Microbioassay of Antimicrobial Agents

HAROLD J. SIMON AND E. JONG YIN

Department of Community Medicine, School of Medicine, University of California, San Diego, La Jolla, California 92037

Received for publication 10 November 1969

A previously described agar-diffusion technique for microbioassay of antimicrobial agents has been modified to increase sensitivity of the technique and to extend the range of antimicrobial agents to which it is applicable. This microtechnique requires only 0.02 ml of an unknown test sample for assay, and is capable of measuring minute concentrations of antibiotics in buffer, serum, and urine. In some cases, up to a 20-fold increase in sensitivity is gained relative to other published standardized methods and the error of this method is less than ±5%. Buffer standard curves have been established for this technique, concurrently with serum standard curves, yielding information on antimicrobial serum-binding and demonstrating linearity of the data points compared to the estimated regression line for the microconcentration ranges covered by this technique. This microassay technique is particularly well suited for pediatric research and for other investigations where sample volumes are small and quantitative accuracy is desired. Dilution of clinical samples to attain concentrations falling with the range of this assay makes the technique readily adaptable and suitable for general clinical pharmacological studies. The microassay technique has been standardized in buffer solutions and in normal human serum pools for the following antimicrobials: ampicillin, methicillin, penicillin G, oxacillin, cloxacillin, dicloxacillin, cephaloglycin, cephalaxin, cephaloridine, cephalothin, erythromycin, rifamycin amino methyl piperazine, kanamycin, neomycin, streptomycin, colistin, polymyxin B, doxycycline, minocycline, oxytetracycline, tetracycline, and chloramphenicol.

Standard agar-diffusion bioassays for antimicrobial agents require greater volumes of blood and other body fluids than can safely be obtained in serial samples from premature infants, diverse body cavities, collections of exudates, or from small animals (5). Other antibiotic assay methods require at least twice the sample volume (1, 3) and do not attain the degree of sensitivity offered by this technique. The microassay technique described in this study was originally developed for clinical pharmacological studies in newborn and premature infants (2). It requires only 0.02 ml of sample material and has been standardized to yield results within a ±5% range of error.

Statistical analysis of the correlation of data points on bioassay standard curves and calculation of serum half-life values of drugs in clinical studies have been speedily attained by a simple adaptation of the data to utilize prewritten programs for the Olivetti Underwood Programma 101, a programmable calculator (7).

MATERIALS AND METHODS

Preparation of standard curves. Measured suspensions of test organisms (Tables 1 and 2) were inoculated into sterile melted agar cooled to and maintained at 50 C. On a critically leveled surface, a continuous pipetting apparatus (B-D Cornwall Continuous Pipetting Outfit) is used to distribute 6-ml volumes of the inoculated agar to sterile glass Petri dish bottoms (Pyrex no. 3162). These are then covered with Brewer Petri dish tops fitted with absorbent cardboard liners (BBL no. 05-264-A), and the agar is allowed to solidify. After the agar has solidified, blank, previously autoclaved, sterile 6.5-mm diameter discs (Schleicher & Schuell Co., Keene, N.H., no. 740E) are placed on the agar surface at 60-degree intervals on a 28 mm radius.

Only freshly prepared plates are used. The bulk cultures of test organisms (5) are prepared biweekly and stored at 4 C. Samples of suspensions are standardized colorimetrically just before use, a procedure taking but a few minutes and contributing significantly to reproducibility of the assay.

Known-potency preparations of antimicrobials to be assayed are diluted in appropriate buffers (4-6) or in a normal human serum pool to provide concentrations of standards within the limits of the testing range (Table 3). These standard concentrations are freshly prepared for each set of determinations.

Quantitative dilutions were made during preparation of the standard concentrations, thus eliminating
the inaccuracies of serial twofold dilutions, as discussed by other investigators (3). The general dilution scheme is similar to that of Bennett et al. (3), except that the final concentrations for the standard curve are prepared from the highest concentration actually used in the standard curve. For example, the dilution scheme for the penicillin G standard curve is as follows: (i) 2.0 ml of penicillin G in diluent at 0.4 units/ml; (ii) 0.6 ml of (i) + 0.2 ml of diluent = 0.3 units/ml; (iii) 0.5 ml of (i) + 0.5 ml of diluent = 0.2 units/ml; (iv) 0.3 ml of (i) + 0.5 ml of diluent = 0.15 units/ml; (v) 0.2 ml of (i) + 0.8 ml of diluent = 0.1 units/ml.

