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A REVIEW OF PROCEDURES FOR THE DETECTION OF RESIDUAL PENICILLINS IN DRUGS

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Introduction and Background

The problem of cross contamination of drugs undoubtedly first arose during the early days of drug production. A brief review of the literature reveals that very few investigational reports or review articles have been published about drug contamination problems, and essentially no analytical procedures that deal with the detection of residual contaminants have appeared in the scientific journals. However, the experiences of the Food and Drug Administration (FDA), through its regulatory activities, indicate that the problem has unquestionably existed for many years and still exists today.

FDA became aware in the early 1960's of the potential health hazards associated with one particular contamination problem when reports were received that young males, reportedly being administered a vitamin preparation, were developing female characteristics. FDA inspections revealed manufacturing practices suspected of causing contamination with diethylstilbestrol. In addition, these manufacturing practices provided evidence of contamination of certain drug preparations with penicillin. Since it was difficult at that time to determine residual diethylstilbestrol, various samples were collected for the analysis of penicillin content to support the evidence of poor manufacturing practices; the laboratory findings from the Division of Antibiotics confirmed the presence of penicillin in several non-antibiotic preparations.

As a result of the new findings of penicillin contamination, the Commissioner of Food and Drugs convened an "Ad Hoc Committee on Penicillin Contamination" to evaluate this problem and make appropriate recommendations; the summary of the proceedings of the Committee was issued December 11, 1964. Amendments to drug regulations for current good manufacturing practices (GMPs) were published January 29, 1965 (1) for the control of cross contamination by penicillin. Immediately work was initiated to develop appropriate methods for the detection and quantitation of residual penicillin as a contaminant in other antibiotic and non-antibiotic drug preparations, and in October 1965 the Division of Antibiotics and Insulin Certification, now the National Center for Antibiotics Analysis (NCAA), published methods to accomplish this task (2).

Since the booklet "Procedures for Detecting and Measuring Penicillin Contamination in Drugs" is now outdated and no longer available for distribution, these original methods are outlined and presented in Appendix 1. The methods have been revised to reflect the latest analytical techniques and have been updated and summarized to provide a more concise reference source. In addition, a new section, "Analytical Approach," has been added in an attempt to give a clearer picture of the capabilities and limitations of the basic analytical procedures.

All of the original developmental work on penicillin contamination methods was conducted with penicillin G (benzylpenicillin) as the investigative agent. With the introduction of semi-synthetic penicillins and cephalosporins into the market, questions arose about the adequacy of these methods to

detect residues of these new drugs. However, no formal evaluations were made of the capabilities of the methods until November 1974 when Herbst et al. (3) published the results of the recovery of residual levels five beta-lactams obtained by using the amyl acetate extraction for erythromycin and the chloroform extraction method for tetracycline. The evaluation revealed that the two extraction procedures could detect, to varying limited degrees, residual phenethicillin, methicillin, nafcillin, and oxacillin in spiked aliquots of erythromycin estolate and tetracycline hydrochloride, respectively. The methods were quite limited in their abilities to detect residual cephalothin (lowest level detectable was about 5 ppm) and were totally unsatisfactory for the detection of residual ampicillin (none was recovered).

New Analytical Methods

Because of the inadequacies of the amyl acetate extraction method and a pressing need for a satisfactory method for the detection of ampicillin in erythromycin products, Herbst et al. (3) developed a simple chloroform extraction procedure which provided good recoveries of residual penicillin and ampicillin from erythromycin bulk and erythromycin stearate bulk.

The chloroform extraction method for erythromycin products, originally published in FDA BY-Lines, November 1974, is reprinted in Appendix II. Most erythromycin salts and esters can be tested directly, since little hydrolysis occurs to produce the active base, and Sarcina lutea ATCC 15957 exhibits a high level of resistance to erythromycin. The chloroform extraction method may be useful for those erythromycin forms that cannot be tested by the direct method (not all erythromycin products and forms were tested by this method).

As a result of the 1974 evaluation by Herbst et al. of the original extraction methods for penicillin G, it became quite clear that there was a need for a new procedure to detect residual ampicillin. Herbst approached this problem in a different manner: she applied the techniques of chromatography and developed a fairly simple method that could detect as little as 2 ppm of ampicillin in tetracycline by bioautography (4). The bioautographic method for the detection of residual penicillin was modified and expanded, and in July 1975 Herbst published a procedure for the detection of residual ampicillin and penicillin in demeclocycline and chlortetracycline (5).

At this point, the real potential and the positive attributes of the bioautographic test system were clearly demonstrated. Most significantly, the chromatographic separation of components provided an identification of the contaminant(s) that had not been possible with the older methods. In addition, the bioautograph was characterized by a certain degree of simplicity and rapidity that allowed for rapid screening. Further investigations by Herbst into a reverse-phase thin layer bioautographic system, employing techniques originally described by Biagi et al. (6), resulted in the development of a bioautographic method that could detect and identify residual penicillin G and ampicillin in 7 members of the tetracycline family and in penicillamine (7). The procedure exhibits a limit of detectability in the range of 0.5 - 1.0 ppm of penicillin or ampicillin in the tetracyclines and penicillamine.

The bioautographic method is reprinted in Appendix III to provide, as much as possible, a more complete, single reference source.

Summary

It is quite clear that the development of new analytical procedures for residue detection has not kept pace with the development of new drugs. There still exists a very real need for valid analytical methods for the detection, quantitation, and identification of residues of the beta-lactam antibiotics as contaminants. However, significant advances have been made and protracted efforts have been exerted to establish procedures by which laboratories can monitor cross contamination; the methods described here provide some basic analytical tools for this quality control testing. The establishment of newer bioautographic methods of analysis eventually may provide, as much as possible, the simplicity, sensitivity, and specificity needed for the ideal regulatory method.

APPENDIX I

I. Microbiological Procedures

The basic microbiological assay method employed for the detection of residual penicillin is the classic Sarcina lutea ATCC 9341 cylinder plate procedure first described by Burke (8) in 1947. This microbiological method has been widely used by laboratories over the past 25 years for the quantitation of penicillin in milk and dairy products, animal tissues and body fluids, and in penicillin-medicated animal feeds. The S. lutea method is clearly outlined in Official Methods of Analysis of the Association of Official Analytical Chemists (A0AC) (9) and the Code of Federal Regulations (CFR) (10).

Methods for the detection of penicillins in drugs employ, in addition to S. lutea ATCC 9341, an erythromycin-resistant strain of this organism (S. lutea ATCC 15957 resistant to 1.5 mg/ml of erythromycin) for the assay of erythromycin drug forms and a streptomycin-resistant strain (S. lutea ATCC 9341a resistant to 50 mg/ml of streptomycin) for the assay of streptomycin and dihydrostreptomycin drug forms. Also included for residue detection work are Staphylococcus aureus ATCC 6538P for testing amphotericin and nystatin products and a novobiocin-resistant strain (Staph. aureus ATCC 12692 resistant to 5 µg/ml of novobiocin) for testing the novobiocin dosage forms.

II. Analytical Approach

The sample preparation procedure for most non-antimicrobial drug products (and a few antibiotic forms) is a fairly simple process whereby aqueous solutions or suspensions of the test sample are prepared with minimal quantities of diluent followed by adjustments in pH to essential neutrality when necessary. Since there are such a large variety of drug forms that may be tested, only this very general approach can be taken to arrive at an appropriate test method for a specific drug product. In cases in which no interference problems are created by active ingredients and there is no non-specific inhibition of the growth of the test organism, the solutions or suspensions may be tested directly without additional sample preparation or method modification.

In those instances in which ingredients of the drug product cause non-specific inhibition of growth and/or enhance or reduce the activity of the residual penicillin contaminant, analytical problems may be eliminated by preparing larger dilutions of the sample material (with, of course, a resultant decrease in test sensitivity). If larger dilutions of test samples do not eliminate the problems, additional

modifications may be made in the assay to reduce, block, or destroy the activities of specific compounds (examples of some specific modifications are presented in a separate section). Should active ingredients enhance or reduce the activity of the residual contaminant, the error in quantitation of the residue may be reduced significantly by preparing standard doses in a special diluent which is equivalent in content to the test solution or suspension. Diluents of this type are made by adding a comparable penicillin-free product (or individual ingredients) to the normal aqueous diluent so that the special diluent contains comparable contaminant-free ingredients in the same ratio and the same concentration as the test solution or suspension.

Finally, if interference and/or non-specific inhibition problems continue to exist after appropriate assay modifications have been made, certain extraction methods may be employed that selectively separate the residual contaminant from the other ingredients of the drug product (specific extraction methods are presented in a separate section).

The recommended analytical approach, at least in terms of simplicity and analytical time, may be summarized as follows:

(1) Use a direct test method if possible.

(2) Prepare additional dilutions of samples to eliminate minor interference problems.

(3) Make specific modifications of the assay method to eliminate the more significant interference problems.

(4) Proceed with a specific extraction method accompanied by appropriate recovery and control samples.

Bioautographic methods may be used as supplements to simpler test procedures to provide sufficient data for a positive identification of a residual drug contaminant.

Microbiological methods do not, per se, provide any significant degree of specificity in the analysis of drug residues. The methods described in Appendix I rely on the inactivation of a test solution by a beta-lactamase to provide evidence of the presence of a residual beta-lactam antibiotic and to distinguish the activity produced by this residue from the antimicrobial activity that may be produced by other non-penicillin and non-cephalosporin active agents. This beta-lactamase inactivation serves only to characterize the residue as a beta-lactam antibiotic; it is not adequate to provide a positive identification of the residual active agent.

The text “Methods in Enzymology” (11) states that many different enzymes are capable of catalyzing the hydrolysis of the beta-lactam ring and that beta-lactamases from at least 25 different strains of bacteria have been purified and studied. Since beta-lactamase preparations are not standardized and are available from many different sources, care must be exercised by the laboratory to employ a suitable beta-lactamase preparation of sufficient titer to provide proper inactivation under specified analytical conditions. (Chapter 5 of "Methods in Enzymology" discusses beta-lactamase assays and serves as an excellent reference.)

