A BROTH DILUTION METHOD OF ASSAYING STREPTOTHRICIN AND STREPTOMYCIN

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The agar dilution (Waksman, 1943) and the agar diffusion (Foster and Woodruff, 1943b) methods of assay of streptothricin and streptomycin are the procedures most commonly employed and have led to what Waksman calls "dilution units" (Waksman, 1943) and "diffusion units" (Schatz, Bugie, and Waksman, 1944). However, in addition, Waksman speaks of "*Escherichia coli* units," or "*Bacillus subtilis* units," or "*Staphylococcus aureus* units," and the picture is further complicated by the possibility of attempting to compare *Escherichia coli* dilution units and *Staphylococcus aureus* diffusion units, etc. To assign more than one activity value to a given substance leads to confusion. Foster and Woodruff's (1943b) choice of an arbitrary unit based on *Bacillus subtilis* inhibition in a cup plate test has value as a reference unit and we have used it as such in the determination of the toxicity of streptothricin (Rake, Hamre, Kavanagh, Koerber, and Donovick, 1945). No absolute value can be given to such a unit in terms of antibacterial activity because this activity varies from day to day with the conditions of the test (Foster and Woodruff, 1943a; Waksman and Reilly, 1944). Since crystalline preparations of streptothricin and streptomycin have now been described (Fried and Wintersteiner, 1945), it should be possible in the near future to define the unit of each of these substances in absolute terms, i.e., a fixed number of units per milligram of crystalline material. For the present, however, the use of an arbitrary reference unit would appear to be most valuable. This entails the use of a reference standard solution to be tested together with samples of unknown potency each day.

Foster and Woodruff (1943b) stated that assay values obtained by the agar diffusion method, using a reference standard, for replicate streptothricin samples showed a variability between 5 and 15 per cent on a given day, whereas on different days this variability might be up to 15 to 25 per cent. Using the same method of assay, Waksman and Reilly (1944) found that a given concentration of streptothricin or streptomycin caused zones of inhibition the diameters of which varied with the period of incubation. Secondary and tertiary zones were formed in streptomycin plates. Hence this assay method required the choice of a standard period of incubation as well as a standard preparation for comparison.

It has been found expedient in this laboratory to employ a method of assay for streptomycin and streptothricin which is a modification of a procedure which has been used here in the assay of penicillin. The test organism was chosen by plating out a number of cultures on beef extract agar containing varying amounts of

streptothricin. The organisms tested included three strains of *Escherichia coli*, and one strain each of *Staphylococcus aureus*, *Aerobacter aerogenes*, *Klebsiella pneumoniae*, and *Salmonella enteritidis*. It was found that *Klebsiella pneumoniae* was somewhat more sensitive to the action of streptothricin than were any of the other organisms. Another point of importance was that our *Klebsiella* strain appeared to contain a smaller proportion of resistant cells than the other organisms. Consequently the latter organism was chosen for further study and conditions optimal as to the age and dilution of the culture to be used in the test were investigated, as were the effects of the test culture medium and period of incubation. The test at present is performed as follows:

Test culture. The test strain of *Klebsiella* is carried in beef heart broth,[1] a culture incubated for 6 hours at 37 C being diluted 10^{-6} for the tests.

The unit and the standard solution. In order to avoid the introduction of still another unit into the literature we have utilized the reference unit of Foster and Woodruff (1943b). We are grateful to Dr. J. W. Foster of Merck & Co., Inc., for supplying us with a sample of streptothricin solution to which they had assigned a value of 120 u per ml. Using this solution as a standard, we standardized one of our streptothricin preparations by repeated replicate assay, employing the method of assay described here.

The standard streptothricin, a freeze-dried preparation, is stored in a vacuum desiccator at 3 C. A solution containing ca. 37 u per ml is distributed in vials and stored at −70 C. At 10-day to 2-week intervals the contents of one vial are diluted to give ca. 2 u per ml. This solution is used as the daily standard for two weeks when a new solution is prepared from a vial of freshly thawed material. A new sample of standard solution is utilized at these intervals to avoid errors involved in any decrease in activity which may gradually occur at 3 C in some preparations. During the interim the standard solution is stored at 3 C.

It was desired to determine whether different units and consequently separate standards were required for assaying streptothricin and streptomycin solutions. In this connection, samples of streptomycin were exchanged with Dr. S. A. Waksman for check assays. We wish to express our appreciation to Dr. Waksman for having this work done. The Waksman group used a streptomycin standard in the cup plate method of assay with *Bacillus subtilis* as the test organism. We employed our streptothricin standard in the broth dilution method described below with *Klebsiella pneumoniae* as the test organism. The assays of Dr. Waksman and ours checked within about 10 per cent which for the number of tests run is within the limits of error of either method of assay. Consequently it was decided in this laboratory to use the streptothricin unit and standard, for the time being at least, to measure streptomycin activity. A streptomycin standard was later employed for the assays.