The actual dilutions could be made on smaller scales, if desired, as, in a typical five-point standard curve, the minimal volumes required for the assay are 0.72 ml of the standard reference concentration and 0.12 ml for each of the other concentrations used to plot the standard curve.

Standard curves are determined by using two plates for each of the concentrations to be tested. Each plate contains three discs impregnated with the reference standard concentration alternating with three discs of one of the other concentrations in the

---

**Table 1. Standardization of test organisms used in microbioassay**

| Organism                         | Cells/ml in water | Equivalent transmission | Standard suspension |
|----------------------------------|-------------------|-------------------------|---------------------|
| Sarcina lutea ATCC 9341          | 6 × 10⁶            | 34                      | 0.5                 |
| Staphylococcus aureus Bristol A 9596 | 4 × 10⁶          | 90                      | 0.4                 |
| Bacillus cereus var. mycoides ATCC 11778 | 3 × 10⁷   | 15                      | 0.5                 |
| B. subtilis ATCC 6633            | 1.5 × 10⁶         | 39                      | 1.0                 |
| Bordetella bronchiseptica ATCC 4617 | 1.6 × 10⁶       | 50                      | 0.1                 |

* Transmission reading taken in a Lumetron photoelectric colorimeter, model 401. Values in parentheses are expressed in nanometers.

b Expressed in milliliters per 100 ml of agar.

---

**Table 2. Preparation of microbioassay standard plates**

| Drug                  | Phosphate buffer | Test organism                  | Assay agar, medium |
|-----------------------|------------------|--------------------------------|--------------------|
| Penicillins           |                  |                                 |                    |
| Penicillin G          | 6.0              | Sarcina lutea ATCC 9341         | 1 (0263-01)        |
| Ampicillin            |                  | Staphylococcus aureus Bristol A9596 | 1 (0263-01)b   |
| Methicillin           |                  | Sarcina lutea ATCC 9341         | 1 (0263-01)        |
| Oxacillin             |                  | Bacillus subtilis ATCC 6633     | 5 (0277-01)        |
| Cloxacillin           | 6.0              | B. cereus var mycoides ATCC 11778 | 1 (0263-01)      |
| Aminoglycosides       |                  |                                |                    |
| Neomycin              | 8.0              |                                |                    |
| Kanamycin             |                  |                                |                    |
| Streptomycin          |                  |                                |                    |
| Tetracyclines         |                  |                                |                    |
| Tetracycline          | 6.0              |                                |                    |
| Doxycycline           |                  |                                |                    |
| Oxytetracycline       |                  |                                |                    |
| Minocycline           |                  |                                |                    |
| Cephalosporins        |                  |                                |                    |
| Cephalothin           | 6.0              |                                |                    |
| Cephaloridine         |                  |                                |                    |
| Cephaloglycin         |                  |                                |                    |
| Cephalixin            |                  |                                |                    |
| Polypeptides          |                  |                                |                    |
| Colistin              | 6.0              |                                |                    |
| Polymixin B           | 6.0 (10%)        |                                |                    |
| Chloramphenicol       | 6.0              |                                |                    |
| Rifamycin AMP         | 6.0              |                                |                    |
| Erythromycin          | 8.0              |                                |                    |
| Polypeptides          |                  |                                |                    |
| Colistin              | 6.0              |                                |                    |
| Polymixin B           | 6.0 (10%)        |                                |                    |
| Chloramphenicol       | 6.0              |                                |                    |
| Rifamycin AMP         | 6.0              |                                |                    |
| Erythromycin          | 8.0              |                                |                    |

a Difco (Bacto).

b With agar concentration increased to 2.5% of total volume.