The beta-lactamase inactivation of the test solution may produce the following results:

(1) Complete inactivation (a positive test): The loss of activity of a treated versus an untreated aliquot of the sample solution indicates that a beta-lactam residue was present. A quantitative determination of the residue can be made in terms of an appropriate standard.

(2) Incomplete inactivation (a presumptive test): A significant reduction in the activity of treated versus untreated aliquots of the sample solution indicates that a beta-lactam residue was present but that other active agents or ingredients have caused interference problems. Accurate quantitation of the beta-lactam residue is not possible under these conditions.

(3) No inactivation (a false test): No significant reduction in the activity of treated versus untreated aliquots of the sample solution indicates that interfering agents or ingredients may be masking the presence of a beta-lactam residue. No qualitative or quantitative analysis can be made of the residue.

(4) No activity (a negative test): The absence of zones of inhibition from treated and untreated aliquots of the sample solution indicates that no beta-lactam residue(s) has been detected (within the limitations of the test method).

III. Assay Methods

Note: The following methods are intended only for the assay of residual penicillin G. No evaluations have been made of the adequacy of these procedures for the assay of other beta-lactam antibiotics.

All procedures should be performed in a clean environment, using chemically clean glassware and equipment. (The use of disposable plastic, paper, and glass products is recommended.) Since residues of the penicillins may be unstable in aqueous solution, sample solutions and suspensions should be assayed soon after preparation and should not be stared for prolonged periods of time.

Culture Media

(Deionized water may be used for the preparation of media.)

(a) Medium 1: Dissolve 6.0 g of pancreatic digest of gelatin, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of anhydrous glucose, and 15 g of agar in water, and dilute to 1 liter. Adjust the pH to 6.5-6.6 after sterilization. (Difco Penassay Seed Agar and BBL Seed Agar have been found satisfactory.)

(b) Medium 2: Dissolve 6.0 g of pancreatic digest of gelatin, 3.0 g of yeast extract, 1.5 g of beef extract, and 15 g of agar in water, and dilute to 1 liter. Adjust the pH to 6.5-6.6 after sterilization. (Difco Penassay Base Agar and BBL Base Agar have been found satisfactory.)

(c) Medium 4: Dissolve 6.0 g of pancreatic digest of gelatin, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of anhydrous glucose, and 15 g of agar in water and dilute to 1 liter. Adjust the pH to 6.5-6.6 after sterilization. (Difco and BBL Yeast Beef Agar have been found satisfactory.)

(d) Medium 23: Use medium 1 sterilized and cooled to 50°C. Aseptically add sufficient sterile erythromycin solution to give a final concentration of 600 µg of erythromycin per ml of medium. (Sterile erythromycin solution may be prepared by filtering a solution containing 10 mg/ml of erythromycin in distilled water through a membrane filter of 0.22 micron porosity.)

(e) Medium 33: Use medium 1 sterilized and cooled to 50°C. Aseptically add sufficient sterile sodium novobiocin solution to give a final concentration of 10 µg/ml of novobiocin activity. (Sterile sodium novobiocin solution may be prepared by filtering a solution containing 2.5 mg/ml of sodium novobiocin in distilled water through a membrane filter of 0.22 micron porosity.)

Reagents

(a) Solution 6 (10% potassium phosphate buffer, pH 6.0): Dissolve 20 g of dibasic potassium phosphate and 80 g of monobasic potassium phosphate in distilled water and dilute to 1 liter.

(b) Solution 11 (10% potassium phosphate buffer, pH 2.5): Dissolve 100 g of monobasic potassium phosphate in approximately 800 m1 of distilled water, add 0.2 ml of concentrated HC1, and dilute to 1 liter. Adjust with 18N phosphoric acid or 10N potassium hydroxide so that the pH is 2.0-2.8 after sterilization.

(c) Solution 12 (10% potassium phosphate buffer, pH 7.0): Dissolve 100 g of monobasic potassium phosphate in distilled water and dilute to 1 liter. Adjust with 18N phosphoric acid or 10N potassium hydroxide so that the pH is 6.95-7.05 after sterilization.

(d) Penicillinase (beta-lactamase): Preparations are available from Difco Laboratories Box 1058A, Detroit, MI 48232; BBL, Div. of BioQuest, PO Box 243, Cockeysville, MD 21030; ICN Nutritional Biochemicals, 26201 Miles Roads, Cleveland OH 44128; Schwarz/Mann, Orangeburg, NY 10962; Calbiochem, 10933 N. Torrey Pines Road, LaJolla, CA 92037.

Apparatus

(a) Cylinders: Use stainless steel or corrosion-resistant cylinders, 8 ± 0.1 mm o.d., 6 ± 0.1 mm i.d., and 10 ± 0.1 mm long. Available from S&L Metal Products Corp., 58-29 5th Drive, Maspeth, NY 11378.

(b) Cylinder dispenser: Cylinders may be placed on plates with a Shaw Dispenser, available from E.C. Condit, PO Box 75, Middle Haddam, CT 06456, or Arthur E. Farmer, PO Box 1785, Trenton, NJ 08618.

(c) Stainless steel plates: 3-1/4" for 100 mm Petri dishes (templates). Available from Lab-Line Instruments, Inc., Lab-Line Plaza, Melrose Park, IL 60160. Catalog No. 1310.

(d) Petri dishes (plates): Plastic or glass Petri dishes, 20 x 100 mm, may be used. Covers should be of suitable material.

Stock Cultures - Preparation of Test Organism Suspensions

Maintain test organisms on agar slants containing 10 ml of medium 1. Incubate slants 18-24 hr at 37°C and store under refrigeration (2-10°C) no longer than 2 weeks. Use sterile glass beads to transfer the growth from the surface of a fresh agar slant to the surface of a Roux bottle containing 250 ml of the appropriate solidified culture medium. (S. lutea. ATCC 9341, S. lutea, ATCC 9341a, and Staph. aureus ATCC 6538P are prepared on Roux bottles of medium 1. S. lutea, ATCC 15957 is grown on medium 23, and Staph. aureus ATCC 12692 is grown on medium 33.) Spread the culture over the entire surface of the Roux bottle with the aid of sterile glass beads and incubate the Roux bottle at 37°C for 24 hr. Wash the growth from the surface of the Roux bottle with 50 ml of sterile isotonic saline solution. Store suspensions of the test organisms no longer than 2 weeks under refrigeration.

(All cultures are available from American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.)

Preparation of Plates

Prepare a base layer by adding 10 m1 of liquefied medium to each plate and allowing it to solidify on a flat, level surface. (If nonporous covers are used, leave each cover slightly ajar to prevent accumulation of condensed moisture.) Prepare the seed layer by adding 4 ml of liquefied medium (50°C) which has been previously inoculated with the test organism. Distribute the medium evenly by carefully tilting the plates from side to side with a circular motion, and allow to harden. For the S. lutea assays use medium 1 for the base layer and medium 4 for the seed layer. For the Staph. aureus assays, use medium 2 and medium 1 for the base and seed layers, respectively. Place six cylinders on the solidified agar surface so that they are at approximately 60° intervals on a 2.8 cm radius. Templates may be used in place of cylinders. Use the plates the same day they are prepared.

Before performing the assay, determine by the preparation of trial plates the optimum concentration of test organism suspension to be used. Plates should be prepared with medium containing varying amounts of the test suspension to obtain zones of adequate size and sharpness and to provide adequate test sensitivity. As a general guide, the reference dose in each assay method should produce zones approximately 17-21 mm in diameter and the lowest standard dose should produce zones approximately 10-12 mm in diameter.

Preparation of Standard Solutions

Accurately weigh, in an atmosphere of 50% relative humidity or less, a quantity of USP sodium penicillin G Reference Standard. Dissolve this weight in enough 1% pH 6.0 phosphate buffer to give a

solution of known concentration of 100-1000 units/ml. Keep the solution under refrigeration and use for no longer than 2 days. (Note: 1 unit of penicillin is the activity contained in 1 μg of sodium penicillin G, or 1 mg contains 1667 units.) Dilute appropriate aliquots of the stock solution with enough 1% phosphate buffer, pH 6.0, to yield 3-6 standard dose levels within the specified linear dose range. All dilutions of standard are prepared in 1% phosphate buffer, pH 6.0, unless other specific modifications of diluent are warranted or indicated.

(a) For assays using S. lutea ATCC 9341, S. lutea ATCC 9341a, S. lutea ATCC 15957, and Staph. aureus ATCC 12692: Prepare standard doses over a range of 0.01 to 0.2 unit/ml. Suggested doses are 0.01, 0.02, 0.04, 0.08, and 0.16 unit/ml with the 0.04 unit/ml concentration as the reference dose.

(b) For assays using Staph. aureus ATCC 6538P: Prepare standard doses over a range of 0.03 to 0.8 unit/ml. Suggested doses are 0.05, 0.1, 0.2, 0.4, and 0.8 unit/ml with the 0.2 unit/ml concentration as the reference dose.

In order to determine test sensitivity most accurately, additional doses of standard may be prepared at levels clustered around the lowest suggested dose. For cylinders with an o.d. of 8 ± 0.1 mm, zone measurements should be limited to 9 mm, i.e., any detectable zones less than 9 mm should be considered negative. For templates containing holes with an o.d. of 5 mm, zone measurements should be limited to 6 mm or greater.

Determination of the Standard Dose Response

Note: The assay design described below is a simplified version of assay design for the single level unknown-standard curve described in the Code of Federal Regulations (10) and in Official Methods of Analysis of the AOAC (9). This simplified version is also described in sections 42.228 and 42.272 of Official Methods of Analysis. Alternative assay designs, such as those described by Kavanaugh (12), are satisfactory for determining the response of the test organism to standard dose levels of the drug.

Fill all standard solutions into each plate, a different solution into each cylinder. Replicate the fill of standard solutions on 10 or more plates. Incubate the plates overnight at the appropriate temperature (S. lutea assays at 30°C and Staph. aureus assays at 32-35°C). Remove the cylinders (or templates) and measure the diameters of the zones of inhibition as accurately as possible (in most cases, it is possible to estimate the zone diameters to the nearest 0.1 mm).