Since not all organisms respond to these two substances in identical fashion (Schatz, Bugie, and Waksman, 1944), the use of a streptothricin standard for assaying streptomycin solutions must be limited to those cases where the test

[1] Difco yeast beef broth has in recent months been found to be superior for this purpose.

organism and test procedures are such that either substance may be employed as the standard without causing discrepancies to arise in the assay of the other. This appears to be the case in our test procedure employing *Klebsiella pneumoniae*. Further studies on this question are continuing.

The test. For the tests a 6-hour culture of *Klebsiella pneumoniae* is diluted 1×10^{-6} in a broth containing only 1 per cent tryptone (pH 7.2), and 2-ml quantities are dispensed with a sterile automatic syringe into clear, sterile tubes measuring 12×100 mm.

Streptomycin or streptothricin solutions to be assayed are diluted to contain between approximately 1.0 and 3.5 **u** per ml. If the solutions are thought to be contaminated, small volumes, to allow even heating, are placed in a boiling water bath for two minutes and are then cooled at once in cold water. Other studies have shown that heating the samples for this period of time does not affect significantly the assay value of most preparations.

By means of acid-cleaned, sterile, 0.2-ml Kahn pipettes, the following volumes[2] of the properly diluted samples are added to 2 ml, respectively, of the 1×10^{-6} dilution of the *Klebsiella pneumoniae* culture: 0.1, 0.09, 0.08, 0.07, 0.06, 0.05, 0.045, 0.04, 0.035, and 0.030 ml. The standard solution (1.85 **u** per ml) is run in quadruplicate daily. Unknowns are run in single or duplicate tests.

The racks holding the tubes of diluted culture are kept in the icebox (4 C) until the time when the tests are to be run, and at that time are removed 6 or 8 racks at a time. After the antibiotic solution has been added, the racks of tubes are returned to the icebox until all the tests have been completed, and then they are placed into the 37 C incubator at the same time, and remain there for 15 to 17 hours.

In reading the tests, each rack is shaken vigorously and then held before a bright light (a fluorescent, daylight lamp is especially desirable) and the degree of growth recorded as (−) for none, (±) for a trace, and (+) for almost full or full growth on the basis of visible turbidity. The end point is considered to be the minimum volume of antibiotic solution causing complete inhibition. In the case of a (−) (±) (+) sequence the mid-point between the volumes causing the (−) and (±) tubes is considered the end point.

Since the volumes of antibiotic solution added are small compared to the volumes of broth culture (5 per cent in the extreme), the final volumes are considered to be constant. Hence the ratio of activity of an unknown solution to that of the standard is inversely proportional to the respective volumes required to cause complete inhibition of the test organism, i.e.,

$$\frac{Ux}{Uc} = \frac{Vc}{Vx} \text{ where}$$

Uc = **u** per ml in the standard solution;
Ux = **u** per ml in the unknown solution;

[2] Since this manuscript was submitted the volumes added have been changed to 0.10, 0.088, 0.077, 0.068, 0.059, 0.052, 0.046, 0.040, 0.035 and 0.030 ml, respectively. These intervals are calculated to give volumes decreasing by equal percentages.

Vc = Volume of standard solution required to cause complete inhibition;

Vx = Volume of unknown solution required to cause complete inhibition;

or

$$Ux = \frac{Uc \times Vc}{Vx}.$$

In order to determine the accuracy of the test a preparation of streptothricin (STCPII) known to be relatively stable was assayed repeatedly, 4 tests per day, over a period of 2 months. The results of these tests are shown in table 1.

TABLE 1

Assays on STCPII

DATE	AVERAGE* STANDARD† END POINT	AVERAGE* STCPII END POINT	u/mg‡ STCPII				AVERAGE
	ml	*ml*					u/mg‡
12/19/44 (STCPII).....	0.067	0.063	60.7	60.7	60.7	52.0	58.5
12/20/44	0.07	0.07	54.3	54.3	54.3	54.3	54.3
12/21/44	0.063	0.069	52.7	52.7	49.2	61.5	54.0
12/22/44	0.069	0.075	46.8	53.6	53.6	46.8	50.2
12/26/44	0.063	0.063	48.6	56.7	56.7	56.7	54.7
1/12/45	0.054	0.053	48.8	58.6	58.6	58.6	56.2
1/15/45	0.064	0.07	49.7	49.7	49.7	49.7	49.7
1/17/45	0.065	0.065	50.5	50.5	58.8	58.8	54.7
2/ 5/45 (STCPII)	0.073	0.063	62.0	53.2	74.4	53.2	60.7
2/ 6/45	0.08	0.07	59.2	69.0	59.2	51.7	59.8
2/ 8/45	0.084	0.071	62.2	62.2	58.0	62.2	61.2
2/ 9/45	0.085	0.08	60.2	60.2	50.2	56.4	56.8
2/26/45	0.078	0.093	41.7	43.8	46.3	49.8	45.4
2/22/45 (STCPII)	0.08	0.079	53.5	57.1	53.5	53.5	54.4
2/23/45	0.078	0.076	55.5	55.6	55.5	52.1	54.7
2/26/45	0.078	0.08	46.3	52.2	55.6	55.6	52.4

2.945 mg / 100 ml

3.095 mg / 100 ml

2.98 mg / 100 ml

* Average of quadruplicate tests.

† Standard solution contained 1.60 units of streptothricin per ml.

