of the calibration line. Although interpolation from the *d versus* log *C* plot on semilogarithmic paper is more conveniently done manually than from a plot of X^2 *versus* log *C*, this is not true for the computer.

The data in Tables I and II were subjected to an analysis of variance. Sources of variation were considered to be dose, plate, thickness of base layer, and measurement of zone size. The first three sources were significant ($p < 0.0005$); no interaction terms were significant. The first must be significant in order to have a test. A significant plate-to-plate variation indicates the necessity for correcting the variation in high accuracy assays. Significant variation caused by different thicknesses of agar layer was expected. The standard deviation of measurement was 0.05 mm. There was no difference in operators. Although the plate-to-plate differences in zone sizes were small, the analysis of variance found them to be significant because of the high resolution and consistency of the measurements.

The accuracy of diffusion methods is severely limited by several factors about which little, if anything, can be done. Among these is the inability of the operator to determine reliably the edge of the zone to better than 0.02 mm, even with a very favorable system. If the uncertainty in measuring zone size is independent of zone size, the percentage of uncertainty in the potency estimate is independent of potency and decreases as the slope of the calibration line increases.

Equation 2, not Eq. 1, is the dose–response line. Equation 1 is only a convenient empirical calibration line for short-range assays. The low precision of measuring distance diffused limits the accuracy of diffusion assays. Zone edges of certain essential assay systems never are sharp. Add to these the generally poor control over operations and accuracy becomes considerably poorer than that obtained here.

Diffusion assays are particularly susceptible to technique. Careless, indifferent, or improperly trained technicians produce assays of low accuracy and precision. Contributing to the degradation of technique is the belief that diffusion assays are biological and, as such, are inherently variable. Therefore, care is not needed because of the large "biological variation." The evidence to date supports the earlier (13) statement that the causes of "biological variation" in microbiological assaying have primarily a macro- and not a microbiological source.

Two-Dose Assays—The first official FDA microbiological assay for penicillin was the two-dose cylinder-plate method (1, 2). Both standard and sample are present on each plate at two concentrations. The ratio of concentrations is the same for standard and sample and can be either 1:2 or 1:4. Each plate has four zones, and four plates constitute the assay of a sample. Each plate contains a complete calibration line and, thus, becomes a complete assay of the sample.

This assay is statistically symmetrical; standard and sample are treated alike. The procedure for calculating the potency of the sample is that for a parallel line assay in which standard and sample lines have identical slopes. The identity of the slope is assured by using the responses from the standard and sample to compute a mean slope. The potency of the sample is the mean of the four potencies obtained from the four plates.

The data on standard curves given in Table II can be considered to be a two-dose assay in which a standard and two samples are on each plate. Three plates, not four, constitute assay of the samples in this example. For purposes of computation, the zone diameters on the first line under each plate number was assigned to the standards, the next row to Sample 1, and the third row to Sample 2. Thus, three potencies for each sample were obtained, one from each plate. The mean of the three values was reported as the potency of the sample in Table V.

All potencies differed from the true value of 8 by more than 1.5%. The standard deviations of the assays performed on plates with a 20-ml base layer were substantially less than those of plates with 10 ml of base layer. The assays on plates with a 20-ml base layer had the smaller errors. Comparison with the assays of the 8-μg/ml samples given in Table IV shows the latter to have the smallest errors.

One difference between the two types of assays is the amount of replication. Nine responses (one concentration) of sample were used in the single-dose method whereas only six (three at two concentrations) were used in the two-dose assay. The number of responses of standard was also smaller in the two-dose method. An assumption underlaying the two-dose method was the identity of slopes of the two dose–response lines. Departure from ideality was assumed to be caused by variances and not biases. Only two of the 12 pairs of slopes were identical. The ratio of the slope of the standard line to the sample line ranged from 0.94 to 1.3 for the plates with 10 ml of agar base layer and from 0.97 to 1.1 for the 20-ml plates.

The two-dose method has the theoretical advantage that each petri dish is a complete test; thus, the influence of plate-to-plate variations in zone size upon precision and accuracy is removed. Plate-to-plate variations in zone diameter could be large at the time (before 1945) when the method was developed. The method was not widely used and was soon abandoned in favor of the single-dose assay. One reason for abandoning it was the lack of identity of slopes of standards and samples, as illustrated by the results of this investigation. Were the differences in slopes real was the unanswerable question. Results of this investigation, which was done under much more favorable conditions than existed in 1945, indicate that the method was inferior to the single-dose method.