Determine the mean response for each standard dose level. Plot these mean values on semilogarithmic graph paper, using the logarithmic scale for the standard solutions (doses) and the arithmetic scale for the mean zone diameters. When the mean responses for the standard doses plot linearly against the log doses, determine the line of best fit by the method of least squares. The slope (b) of the plotted regression line is the increase in zone size for each 10-fold increase in drug concentration (the increase per log dose or the change per log cycle). If the doses are spaced at equal intervals on the logarithmic scale, the predicted responses at the extreme ends of the line of best fit can be computed directly by using the simplified formulas with the appropriate coefficients as described in USP XIX (13). The reference point is the response to the reference dose of standard as predicted by the regression line.

Alternatively, calculate the regression coefficient, b (the slope), and the Y-intercept, a, for the regressions equation $y = a + b (\log x)$ and determine the logarithmic curve fit.

(See Table 1 for the general formulas and examples of the calculations employed.)

Determination of Potency

Use three plates for each sample solution. On each plate, fill two cylinders with the reference dose of standard, two cylinders with the sample solution, and two cylinders with an aliquot of the sample solution that has been treated with penicillinase. Incubate the plates overnight at the appropriate temperature. Remove the cylinders (or templates) and measure the diameters of the zones of inhibition as accurately as possible.

A positive test for the presence of a penicillin residue is indicated by the production of zones of inhibition by the sample solution and the absence of any detectable zones of inhibition by the penicillinase-treated portion of the sample solution. If a clear-cut positive test is not obtained, do not proceed with the determination of potency. If a positive test is obtained, determine the mean responses for the sample and the reference dose of standard. If the sample gives a larger average zone diameter than the reference dose, add the difference between them to the reference point. If the sample gives a smaller average zone diameter than the reference dose, subtract the difference between them from the reference point. Using the corrected value for the sample, determine the concentration of drug per milliliter of solution from the plotted regression line.

Alternatively, determine the log relative potency $M' = (y_u - y_s)/b$, where y_u and y_s are the mean responses of unknown and standard, respectively, and b is the slope of the regression line. Antilog M' = potency of the assay solution relative to the standard; and (antilog M') x 100 = potency of the assay solution as a percentage of the reference dose of standard.

(See Table 1 for examples of calculations.)

Multiply the concentration per milliliter of solution by the appropriate dilution factor to obtain the concentration of drug per unit or sample size tested.

Preparation of Samples

Treat a 2-5 ml aliquot of each prepared sample solution with 1-2 drops of penicillinase and shake the solution well. Test the treated and untreated aliquots as directed under Determination of Potency.

A. Direct Method (Method 1)

Dissolve or suspend 0.5 g of bulk powder (or the equivalent of 3 crushed tablets or the contents of 3 capsules) in 10 ml of 1% phosphate buffer, pH 6.0. Adjust the pH to neutrality, and clarify, if necessary, by filtration or centrifugation. Prepare solutions or suspensions (or injectable powders reconstituted as directed in the labeling) by diluting 1 part sample with 4 parts 1% phosphate buffer, pH 6.0.

B. Extraction Methods

Method 2: Dissolve or suspend 1.0 g of bulk powder (or the equivalent of 2 crushed tablets or the contents of 2 capsules) in 18 ml of distilled water. Prepare solutions or suspensions (or injectable powders reconstituted as directed in the labeling) by diluting 2 parts with 18 parts distilled water. Transfer 9 ml of the aqueous solution to be tested to a separatory funnel and add 20 ml of chloroform. Add 1 ml of 10% phosphate buffer, pH 2.5, shake, allow the layers to separate, and draw off the chloroform layer into a second separatory funnel. (If the pH of the aqueous solution is greater than 3.0, readjust to pH 2.5; the sample should not remain at pH 2.5 longer than 5 min.) Add a second 20 ml portion of chloroform, re-extract, and combine the extracts. Wash the combined extracts with one or more 10 ml portions of 1% phosphate buffer, pH 2.5, and discard the buffer washes. Extract the penicillin from the chloroform with 10 ml of 1% phosphate buffer, pH 6.0.

Method 3: Dissolve or suspend 1.0 g of bulk powder (or the equivalent of 2 crushed tablets or the contents of 2 capsules) in 18 ml of distilled water. Prepare solutions or suspensions (or injectable powders reconstituted as directed in the labeling) by diluting 2 parts with 18 parts distilled water. Transfer 9 ml of the aqueous solution to be tested to a separatory funnel and add 20 ml of amyl acetate. Add 1 ml of 10% phosphate buffer, pH 2.5, shake, allow the layers to separate, and draw off the aqueous layer into a second separatory funnel. (If the pH of the aqueous solution is greater than 3.0, readjust to pH 2.5; the sample should not remain at pH 2.5 longer than 5 min.) Add a second 20 m1 portion of amyl acetate, re-extract, and combine the extracts. Wash the combined extracts with one or more 10 ml portions of 1% phosphate buffer, pH 2.5, and discard the buffer washes. Extract the penicillin from the amyl acetate with 10 ml of 1% phosphate buffer, pH 6.0.

Note: To prepare cycloserine bulk and cycloserine capsules by this procedure, use 1.5 ml of (1 + 3) phosphoric acid in place of 1% phosphate buffer, pH 2.5.

Method 4: Use 0.5 g of bulk powder, the equivalent of 2 crushed tablets or the contents of 2 capsules, or 1 dose of an injectable solution. Transfer the sample to be tested to a small beaker, add 10 ml of distilled water, and mix thoroughly. Adjust the pH to 2.5, if necessary, by adding 1N HCl dropwise. Clarify the solution, if necessary, by filtration through a sintered glass filter. Transfer the solution to a glass-stoppered centrifuge tube containing 10 ml of chloroform, shake, and centrifuge briefly. Aspirate the aqueous layer and wash the chloroform by swirling with 5 ml of 0.1% phosphate buffer, pH 2.5. Discard the aqueous layer and repeat the washing procedure. Add 8 ml of 1% phosphate buffer, pH 7.0, shake, and centrifuge briefly. Transfer the aqueous layer to a beaker. Add 1 ml of a 30% aluminum chloride solution. Adjust the pH to 6.0–7.0 by adding NaOH dropwise, centrifuge, and decant the supernatant fluid for assay.

Method 5: Use a single dose of a syrup or an aqueous suspension. Transfer the sample to be tested to a small beaker and adjust the pH to 2.5 by adding 1N HCl dropwise. Quickly pour the solution or suspension into a glass-stoppered centrifuge tube containing 10 ml of amyl acetate, shake, and centrifuge briefly. Transfer the amyl acetate layer to a second glass-stoppered centrifuge tube containing 8 ml of 1% phosphate buffer, pH 7.0, and 5 ml of carbon tetrachloride. Shake and centrifuge briefly. Transfer the aqueous layer (the upper phase) to a small beaker and add 1 ml of 30% aluminum chloride solution. Adjust the pH to 6.0-7.0 by adding NaOH dropwise, centrifuge, and decant the supernatant fluid for assay.

Method 6: Dissolve or suspend 1 g of sample in sufficient distilled water to make approximately 18 ml. Filter, if necessary; transfer 9 ml of the solution to a centrifuge tube containing 10 ml of amyl acetate and add 2 ml of (1 + 3) phosphoric acid. Mix thoroughly, but gently, and centrifuge for 15 min. Draw off the amyl acetate (upper layer) and place in a second centrifuge tube containing 10 ml of carbon tetrachloride and 10 ml of 1% phosphate buffer, pH 6.0. Shake and centrifuge. Draw off the aqueous buffer layer and assay (if the buffer layer is not on the top, add 5 ml of chloroform, shake, and centrifuge).

Note A: To prepare sodium novobiocin bulk, capsules, and tablets by this procedure, use 10% phosphate buffer, pH 6.0, in place of 1% phosphate buffer, pH 6.0.

Note B: To prepare sterile vancomycin and vancomycin HCl bulk by this procedure, use 1-2 drops of a 20% solution of phosphotungstic acid in place of 2 ml of (1 + 3) phosphoric acid.

Method 7: Suspend or dissolve 1 g of the sample in sufficient 1% phosphate buffer, pH 6.0, to make 20 ml. Filter, if necessary; transfer 10 ml of the filtrate to a separatory funnel and wash 3 times with 20 ml portions of ethyl ether. Discard the ether washes and use the aqueous buffer layer as the assay solution.

(See Table 2 for an alphabetical listing of the methods by drug.)

Special Assay Modifications

The Sarcina lutea assays may be modified as follows to eliminate specific interference problems and to compensate for positive or negative bias:

(1) For products containing sulfonamides: Add 2 ml of a 0.5% aqueous solution of para-aminobenzoic acid to each 100 ml of medium used as the base layer.

(2) For products containing mercuric nitrates and merthiolates: Add thioglycollic acid to the assay medium used as the seed layer to give a final concentration of 0.03%.

(3) For products containing citrates: Add 10 ml of an aqueous solution of 2M magnesium chloride to each liter of media used as the base and seed layers so that the assay media contain a final concentration of 0.02M magnesium (developed from the work of Wood et al. (14) in their studies of the inhibitory effects of citrates).

(4) For streptomycin or dihydrostreptomycin products: Prepare a special diluent by dissolving 1 g of streptomycin in 20 ml of 1% phosphate buffer, pH 6.0, and use this special diluent in place of 1% pH 6.0 phosphate buffer.