‡ Units per milligram.

Statistical analysis[3] of these results showed that one would expect a standard deviation from day to day of ±7.5 per cent. This means that if 4 tests were run on a given sample on 1 day, the calculated potency for that day would be within ±7.5 per cent of the true potency 67 times out of 100.

The standard deviation for a single test was found to be ±10.3 per cent. To obtain the highest possible standard deviation for a single test an analysis was made using from each duplicate test only the result which deviated most from the average. The standard deviation for this group was ±12.7 per cent.

The data further showed that when tests are done in duplicate, the standard

[3] The authors are indebted to Mr. Ross Blue of E. R. Squibb & Sons for this statistical study.

deviation within any given day would be ±4.4 per cent. Hence there is more deviation from day to day than there is in a group of 4 tests run on a single day.

DISCUSSION

Since this test can detect as little as 1.0 to 1.5 units of streptothricin or streptomycin per ml with considerable accuracy, it is more sensitive than the agar diffusion method (Foster and Woodruff, 1943b) and, judging from the data available, is perhaps more accurate. On the other hand, it has the disadvantages of requiring that solutions to be assayed must be sterile and that, as the range of the test is only threefold, such solutions must contain between approximately 1.0 and 3.0 **u** per ml.

One trained technician can prepare the dilutions and assay as many as 60 samples or replicates a day without difficulty.

TABLE 2

Activity of streptothricin in various lots of tryptone broth

DIFCO LOT NO.	UNITS STREPTOTHRICIN CAUSING INHIBITION IN 2 ML BROTH CULTURE		COMMENTS
	1st test	2nd test	
373418	0.174	0.174	Satisfactory
373419	0.253	0.221	Satisfactory
373420	>0.316	>0.316	Unsatisfactory
373421	0.316	0.308	Growth very light. End point difficult to determine
373422 (tryptose)	0.189	0.253	
373423 (tryptose)	>0.316	>0.316	Unsatisfactory
372731	0.111	0.111	Satisfactory
361955	0.205	0.158	Satisfactory

Certain aspects of this test are still under investigation. It has been found that the volume of a standard streptothricin solution required to cause complete inhibition will vary somewhat with the lot of tryptone used in the broth. We have found it of value to obtain samples of 8 or 10 lots of tryptone at a time from our supplier and to subject them to test. A large amount of the most satisfactory lot is then ordered and set aside for this test. Table 2 gives typical results of such a test of 9 lots of tryptone (or tryptose) obtained through the courtesy of Dr. H. G. Dunham, Director of Difco Laboratories, Inc., Detroit, Michigan.

A modified test consisting of only 5 tubes containing 2 ml of 1×10^{-6} dilution of culture, to which are added 0.10, 0.077, 0.059, 0.046, and 0.035 ml of antibiotic solution, respectively, is also under investigation. Although this test is not so accurate as the 10-tube test, it apparently is accurate enough for many purposes, and one technician can run approximately twice as many of these tests per day as of the 10-tube tests.

Also still under study is the effect of body fluids on the assay results. When known amounts of streptomycin are added to normal human or other sera which appear to have no antibacterial activity per se, the assay results have been found

to be 30 to 40 per cent higher than similar aqueous streptomycin solution controls. Urine samples thus prepared have given less constant results but usually exceed the aqueous controls by 10 to 20 per cent. Spinal fluids, on the other hand, have little effect on the test results, all those tested thus far falling within the range of experimental error. It is possible that chemical pretreatment of serum and urine samples containing streptomycin may lead to more accurate assays.

SUMMARY

Description is given of a broth dilution method of assay of streptothricin and streptomycin which is apparently more sensitive and somewhat more accurate than the procedures described heretofore for the assay of these substances. Although the test has only a threefold range, it has the advantage that as little as 0.05 **u** per ml of these antibiotics will inhibit *Klebsiella pneumoniae* under the test conditions; thus, allowing for the volumes added, 1 **u** per ml of the original solution of either substance can be measured.

REFERENCES

FOSTER, J. W., AND WOODRUFF, H. B. 1943a Microbiological aspects of streptothricin. II. Antibiotic activity of streptothricin. Arch. Biochem., **3**, 241–255.

FOSTER, J. W., AND WOODRUFF, H. B. 1943b Quantitative estimation of streptothricin. J. Bact., **45**, 408–409.

FRIED, J., AND WINTERSTEINER, O. 1945 Crystalline reineckates of streptothricin and streptomycin. Science, **101**, 613–615.

RAKE, G., HAMRE, D., KAVANAGH, F., KOERBER, W. L., AND DONOVICK, R. 1945 On the toxicity of streptothricin. Am. J. Med. Sci., **210**, 61–66.

SCHATZ, A., BUGIE, E., AND WAKSMAN, S. A. 1944 Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc. Soc. Exptl. Biol. Med., **55**, 66–69.

WAKSMAN, S. A. 1943 Production and activity of streptothricin. J. Bact., **46**, 299–310.

WAKSMAN, S. A., AND REILLY, H. J. 1944 Bactericidal action of antibiotic substances. J. Infectious Diseases, **75**, 150–159.