SUMMARY

Facilities and equipment used in this work were designed for plate assaying and were superior to most. Zone diameters were measured with a resolution of 0.01 mm. Control of other operations was such that the corrected mean zone diameter for a sample on one plate was indistinguishable from the corrected mean sample zone diameters on the other two of the set of three plates.

Acquisition and processing of the data by a computer not only reduced drudgery and error associated with manual operations but also removed practical restrictions upon form of the standard line. A calibration line of the form X^2 *versus* log *C* was better than the usual *d versus* log *C*. The principal assay used was the single-dose design of FDA modified to employ only two standards. The test assay, cephalexin–*B. subtilis*, had such sharp zone edges that most

measurements of diameter were replicated within 0.08 mm or within 2% of potency.

Using the equipment, procedures, and designs described here, proper computational procedures, and a very favorable assay system, a single determination (three plates) may be expected to be within 2% of the true value. Furthermore, no increase in effort or expense is required. As shown by the consideration of the influence of error of zone measurement upon error in sample potency, the tests cannot be greatly improved. The modified method requires fewer plates and less operator time than the standard FDA method. The accuracy of this plate method should be compared with the five- to 10-fold greater accuracy obtainable from an automated method for turbidimetric assays (14). Such differences in inherent accuracy is one reason to prefer turbidimetric methods when they are applicable.

The two-dose design of FDA was a less satisfactory method than the single-dose assay because it seemed to be excessively sensitive to the slope of the calibration line.

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Automated Constant-Current Coulometric Assay System for Ascorbic Acid and Sodium Ascorbate

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Abstract □ The performance of an automated constant-current coulometric system for the assay of ascorbic acid and sodium ascorbate is described. After loading, it is capable of analyzing 25 samples and printing out the titer values with no operator attention for 2.5 hr. Under optimum conditions, ascertained by evaluating various electrochemical parameters, the accuracy and precision (95% ts) were found to be ±0.3%.

Keyphrases □ Ascorbic acid and sodium ascorbate—automated constant-current coulometric assay system □ Coulometry, constant current, automated—analysis, ascorbic acid and sodium ascorbate

Coulometric analysis of ascorbic acid, using both controlled-potential and constant-current methods, was reported previously. The constant-current method with iodine generated at the anode was used for the determination of small quantities (about 0.5 mg) of ascorbic acid with biamperometric ("dead-stop") end-point detection (1). The reproducibility was found to be ±0.1% *SD*. A platinum anode controlled at +1.1 v *versus* the saturated calomel reference electrode was used to oxidize 15–100 mg of ascorbic acid within 0.5–1 hr, with an average accuracy of ±0.7 mg (2).

The constant-current method employing electrogenerated iodine affords the electrochemical equivalent to the USP assay and offers a time advantage over the potentiostatic technique. Therefore, it was chosen for automation. A modular system (Fig. 1) consisting of an electrode station, electrolyte dispenser, current source, sample changer, control module, and

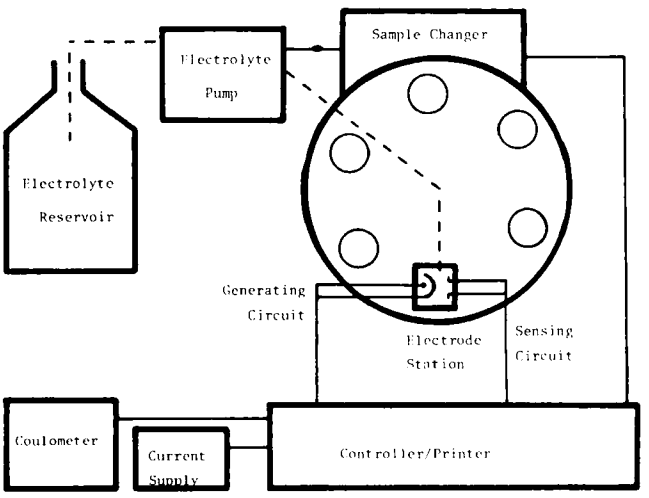

Figure 1—*System block diagram and interrelation of components.*