Table 1. Examples of calculations

Determination of Standard Dose Response

Standard Doses, units/ml	0.015	0.025	0.04	0.08	0.12	n= 5
Log Doses, x	-1.82391	-1.60206	-1.39794	-1.09691	-0.92082	Σx= -6.84164
x^2	3.32665	2.5666	1.95424	1.20321	0.84791	Σx^2 = 9.89859
						$\Sigma(x)^2$ = 46.808

Standard Zones, mm						
Plate 1	10.8	15.1	17.0	20.8	24.1	
Plate 2	11.2	14.0	17.6	20.6	23.8	
Plate 3	11.0	14.5	16.0	20.5	23.2	
Plate 4	10.7	15.1	16.6	19.5	23.0	
Plate 5	11.0	14.5	16.8	20.3	23.3	
Plate 6	10.9	14.2	16.9	20.0	22.7	
Plate 7	10.4	13.9	16.0	20.0	23.1	
Plate 8	10.4	14.6	16.4	20.7	24.0	
Plate 9	12.0	14.0	17.2	21.1	22.5	
Plate 10	11.6	15.1	17.5	19.5	23.3	
Mean Responses, y	11.0	14.5	16.8	20.3	23.3	Σy = 85.9
xy	-20.063	-23.2298	-23.4854	22.2673	-21.4551	Σxy = -110.5006

$$b = \frac{n\Sigma xy - (\Sigma x)(\Sigma y)}{n\Sigma x^2 - (\Sigma x)^2} \quad \text{or} \quad b = \frac{5(-110.5006) - (-6.84164)(85.9)}{5(9.89859) - 46.808} = 13.107$$

$$a = \frac{\Sigma y - b\Sigma x}{n} \quad \text{or} \quad a = \frac{85.9 - (13.107)(-6.84164)}{5} = 35.115$$

$$y = a + bx \quad \text{or} \quad y = 35.115 + 13.107x \text{ and } x = \text{antilog}\left[\frac{y - a}{b}\right]$$

Determination of Potency

	0.04 unit/ml	Unknown 1
Plate 1	16.0	13.0
	17.3	13.6
Plate 2	16.8	13.0
	16.4	12.1
Plate 3	16.0	12.5
	17.1	13.8
	$\bar{y}_s$ = 16.6	$\bar{y}_u$ = 13.0

M' = antilog (-3.6/13.107) = 0.531

Relative Potency = 53.1%

Potency = 0.021 unit/ml

	0.04 unit/ml	Unknown 2
Plate 1	17.0	21.3
	17.1	21.0
Plate 2	16.6	22.0
	17.4	21.5
Plate 3	17.1	20.0
	16.2	20.2
	$\bar{y}_{s=}$ 16.9	$\bar{y}_u$ = 21.0

M' = antilog (4.1/13.107) = 2.055

Relative Potency = 205.5%

Potency = 0.082 unit/ml

Table 2. Methods for sample preparation

Type of sample	Method
Amphotericin B bulk	Direct
Amphotericin B for injection	2
Bacitracin and zinc bacitracin bulk and tablets, sterile bacitracin	6
Candicidin bulk	3
Capreomycin sulfate bulk	3
Chloramphenicol bulk, capsules	7
Chloramphenicol palmitate bulk, oral suspension	Direct
Clindamycin HCl hydrate bulk, capsules	3
Colistimethate sodium bulk	3
Colistin sulfate bulk, powder for oral suspension	3
Cycloserine bulk and capsules	3
Erythromycin bulk, tablets	3
Erythromycin estolate bulk, tablets, capsules, powder for oral suspension	Direct
Erythromycin ethylsuccinate bulk, tablets	Direct
Erythromycin ethylsuccinate injection	3
Erythromycin gluceptate sterile powder	3
Erythromycin stearate bulk, tablets	Direct
Erythromycin lactobionate for injection	3
Gentamicin sulfate bulk	3
Griseofulvin bulk, tablets, capsules, oral suspension	Direct
Kanamycin sulfate bulk, capsules, powder for injection	3
Lincomycin HC1 monohydrate bulk, capsules, syrup, powder for injection	3
Neomycin sulfate bulk, tablets	3
Novobiocin calcium bulk	3
Novobiocin sodium bulk, capsules, tablets	6
Nystatin tablets	Direct
Nystatin bulk, powder for oral suspension	3
Paromomycin sulfate bulk, capsules, syrup	3
Polymyxin B sulfate bulk	3
Streptomycin sulfate bulk, tablets, powder for injection	Direct
Tetracycline bulk, tablets, capsules, injectable dosage forms	4
Tetracycline oral suspensions	5
Troleandomycin bulk, capsules, tablets	3
Vancomycin HC1 bulk and sterile vancomycin	6
Viomycin sulfate bulk and sterile viomycin	3

APPENDIX II

Chloroform Extraction Method for Erythromycin Products

Weigh into a small container approximately 0.5 g of erythromycin, add 10 ml of 1M phosphate buffer solution, pH 8, and transfer the entire contents to a separatory funnel. Add 10 ml of chloroform and shake the mixture well. Allow the layers to separate, and then remove and discard the lower chloroform layer. Repeat this procedure with two more 10 ml portions of chloroform. Remove the aqueous layer, divide into two portions, and add penicillinase to one portion. Assay these solutions by the erythromycin-resistant Sarcina lutea (ATCC 15957) cylinder plate method (2).

Aliquots of erythromycin base and erythromycin stearate were spiked with aqueous solutions of ampicillin or penicillin G in amounts approximating residual levels. These spiked samples were then subjected to the described extraction method. The results are tabulated below.

Recoveries of ampicillin and penicillin G from erythromycin base and erythromycin stearate

Antibiotic	Levels tested, ppm	Lowest level detected, ppm
Ampicillin	2.0, 1.0, 0.5, 0.25, 0.125	0.125
Penicillin G	2.0, 1.0, 0.5, 0.25, 0.125	0.25

(Reprinted from FDA By-Lines, November 1974.)

APPENDIX III

Bioautographic Method

Apparatus and Reagents

(a) Thin layer chromatography plates: Analtech, Inc., Blue Hen Industrial Park, 75 Blue Hen Drive, Newark, DE 19711.

(b) Silica gel GF: Brinkman Instruments, Inc., Cantiague Road, Westbury, NY 11590.

(c) Silicone: Silicone DC 200, 350 cs (Applied Science Laboratories Inc., P.O. Box 440, State College, PA 16801).

(d) Ethyl ether: Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442.

(e) Acetone: Fisher Scientific, Fairlawn, NJ.

(f) Sodium acetate (crystal): J.T. Baker Chemical Co., Phillipsburg, NJ.

(g) Filter paper: Analytical, No. 588 (Schleicher & Schuell, Inc., Keene, NH 03431)

(h) Barbital: Fisher Scientific, Fairlawn, NJ.

(i) Bioassay trays: Plastic (Vanguard International Inc., Box 312, Red Bank, NJ 07701).

(j) Sarcina lutea: ATCC 9431 (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20841).

(k) 2,3,5-Triphenol tetrazolium chloride: Fisher Scientific, Fairlawn, NJ.

(1) Methanol: Fisher Scientific, Fairlawn, NJ.

(m) Dextrose, 2%: Mallinckrodt Chemical Works, St. Louis, MO 63160.

Preparation of TLC Plates

Impregnate 20 x 20 cm thin layer chromatography plates, precoated with silica gel GF, with silicone by developing the plates in a solution of 200 ml of silicone and ethyl ether (5 + 95, v/v) in a covered developing chamber. After overnight development, remove the plates from the chamber and allow them to air dry at room temperature.

Preparation of Samples

Using glass-stoppered volumetric flasks to prevent evaporation of the solvent, prepare standard solutions of ampicillin in acetone to contain 0.1, 0.05, 0.025, 0.0125. and 0.00625 μg/ml. Prepare a similar set of penicillin G standards. Place 25 mg portions of chlortetracycline, demeclocycline, methacycline, minocycline, oxytetracycline, tetracycline, and doxycycline hyclate in 7 ml glass-stoppered weighing vials. Add 1 ml of each of the standard solutions to the vials containing the tetracyclines; then stopper the vials and shake gently for a few seconds to form a suspension of the drugs. Follow this same procedure for a second set of samples, using the penicillin G standards. In addition, place 250 mg portions of penicillamine capsules in 16 x 125 mm screw-cap glass test tubes and spike with 10 ml of each of the standard solutions of penicillin G.

Chromatographic Procedure

Mark the silicone-impregnated plates with a finish line horizontally about 1 cm from the top of the plate. Using a 10 μl tip and holder, spot 10-100 μl aliquots on a line 2 cm from the bottom of the plates. (Various aliquots were used in an attempt to determine the maximum amount which could be spotted and still maintain sufficient separation of the contaminant from the antibiotic.) Deliver these aliquots 10 μ1 at a time and dry the spots with a gentle air flow in order to keep them as small as possible. Prepare an aqueous buffer by placing 19.4 g of sodium acetate (crystal) in 1 liter of distilled water and lowering the pH to 7.4 by adding small amounts of barbital while stirring. Filter this buffer through an analytical filter paper to remove any undissolved barbital. Place the impregnated plates in an unsaturated tank containing 100 ml of a solvent consisting of the barbital-acetate buffer and acetone (94 + 6, v/v). Allow the solvent front to move to the finish line; remove the plates and air dry them at room temperature. The drying process may be hastened by incubating the plates at 54°C for approximately 10 min.

Bioautographic Procedure

Place the dried chromatographic plates in plastic bioassay trays (23 x 23 cm, with lids) and fasten them to the bottom of the plastic trays with double-sided tape to prevent the plates from floating when the liquid agar medium is added. Flood each plate with 100 ml of antibiotic medium #4 (yeast beef agar) previously inoculated with 0.02 ml of a suspension of Sarcina lutea ATCC 9431. When the agar has set (about 20 min) cover the bioassay trays, place them in a plastic bag with a piece of damp paper, seal, and refrigerate them for approximately 1 hr. Incubate the trays with the sealed bag intact at 30°C overnight. After 16-18 hr of incubation, make the areas of inhibition visible by spraying the plates with a solution of 2% 2,3,5-triphenol tetrazolium chloride + 2% dextrose in 50% methanol.

(This procedure in described in the article, "Detection of Penicillin G and Ampicillin as Contaminants in Tetracyclines and Penicillamine", by Doris Herbst, Journal of Pharmaceutical Sciences, November 1977.)

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PENICILLIN CONTAMINATION IN FOOD AND DRUGS

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Practical laboratory guidelines are presented in this paper to assist analysts in understanding, performing, and interpreting the microbiological cylinder plate method routinely used for the detection of penicillin in foods and drugs. Methods for detecting residual penicillin in various foods and drugs have been published by the Association of Official Analytical Chemists and the FDA (1-3). The mechanics and techniques of these methods are described here and practical information is provided for performing reliable assays.

The cylinder plate assay is a microbiological test which is used to detect and quantitate active antibiotic. An antibiotic in solution, when added to a cylinder placed on agar, will diffuse from the cylinder into the agar at a specific rate. A bacterial suspension homogeneously mixed in an agar layer will grow at a specific rate. When an antibiotic is placed on an agar layer containing a sensitive test organism in its beginning growth phase, the antibiotic will inhibit the growth of the organism. This inhibition is shown by the appearance of a clear, round area (zone of inhibition) surrounding the cylinder in which the antibiotic has been placed, after suitable incubation to allow for growth of the organism. High concentrations of antibiotic will diffuse farther into the agar than low concentrations so that the high concentrations will demonstrate larger clear, round zones. Zone diameters (in mm) of the known amounts of antibiotic are compared with the diameters of the zones of inhibition of the samples being tested. By comparing the diameters of the zones of inhibition, active antibiotic can be quantitated in sample analysis.

General Instructions

1. Use chemically-clean, penicillin-free glassware and equipment. Heat all glassware and cylinders in a 200-220°C dry-air oven for 2 hr to inactivate any penicillin residue that might be present.

2. Prepare samples and standards separately; prepare samples first, set aside, and then prepare standards or prepare each at different benches. Prevent laboratory contamination by never preparing both at the same time at the same bench.

3. When using blenders for sample and standard preparation, wash blender jars several times by adding enough water to cover the blades, and then blend. After several washes, when blended wash is free from cloudiness, dispense enough 1% phosphate buffer, pH 6, to each blender jar to cover the blades. Blend, and remove a portion to be tested as a blender control. The blender control must be free of penicillin activity.

4. Do not allow prepared samples and standards to set at room temperature longer than 2 hr. refrigerate until time to fill the cylinders.

5. If using a cylinder dropping device, routinely clean the interior of the columns and cylinder container with 70% ethanol to prevent bacterial contamination. Cotton swabs soaked in 70% ethanol can be used to clean the columns.

6. Use a humidified low temperature (26-30°C) incubator for plate incubation.

7. Ten per cent phosphate buffer, pH 6, can be made in advance, sterilized, and stored at room temperature. Formula for 10% phosphate buffer, pH 6 (±0.1): Dissolve 80 g monobasic potassium phosphate and 20 g dibasic potassium phosphate in distilled water and dilute to 1 L with distilled water.

8. Prepare sufficient 1% phosphate buffer, pH 6, so that all sample and standard dilutions on any one day are taken from the same batch of diluent.

9. Generally, zones of inhibition less than 9.0 mm are not recorded as zones, if cylinders 8 mm in diameter are used. They can be recorded as negative.

10. A standard curve is run and the sensitivity of the test is determined each day on which samples are tested. The test sensitivity is the least amount of penicillin which can be detected with 9.0 mm zone diameters. The test sensitivity for penicillin, using Sarcina lutea, as the test organism, is generally 0.01 unit/ml.

11. The standard doses measured by the zone diameters are plotted on semilog graph paper to determine the linearity of the curve. Generally, the penicillin curve is linear from 0.01 to 0.2 unit/ml. A standard response line which is curvilinear should not be used; additional tests should be scheduled to reassay the samples.

12. If any average sample zone diameter falls outside the average standard zone diameters of the curve, the sample must be rediluted so that the results fall within the standard curve zones.

13. The enzyme penicillinase, available from various biological manufacturing houses, is used to identify penicillin by inactivating active penicillin. Check the penicillinase activity by adding an appropriate amount (ca 0.1 ml/ 5 ml sample) to a known amount of penicillin (e.g., 0.1 or 0.05 unit/ml). The penicillinase must inactivate the penicillin so that no zone of inhibition is produced. Use this same ratio (ca 0.1 ml/ 5 ml sample) for identifying penicillin in samples.

14. Stainless steel cylinders should be rinsed thoroughly with water after each use. Cylinders can be completely covered with water, boiled or autoclaved first, and then thoroughly rinsed with distilled water and sterilized. Detergents, other than mild laboratory cleaning agents, should not be used on cylinders. A 10% solution of nitric acid is recommended for washing cylinders. Put autoclaved or rinsed cylinders in a convenient size container, cover the cylinders with a 10% solution of nitric acid, boil on a hot plate in a hood for 10 min, rinse thoroughly with distilled water, and sterilize.

15. Check the following list and make the necessary adjustments when 0.01 unit/ml in not detected.

a. Check incubator temperature and possibly lower it.
b. Decrease amount of inoculum used.
c. Dispense inoculated agar into plates just prior to using them or dispense inoculated agar and refrigerate plates until use.
d. Fill solutions into cylinders on prepared plates. Allow solutions to diffuse into agar for ca 1

hr before incubating plates. Prediffusion is best at refrigeration temperature although room temperature is sufficient. Prediffusion allows solutions to start diffusing into agar before the test organism starts growing.

e. Make fresh batch of diluent control. For diluent controls other than 1% phosphate buffer, pH 6, new ingredients should be used (e.g., nonfat dry milk, whole milk, etc.).
f. Make fresh penicillin G stock solution. If possible, check potency of old standard against standard solution made from new, dry standard contained in vial not continually opened and closed in laboratory.

16. Check the following list and make the necessary adjustments when there is significant microbiological variation between plates, i.e., the zone diameters of 1 standard concentration vary significantly among plates:

a. Thoroughly mix liquid agar containing test organism.
b. Dispense into each plate 4 ml liquid agar containing test organism. Distribute agar evenly on each plate.
c. Fill each cylinder at least 3/4 full with solutions being tested. Make sure bubbles are not trapped inside cylinders on surface of agar, beneath antibiotic solutions.
d. Check temperature of all quadrants of incubator. Use quadrants which maintain constant and similar temperatures.
e. Check cleaning procedure used on cylinders. Dirty cylinders can inhibit or enhance microbiological activity, causing significant zone variations.

Preparation of the Test Organism

The test organism used in the bioassay method for detecting penicillin is Sarcina lutea (ATCC 9341) and can be obtained from the American Type Culture Collection, Rockville, MD. S. lutea forms smooth yellow convex colonies with regular edges. The magnified edges of the zones of inhibition are slightly hazy but can be read to the nearest 0.5 mm.

Prepare lyophilized cultures as directed on the attached labels. Sometimes overnight shaking and several days' incubation are needed to initiate growth of the freeze-dried cultures. When growth is obtained, transfer the organism to a seed agar (Antibiotic Medium 1) slant and incubate at 30-35°C for ca 24 hr. Maintain the organism on seed agar slants.

The following procedure should be followed when making each new suspension of the test organism:

1. Inoculate the entire surface of 2 dry seed agar slants (ca 10 ml agar in 20 X 150 mm screw-cap tubes) with the test organism and incubate at 30-35°C for ca 24 hr.

2. Transfer the growth from 1 slant, with the aid of sterile glass beads and 3-4 ml USP sterile physiological saline, to the dry surface of a Roux bottle containing 250-300 ml seed agar. Cover the entire surface of the Roux bottle by rotating the beads back and forth. Incubate the Roux bottle at 30-35°C for ca 24 hr.

Maintain the second slant at 2-10°C. In 2 weeks, transfer the organism from the refrigerated slant to 2 new seed agar slants; keep 1 slant for maintenance and use 1 slant for the new suspension.

3. Harvest the bacterial growth by aseptically adding 50 ml USP sterile physiological saline to the Roux bottle. Rotate the beads back and forth until the organism is washed from the agar surface. Aseptically transfer the bacterial suspension to a suitable sterile container, e.g., 125 ml Erlenmeyer flask.

4. Determine the amount of suspension to be added to the seeded agar layer as follows:

Add various amounts of inocula (e.g., 0.05, 0.1, and 0.2 ml) to separate 100 ml aliquots of liquid yeast beef agar (48-50°C). Prepare 2-3 plates for each level of inoculum. Fill all the cylinders with the penicillin standard (0.05 unit/ml) and incubate the plates at 26-30°C for 16-18 hr. Choose the amount of inoculum which gives the clearest, sharpest zones of inhibition measuring17-21 mm in diameter. Use this amount of inoculum whenever using this suspension for assaying penicillin samples.

5. The analyst may wish to check the purity of the suspension by streaking it for isolation on agar plates.

6. The bacterial suspension can be used for 2 weeks with the same amount of inoculum if stored at 2-10°C. Continue to transfer the organism to a fresh seed agar slant every 2 weeks even though a new suspension is not needed.

Preparation of the Agar Plates

Media

Two types of agar are used in the preparation of plates for penicillin assay.

(1) Antibiotic Medium 1. Seed agar consists of (g) peptic digest of meat 6.0, pancreatic digest of casein 4.0, yeast extract 3.0, beef extract 1.5, dextrose 1.0, agar 15.0, and distilled water to yield 1 L. Final pH 6.5-6.6 after sterilization.

(2) Antibiotic Medium 4. Yeast beef agar consists of (g) peptic digest of meat 6.0, yeast extract 3.0, beef extract 1.5 g, dextrose 1.0, agar 15.0, and distilled water to yield 1 L. Final pH 6.5-6.6 after sterilization.

Prepared Difco Penassay seed agar and BBL seed agar can be substituted for Medium 1. Prepared Difco yeast beef agar and BBL yeast agar have been found to be satisfactory substitutes for Medium 4.

Equipment

Glass petri dishes (100 X 20 mm) with porcelain covers glazed on the outside or cover lids with filter pad inserts are satisfactory for absorbing the water of syneresis. Also, plastic petri dishes and covers can be used if the covers are raised slightly to allow water evaporation. Some plastic lids are constructed with raised ridges for this purpose.

General Instructions

A flat, level surface at a convenient height must be used for pouring plates. The surface should be located in an area free from antimicrobial and bacterial fallout. Antimicrobial contamination will produce clear inhibitory areas on the agar surfaces, whereas bacterial surface contamination may overgrow the test organism. Either could interfere with the growth of the test organism and the interpretation of the penicillin zones of inhibition.

The first agar layer to be poured into each petri dish is a 10ml base layer of Antibiotic Medium 1. The agar can be poured at >50°C provided that the plates (plastic petri dishes) are not distorted by the excessive heat. The covers can be set ajar so that the steam will escape and the agar layers will solidify quickly. Care should be taken not to disturb the plates until the agar layers have sufficiently hardened (ca 10-15 min). Replace the covers if they are set ajar. Allow more time for the base layers to harden if the covers remain on the dishes.

The second agar layer is a 4 ml seed layer of Antibiotic Medium 4 containing the test organism. The agar temperature must not exceed 50°C when the test organism, S. lutea, is added. Mix the agar and test organism thoroughly. All the plates must be seeded before the liquid agar solidifies or the agar layers may not be level, the test organism may not grow evenly on the agar surfaces, or the test solutions may not diffuse evenly into the agar. Spread the agar evenly in each plate by tilting the plate from side to side in a circular motion. Let the seed layers harden (ca 5 min) before moving the plates.

Twenty-five ml wide-tip pipets can be used to deliver the agar for both the base and seed layers. The seed layer is the most critical layer and care should be taken to dispense 4 ml into each plate. The zones of inhibition will be significantly larger when 3 ml is delivered, and smaller if 5 ml is delivered. With practice, a 25 ml wide-tip pipet can be an efficient means of dispensing 4 ml. The agar is dispensed quickly into each plate and can be evenly spread over the surface before it solidifies.

When there are a large number of plates, several analysts can share in the seeding process. Inoculate a sufficient amount of yeast beef agar to be dispensed into all the plates, thoroughly mix, and then divide the agar into several portions, one for each analyst. Also, one analyst can seed a large group of plates by keeping the inoculated agar in a container of hot water (ca 50°C) which he can have in a convenient place while seeding the plates.

The finished plates should be picked up in a random order. If the agar layers are poured row by row, top to bottom, the plates should be picked up in a crosswise direction. This mixing will distribute all plates evenly throughout the entire group of plates. Those seeded first are mixed with those seeded last, helping to minimize the amount of microbiological variation due to the plates themselves.

The cylinders can be dispensed into each of the plates with the use of the cylinder cup dropping device. If the cylinders tend to “sink” into the agar, the agar needs more time to harden. It is helpful to mark the bottom of each plate (a point of origin) and to rest one cylinder on or near that mark. The point of origin serves as a reference for filling the cylinders on the first day and reading the inhibition zones on the next day.

Fill each cylinder at least 3/4 full with the standard and sample solutions, making sure that bubbles are not trapped beneath the liquids. Small, disposable 2-3 ml glass pipets and rubber aspirator

bulbs can be used to dispense the test solutions into the cylinders. For the order of filling the cylinders and the number of plates to be used for both the standard and sample solutions, see Preparation of the Standard Curve and Preparation of the Sample.

Plastic petri dishes with filled cylinders should be placed in single layers on trays. Trays can then be stacked if they are not sitting directly on the petri dishes. Rubber stoppers, 25-30 mm high, can be placed on the trays to hold the trays above the petri dishes. Plastic petri dishes should not be stacked directly on top of each other but glass petri dishes can be stacked. Stacking plastic petri dishes may affect the constant air circulation between plates and the growth of the organism in each plate. Carefully transfer plates with filled cylinders to a 26-30°C incubator for 16-18 hr. In addition, incubators should not be bumped or the doors slammed, or the solutions may leak from the cylinders and the zones of inhibition may be difficult, if not impossible, to read.

Preparation of the Standard Curve

Antibiotic reference standards may be obtained from the U.S. Pharmacopeia or the National Formulary. The National Center for Antibiotics Analysis in Washington, DC maintains all FDA antibiotic standards listed in the Code of Federal Regulations (CFR) (436.105-106) (4). The FDA penicillin G working standard is the sodium salt, currently with an activity of 1642 units/mg. This standard is used "as is" but other penicillin G salts from different sources require drying before they are dissolved. The analyst should follow the directions which are attached to the standard he uses.

Store dry penicillin standards in a dark desiccator at 2-10°C, unless directions state otherwise. Standards older than 1 year should be discarded. Vials containing ca 200 mg standard are a convenient size for routine laboratory work. About 10 aliquots, each weighing 15-25 mg, can be taken from a single vial. Single vials should be discarded after routinely being opened and closed for 3 months.

A system for recording standard stock weights and related information should be kept. A card file or small notebook is adequate for keeping these laboratory records. Record the following information:

1. Date the standard stock weight was dissolved, and by whom.
2. Type of antibiotic used.
3. Source of antibiotic.
4. Potency of the antibiotic powder.
5. Weight of the standard.
6. Potency of the stock solution to be made.
7. Amount and type of diluent added.

Example:

Sodium penicillin G 9/8/76 LAP

FDA working standard, 1642 units/mg

Total wt	55.8222 g
Flask wt	55.8063 g
Std wt	0.0159 g

Stock to 1000 units/ml

(15.9 mg X 1642 units/mg) / 1000 units/ml = 26.1 ml 1% phosphate buffer, pH 6

Weigh accurately, on an analytical balance in an atmosphere of 50% relative humidity or less, 15-25 mg penicillin G standard into a suitable container (e.g., 125 ml Erlenmeyer flask). Dissolve the sample in a sufficient amount of 1% phosphate buffer, pH 6, to obtain a solution containing 1000 units/ml. This solution can be used for 2 days if stored at 2-10°C.

Measure the diluent accurately with a pipet or buret. Do not try to estimate amounts of diluent for the stock solution with graduated cylinders. Make sure the bulk powder is completely dissolved and the solution is homogeneous by gently shaking and turning the flask so that all its inner surfaces are exposed to the solution. On the day samples are run, further dilute the standard stock solution in the appropriate diluent (to be determined by the type of sample) to 0.1, 0.05, 0.025, 0.0125, and 0.00625 unit/ml. These solutions should be used only on the day they are prepared. The reference concentration is 0.05 unit/ml; 0.00625 unit/ml is generally negative.

Three typical standard curve dilution schemes are given in Table 1. The dilution schemes are determined by the amount of diluent available and the number and types of volumetric flasks available in the laboratory. Enough reference concentration (0.05 unit/ml) should be made to fill all test plates in a single day. Allow 1 ml reference concentration for each plate. The minimum amount needed is ca 0.3 ml/cylinder.

Table 1. Dilution schemes for preparing penicillin G standard curve solutions

Pen. G concn, unit/ml	Pen. G, ml	Total vol., ml, pen. G + diluent	Final concn pen. G, unit/ml
		Dilution Scheme 1	
1000	2.5	100	25
25	2.5	50	1.25
1.25	4	50	0.1
1.25	4	100	0.05
1.25	1	50	0.025
1.25	0.5	50	0.0125
1.25	0.5	100	0.00625
		Dilution Scheme 2	
1000	0.5	100	5
5	5	100	0.25
0.25	4	10	0.1
0.25	20	100	0.05
0.25	1	10	0.025
0.25	0.5	10	0.0125
0.25	0.2	8	0.00625

(Table 1 – Continued)

Dilution Scheme 3			
1000	0.5	10	50
50	0.5	5	5
5	1.5	15	0.5
0.5	1	5	0.1
0.5	10	100	0.05
0.5	1	8	0.0625
0.0625	2	5	0.025
0.0625	1	5	0.0125
0.0625	0.5	5	0.00625

The penicillin standard curve solutions can be assayed by the procedures given in refs. 2 and 3. The following method can also be used. Fill the standard curve solutions, 1 concentration/cylinder on each plate, on a minimum of 10 plates. The cylinders can be filled clockwise starting on the mark of origin (0.1, 0.05, 0.025, 0.0125, 0.00625 unit/ml, and the diluent control). The diluent control should always be tested and should always be negative. If it is positive, there is a zone of inhibition, and the standard curve and samples must be tested again using a negative control diluent.

When the cylinders are filled in this manner, the 10 zones of inhibition are averaged for each concentration. These averages are plotted on semilog paper and are used to calculate the high and low points and the slope of the line. There is no correction factor to allow for plate-to-plate variation when the standard curve is filled in this manner. Therefore, the assay variation must be minimized so that each concentration is reflected by a specific range of zones of inhibition. A statement should be included indicating a modification of the standard curve as written in refs. 2 and 3. The following example states the modifications made.

> "The reference standard is diluted to 1000 units/ml. The standard curve consists of 5 dose levels (0.1, 0.05, 0.025, 0.0125, and 0.00625 unit/ml) which can be assayed together on a single plate. The standard curve in replicated on 10 plates and the average for each dose is used to calculate the curve response."

Calculation of the Standard Curve

The penicillin G standard curve can be calculated mathematically as follows:

1. Average the 10 zones of inhibition for each concentration.

2. Calculate the high and low points by using the following formulas taken from the least squares formulas (1, pp. 805-806). The penicillin doses must be equally spaced on the logarithmic scale for these formulas to apply. L and H are the calculated low and high points; a,b,c,d, and e are the "corrected" or average zone diameters for each standard dose where $a<b<c<d<e$.

a. Use these formulas for a 5-dose curve:

$$L = (3a + 2b + c - e)/5$$
$$H = (3e + 2d + c - a)/5$$

b. Use these formulas for a 4-dose curve:

$$L = (7a + 4b + c - 2d)/10$$
$$H = (7d + 4c + b - 2a)/10$$

c. Use these formulas for a 3-dose curve:

$$L = (5a + 2b - c)/6$$
$$H = (5c + 2b - a)/6$$

3. Determine the slope, b, by using the formula:

$$b = \frac{H - L}{\log h - \log 1}$$

where b = number of mm the calculated response line extends, through 1 complete log cycle; h = the highest antibiotic concentration; and 1 = the lowest antibiotic concentration.

An example of data for a penicillin G standard curve in given in Table 2.

Table 2. Penicillin G standard curve data

Example: Penicillin G standard curve (raw data; diameter, mm, of zones of inhibition)

Date: July 23, 1976

Diluent: 1 part nonfat dry milk + 3 parts 1% phosphate buffer, pH 6

Inoculum: 0.05% S. lutea

Agar layers: 10 ml seed + 4 ml yeast beef agar

(Table 2 - Continued)

Reference concentration: 0.05 unit/ml

Units of pen. G/ml			
0.1	0.05	0.025	0.0125
24.5	20.0	15.5	11.0
24.0	20.0	15.5	11.0
24.0	20.0	15.0	11.0
24.0	19.0	15.0	10.5
25.0	20.0	15.0	11.0
24.0	20.0	14.0	10.0
24.0	20.0	15.0	11.0
24.0	20.0	15.0	10.0
24.0	20.0	14.0	10.0
24.0	20.0	15.0	10.5
Av. 24.2	19.9	14.9	10.6

Diluent control and 0.00625 unit/ml standard tested negative.

H = [(7) (24.2) + (4) (19.9) + 14.9 - (2) (10.6)] / 10 = 24.3
L = [(7) (10.6) + (4) (14.9) + 19.9 - (2) (24.2)] / 10 = 10.5

h = 0.1 unit/ml — log h = 1.0000
1 = 0.0125 unit/ml — log 1 = 0.0969

log h - log 1 = 0.9031

$$b = \frac{24.3 - 10.5}{0.9031} = 15.28$$

The penicillin G standard curve is plotted on semilogarithmic graph paper. The zone diameters are plotted on the arithmetic scale and the antibiotic concentrations are plotted on the logarithmic scale. The calculated response line is drawn equidistant from all points on the line. Therefore, there must be points on either side of the calculated response line. The standard doses are equally spaced on the log scale. The least squares formulas are not applicable to unequally spaced concentrations on the logarithmic scale.

The slope can be figured directly from the plotted response line. It is the difference in mm between any 2 points, representing 1 complete log cycle.

Example:

0.15 unit/ml = 27.1 mm
0.015 unit/ml = 11.8 mm

27.1 - 11.8 = 15.3 mm, b = 15.3 mm

The calculated slope should not be significantly different from the slope determined on graph paper. The mathematically calculated slope is usually more accurate, especially when using a computer. Averaging, the number of significant figures, and actual human errors in plotting and reading the response line cause differences.

The analyst should always plot the standard curve on graph paper. The inspection of the standard response line will indicate potential problems in the day's tests that might be overlooked. Examine the graph and determine whether any of the following conditions (which might invalidate the results) exist.

1. There is significant curvature in the standard response line.

2. The slope of the standard response line is atypical.

3. The sensitivity of the assay in 1% phosphate buffer, pH 6, is >0.01 unit/ml.

Preparation of the Sample

Samples to be examined for penicillin contamination will generally fall into 1 of 3 categories.

1. Drugs manufactured or processed by a company that manufactures or processes penicillin.

2. Drugs made of raw ingredients suspected of containing penicillin residues.

3. Food products suspected of containing penicillin residues.

Penicillin and other antimicrobial agents are used routinely for growth promotion and disease control in food-producing animals. The wide use of these drugs necessitated the establishment of tolerances at levels no higher than those which had been shown to be safe for human consumption. Among other criteria, tolerance levels are based on the availability of analytical methods to detect at least the tolerance levels cited. The CFR (4) states the established tolerance levels for penicillin in Title 21, Part 556, section 556.510. Penicillin detected above the established tolerance levels for certain commodities is considered significant.

Human and animal drugs labeled for antibiotic content are sent to the National Center for Antibiotics Analysis, Washington, DC, to be examined for antibiotic potency. Most of these products are regulated by the antibiotic certification program as described in the CFR, Parts 431-450 (4).

Two publications (2,3) contain the current methods for testing different types of samples for penicillin contamination. Not every type of sample has been mentioned nor are there known working methods for all samples. The analyst must determine the best method and dilution to use by screening the sample before the actual analysis.

A universal method employed by analysts is a direct dilution that may include blending of a sample in l% posphate buffer, pH 6, possibly followed by adjustment to pH 6, and filtration or centrifugation. Special extraction procedures are used for specific products but are not validated for miscellaneous samples. Once the method has been chosen and the samples tested, the analyst must know how to interpret the results. A sample may be negative or positive for penicillin, or produce nonspecific zones of inhibition.

Three types of analytical results may be found.

1. The sample is negative (no detectable penicillin).

a. The reference standard is positive (zones are ca 17-21 mm).
b. The sample is negative (zones are <9.0 mm).
c. The penicillinase is negative (zones are <9.0 mm).

2. The sample in positive.

a. The reference standard is positive (zones are ca 17-21 mm).
b. The sample is positive (zones are ≥9.0 mm).
c. The penicillinase is negative (zones are <9.0 mm).

3. The sample has nonspecific inhibition.

a. The reference standard is positive (zones are ca 17-21 mm).
b. The sample is positive (zones are >9.0 mm).
c. The penicillinase is positive (zones are >9.0 mm).

Zones are approximately the same size in 3b and c.

Many products, especially drugs, contain raw ingredients which are active against S. lutea; the product itself will produce zones of inhibition. This interference must be diluted out before the analyst can definitely say that penicillin activity is absent or present; then the amount of penicillin present should be determined. It cannot be assumed that penicillin is additive to an interfering substance.

The product itself can also enhance or inhibit active penicillin. By adding known amounts of penicillin to an antibiotic-free sample, one can show by the per cent of penicillin recovered whether or not the active penicillin is being inhibited or enhanced by the product. For this reason, it is best to assay a given sample against a penicillin curve in a similar diluent (e.g., l crushed tablet dissolved in 10 ml 1% phosphate buffer, pH 6, should be assayed against a standard curve in a similar diluent, 1 crushed penicillin - free tablet in 10 ml 1% phosphate buffer, pH 6).

Since it is difficult, if not impossible, to test each sample against its own standard curve, the analyst can run several spiked penicillin recovery samples for each type of sample tested. When the recovery technique is used, the samples and recoveries are tested against a penicillin standard curve in 1% phosphate buffer, pH 6. Known amounts of penicillin are added to penicillin-free samples, usually at the levels assigned in the standard curve, 0.1- 0.0125 unit/ml. If 2 recoveries are run and the mean recovery for each is 80% of the theoretical amount, the sample is inhibiting the penicillin activity. If 80% of the penicillin is recovered, a correction factor of 100/80 should be applied when calculating the potency of a positive sample.

Any product not mentioned in either ref. 2 or 3 must be screened (diluted several times) to determine the appropriate dilution factor to use for analysis. An updated card file listing the final working dilutions for products should be kept. Each new product should be added to the card file so that the file

can be used as a guide for future samples. Percent recoveries for spiked penicillin samples can also be listed for each product.

Example of a Sample Analysis

Assume a solution is submitted to the laboratory to be tested for possible penicillin contamination. Make several dilutions, using only 1 or 2 vials of the product, to determine which dilution to use for final analysis. Use the dilution that exhibits no interference from the sample. The following dilution scheme could be used to screen the product.

1. Product as is or undiluted = lX
2. 1 ml product + 4 ml 1% phosphate buffer, pH 6 = 5X
3. 1 ml product + 6 ml 1% phosphate buffer, pH 6 = 7X
4. 1 ml product + 9 ml 1% phosphate buffer, pH 6 = l0X
5. 1 ml product + 14 ml 1% phosphate buffer, pH 6 = 15X

Fill the cylinders clockwise on previously prepared plates by filling the reference standard into the first 2 cylinders, the sample into the next 2 cylinders, and the sample with penicillinase into the last 2 cylinders. Each sample dilution should be filled on 2 plates; there will be a total of 4 zones of inhibition for the reference concentration, 4 zones for the sample, and 4 zones for the sample containing penicillinase for each sample dilution.

The number of plates run for each sample dilution is not fixed. Some laboratories routinely use 2 plates; other laboratories use 3 or more plates. The number of plates used is determined by the total sample volume, the time available, and the materials and supplies needed to conduct the replicate work. A 1-plate assay is acceptable if the sample volume is small. For example, a sample containing 2 ml liquid submitted for penicillin analysis can be tested on 1 plate. Replicating the solution on at least 2 plates is statistically better since the sample is then represented by 2 plates and the microbiological variation can appear on 2 plates.

Continue the sample analysis by using the initial screening results as a guide.

1. If zones are absent both for the sample and the sample with penicillinase at IX, use the lX dilution to test the other vials. Negative results can be reported as "no detectable penicillin" or "The sample was assayed undiluted and was negative for detectable penicillin."

2. If there are zones for the sample and approximately the same size zones for the sample with penicillinase at 1X, 5X, and 7X but no zones at 1OX and above, use a 1OX dilution to test the other vials. Negative results can be reported as "no detectable penicillin" or “The sample was diluted 1OX and no detectable penicillin was found."

3. If there are zones for the sample and approximately the same size zones for the sample with penicillinase at all dilutions through 15X, the following day rerun the sample at 20X and 25X. (Generally a sample is not diluted more than 25X, for if any penicillin is present, it is diluted out at the higher dilutions.) If the interference is diluted out at either dilution, test all the vials at that dilution. Report negative samples as in 2.

If the interference is not diluted out, test all vials at 25X. These results can be reported as "The sample was diluted up to 25X and each time exhibited nonspecific microbiological activity, not inactivated by penicillinase. No detectable penicillin."or "The sample was tested for penicillin by the direct method, 25X in 1% phosphate buffer, pH 6. The sample and the sample with penicillinase exhibited the same size zones of inhibition. Therefore, penicillin could not be detected if any was present in the sample. No detectable penicillin."

4. If there are zones for the sample and smaller zones for the sample with penicillinase (2 mm or more) continue to dilute the sample until there are no zones for the sample with penicillinase. Then if the sample still shows zones, they are penicillin zones. Never report a sample as "possible penicillin activity" if the sample with penicillinase gives zones smaller than the sample zones. Continue diluting the sample until the penicillinase is completely negative. Positive penicillin samples are characterized by zones of inhibition around the sample and no zones of inhibition around the sample with penicillinase, regardless of the specific dilution factor.

If penicillin is found in any sample, repeat the tests with those specific positive units on another day. All positive subsamples should be run again either by another analyst or the same analyst. Samples that are negative do not have to be run again unless the whole day's work has to be repeated.
The second day assays are run on all positive subsamples because of the inherent microbiological variation of the method itself. Upon completion of both days' work, average the findings and report the results.

Calculation of the Sample

The results can be calculated in one of two ways. They can be read from a plotted graph or they can be figured mathematically by logarithms. Items 1 through 6 must be completed before calculating the sample potencies.

1. Record the zones of inhibition on laboratory forms.

2. Reschedule the assay of samples in which the sample zone diameters are larger than the zone diameters of the highest concentration on the standard curve. These samples must be further diluted so that the zone diameters fall within the zone diameters of the standard curve.

3. Average the zone diameters for the standard, sample, and sample with penicillinase for each unit tested.

4. Calculate the difference in mm between the average standard zone and the average sample zone for each unit tested.

5. Plot the standard response line on semilogarithmic graph paper.

6. Place a "0" at the intersect of 0.05 unit/ml and the plotted response line. Mark off positive (+) mm intervals on the response line above "0" and negative (-) below "0", using the mm intervals on the arithmetic scale as a guide.

The following raw data are used to demonstrate the methods for determining sample potencies. The recordings are the diameters of the zones of inhibition in mm. Each sample has been assayed on 2 plates, using the following design: 2 standards, 2 samples, and 2 samples with penicillinase added. The standard response data are recorded In Table 2. Significant standard response figures are listed below.

a = 10. 6 mm
b = 14.9 mm
c.= 19.9 mm
d.= 24.2 mm

Calculated High (H) = 24.3 mm

Calculated Low (L) = 10.5 mm

h = 0.1 unit/ml
1 = 0.0125 unit/ml
b = 15.28

Subsample 1. (1 ml product + 4 ml 1% phosphate buffer, pH 6 = 5X)

Plate	Standard	Product	Product + penicillinase
1	19.5	14.5	neg.
	20.0	14.0	neg.
2	19.0	14.0	neg.
	20.0	15.0	neg.
Av. (mm)	19.6	14.4	neg.

Av. diff. (mm) = 5.2

Subsample 2. (1 ml product + 4 ml 1% phosphate buffer, pH 6 = 5X)

Plate	Standard	Product	Product + penicillinase
1	19.5	22.0	neg.
	19.0	23.0	neg.
2	20.0	22.5	neg.
	21.0	23.0	neg.
Av. (mm)	19.9	22.6	neg.

Av. diff. (mm) = +2.7

Subsample 3. (l ml product + 4 ml 1% phosphate buffer, pH 6 = 5X)

Plate	Standard	Product	Product + penicillinase
1	19.0	11.0	neg.
	19.0	11.5	neg.
2	20.0	12.0	neg.
	21.0	11.0	neg.
Av. (mm)	19.8	11.4	neg.

Av. diff. (mm) = -8.4

Subsample 4. (1 ml product + 4 ml 1% phosphate buffer, pH 6 = 5X)

Plate	Standard	Product	Product + penicillinase
1	19.5	24.0	neg.
	20.0	23.0	neg.
2	20.0	23.5	neg.
	20.5	24.0	neg.
Av. (mm)	20.0	23.6	neg.

Av. diff. (mm) = +3.6

Subsample 5. (1 ml product + 4 ml 1% phosphate buffer, pH 6 = 5X)

Plate	Standard	Product	Product + penicillinase
1	19.0	neg.	neg.
	19.5	neg.	neg.
2	20.0	neg.	neg.
	21.0	neg.	neg.
Av. (mm)	19.9	neg.	neg.

Av. diff. (mm) = not applicable

Subsample 6. (1 ml product + 24 m1 1% phosphate buffer, pH 6 = 25X)

Plate	Standard	Product	Product + penicillinase
1	20.0	16.0	16.0
	21.0	16.5	16.0
2	20.5	17.0	17.0
	21.0	16.0	17.5
Av. (mm)	20.6	16.4	16.6

Av. diff. (mm) = not applicable

Sample potencies can be determined now that the average differences have been figured and the response line has been plotted. The relative potency (antibiotic concentration read from the graph) is determined by reading the intersect of the average difference of a sample and the plotted response line. The potencies for the 6 samples are determined as follows: Relative potency x dilution factor = sample potency.

Subsample No.	Av. diff., mm	Rel. potency, unit/ml	Dil. factor	Sample potency, unit/ml
1	-5.2	0.023	5	0.11
2	+2.7	0.075	5	0.37
3	-8.4	0.014	5	0.07
4	+3.6	0.085	5	0.42
5	no detectable penicillin			
6	sample interference			

A second method, a mathematical method using logarithms, can also be used to determine sample potencies. The slope of the standard response line must be calculated. The difference in mm between the sample and standard zone diameter averages divided by the slope = the log relative potency (M'). The antilog of M'= the relative potency as a per cent of the reference standard. Subsamples 1 through 4 have been calculated using logarithms to demonstrate this method. Equations 1 through 4 have been followed to determine the sample potencies.

1. Average difference/slope = log relative potency (M')
2. Antilog of M' = potency of the unknown relative to the standard
3. Antilog of M' x 100 = potency of the unknown as a per cent of the reference standard
4. (Percent/100) x reference standard (unit/ml) x dilution factor = sample potency

Subsample 1

1. -5.2 / 15.28 = - 0.34031 + 1 = 0.65969 = M'
2. Antilog of 0.65969 = 0.457
3. 0.457 x 100 = 45.7% (per cent of the reference concentration)
4. 45.7 / (100 x 0.05 x 5) = 0.11 unit/ml

Subsample 2

1. +2.7 / 15.28 = 0.17670 = M'
2. Antilog of 0.17670 = 1.50
3. 1.50 x 100 = 150% (per cent of the reference concentration)
4. 150/(100 x 0.05 x 5) = 0.37 unit/ml

Subsample 3

1. - 8.4 / 15.28 = - 0.54974 + 1 = 0.45026 = M'
2. Antilog of 0.45026 = 0.282
3. 0.282 x 100 = 28.2% (per cent of the reference concentration)
4. 28.2/(100 x 0.05 x 5) = 0.07 unit/ml

Subsample 4

1. +3.6 / 15.28 = 0.23560 = M'
2. Antilog of 0.23560 = 1.72
3. 1.72 x 100 = 172% (per cent of the reference concentration)
4. 172 / (100 x 0.05 x 5) = 0.45 unit/ml

Note the following in determining sample potencies:

1. Zone diameters from both plates for each subsample are averaged together.

2. The difference of the sample average from the reference standard average for each subsample is expressed in mm greater or less than the reference standard average.

3. The original potency read from the graph is called the relative potency. It is labeled in terms of the standard, unit/ml for penicillin.

4. The relative potency of a positive average difference is greater than the reference standard, 0.05 unit/ml.

5. The relative potency of a negative average difference is less than the reference standard, 0.05 unit/ml.

6. The sample potency is reported in terms of the product.

a. Solutions and liquid food commodities are reported in units/ml.
b. Powders and dry food commodities are reported in units/g.
c. Capsules are reported in units/capsule.
d. Tablets are reported in units/tablet.

7. The difference in potencies found between the graph and logarithm method is due to the accuracy of the logarithms used and the human errors in plotting and reading from the standard response line.

It is important to remember that when penicillin is present, it generally will be recovered on the second day and to the same degree as the first day. Exceptions to this rule would include dried bulks, tablets, and capsules which are not necessarily homogeneous. It is also possible to find penicillin in only 2 of 10 vials of a similarly coded solution. When tested a second day, those same 2 vials should be positive again.

Typical ways of reporting or summarizing analytical data are:

1. 10/10 solution vials examined for penicillin activity
   1/10 vials found positive for penicillin

a. Vial 1	Day 1		0.102 unit/ml
	Day 2		0.089 unit/ml
	Av. of 2 days	=	0.096 unit/ml

Activity identified as penicillin with penicillinase.

Vials 2 through 10, no detectable penicillin.

b. Day 1	vial 4		0.102 unit/ml
Day 2	vial 4,	aliquot 1	0.085 unit/ml
Day 3	vial 4,	aliquot 2	0.080 unit/ml
	Av. of 3 aliquots	=	0.089 unit/ml

Activity identified as penicillin with penicillinase.

Vials 1-3 and 5-10, no detectable penicillin.

2. 20/20 subsamples of nonfat dry milk examined for penicillin activity
   1/20 subsamples found positive for penicillin

7/4/76	Sub. 4 Wt 1	0.530 unit/g
	Sub. 4 Wt 2	0.580 unit/g
7/5/76	Sub. 4 Wt 3	0.460 unit/g
	Sub. 4 Wt 4	0.450 unit/g
	Av. of 4 assays =	0.505 unit/g

Subsamples 1-3 and 5-20, no detectable penicillin.

3. 9/150 capsules examined for penicillin activity
   3/3 capsules (Day 1): results rejected due to curvature in standard response line
   6/6 capsules found positive for penicillin

a.	Day 1	Cap. 1	0.530 unit/capsule
		Cap. 2	0.480 unit/capsule
		Cap. 3	0.510 unit/capsule
	Day 2	Cap. 4	0.140 unit/capsule
		Cap. 5	0.150 unit/capsule
		Cap. 6	0.162 unit/capsule
	Day 3	Cap. 7	0.170 unit/capsule
		Cap. 8	0.162 unit/capsule
		Cap. 9	0.150 unit/capsule
		Av. of 6 capsules =	0.155 unit/capsule

Activity identified as penicillin with penicillinase.

b.	Day 2	Cap. 1	0.140 unit/capsule
		Cap. 2	0.150 unit/capsule
		Cap. 3	0.162 unit/capsule
	Day 3	Cap. 4	0.170 unit/capsule
		Cap. 5	0.162 unit/capsule
		Cap. 6	0.150 unit/capsule
		Av. of 6 assays =	0.155 unit/capsule

Activity identified as penicillin with penicillinase.

4. 6/50 tablets examined for penicillin activity
   3/6 tablets found positive for penicillin
   3/6 tablets, no detectable penicillin

10/18/76	Analyst A	Tab. 1	0.08 unit/tablet
		Tab. 2	0.10 unit/tablet
		Tab. 3	no detectable penicillin
10/20/76	Analyst B	Tab. 4	no detectable penicillin
		Tab. 5	no detectable penicillin
		Tab. 6	0.12 unit/tablet
		Av. of 3 positive tablets =	0.10 unit/tablet

Activity identified as penicillin with penicillinase.

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*This publication is no longer available. It has been replaced by Carter, G.G. (Nov. 1977) FDA By-Lines 8, 119-137.

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