c Diluted with an equal volume of acetone.
Table 3. Data concerning standard curve dilutions

| Drug            | Final conc for buffer standard curve* | Final conc for standard curve* |
|-----------------|--------------------------------------|--------------------------------|
| Penicillin G    | 0.1, 0.15, 0.2, 0.3, 0.4             | 0.1, 0.15, 0.2, 0.3, 0,4        |
| Ampicillin      | 0.025, 0.05, 0.1, 0.2                | 0.025, 0.05, 0.1, 0.2           |
| Methylthionine  | 0.15, 0.2                            | 0.15, 0.2                       |
| Oxacillin       | 1, 1.5, 2, 3, 4                      | 1.1, 1.5, 2, 3, 4               |
| Cloxacillin     | 0.5, 0.75, 1, 1.5, 2                 | 1.1, 1.5, 2, 3, 5, 6            |
| Neomycin        | 0.25, 0.5, 1, 2, 4, 4, 8             | 0.25, 0.5, 1, 2, 5, 10           |
| Kanamycin       | 0.25, 0.5, 0.75, 1, 2                | 0.5, 0.8, 1, 1.5, 2             |
| Streptomycin    | 0.125, 0.25, 0.5, 1, 2               | 0.25, 0.5, 1, 1.5, 2            |
| Tetracycline    | 0.2, 0.5, 1, 2, 4                    | 0.2, 0.5, 1, 2, 4               |
| Doxycycline     | 0.25, 0.5, 1, 1.5, 2                 | 0.25, 0.5, 1, 1.5, 2            |
| Oxytetracycline | 0.25, 0.5, 1, 1.5, 2                 | 0.25, 0.5, 1, 1.5, 2            |
| Minocycline     | 0.125, 0.25, 0.5, 1, 2               | 0.125, 0.25, 0.5, 1, 2          |
| Cephalothin     | 0.75, 1, 2, 3, 4                     | 1.5, 2, 2.5, 3, 4               |
| Cephaloridine   | 0.5, 0.8, 1, 1.5, 2, 2               | 0.5, 0.8, 1, 1.5, 2, 2          |
| Cephaloglycin   | 0.08, 0.15, 0.25, 0.5, 1, 2          | 0.125, 0.25, 0.5, 1, 2          |
| Cephalixin      | 0.25, 0.5, 0.75, 1, 2                | 0.25, 0.5, 0.75, 1, 2           |
| Colistin        | 1, 1.5, 2, 4, 6                      | 1.1, 1.5, 2, 4, 6               |
| Polymyxin B     | 0.25, 0.5, 1, 2, 4                   | 0.25, 0.5, 1, 2, 4              |
| Rifamycin AMP   | 0.1, 0.15, 0.25, 0.5, 1               | 0.1, 0.15, 0.25, 0.5, 1.1       |
| Erythromycin    | 0.025, 0.05, 0.1, 0.2                 | 0.025, 0.05, 0.1, 0.2           |
| Chloramphenicol | 20, 30, 40, 50, 60                    | 20, 30, 40, 50, 60              |

* Standard reference concentrations are italicized. Values are expressed in micrograms per milliliter except for penicillin G, which is expressed in Oxford units per milliliter.

standard curve. In a five-point standard determination, 36 readings are thus obtained for the reference standard and 6 readings for each of the other test concentrations employed to determine the slope of the standard curve.

Two-hundredth-milliliter volumes of test materials are pipetted onto discs through 0.02-ml constricted micropipettes (H. E. Pedersen Laboratories, Copenhagen, Denmark), or through 0.02-ml disposable capillary micropipettes (Drummond Microcaps). Plates are incubated at 35 C for 16 to 18 hr, and zones of inhibition are measured with a Fisher-Lilly Antibiotic Zone Reader (no. 7-906V2).

Assay of unknown concentrations. Two-hundredth-milliliter volumes of unknown samples to be assayed are diluted, if necessary, in appropriate phosphate buffers to concentrations estimated to fall within the limits of the microbioassay. By using duplicate plates, three alternate discs are impregnated with the sample to be tested and three with the reference standard concentration, and the plates are incubated. Zone diameters are measured and averaged; the averages are corrected to the mean standard reference concentration to allow for variations among plates, referred to the standard curve, and concentrations of the unknowns are calculated.

Human serum samples containing unknown concentrations of an antimicrobial are assayed as above, but a normal human serum pool is used as diluent. The reference standard concentration is also prepared in a normal human serum pool. All serum results are referred to standard curves established with serum diluents.

Human serum pools are prepared from serum collected from healthy fasting volunteers, who have not received any medications, including antimicrobials, for the past month. Serum pools are frozen until use. Accurately measured amounts of powdered antimicrobial standards are diluted initially in buffer diluents, but succeeding quantitative dilutions use the normal human serum pool as diluent so that the highest concentration of the standard curve is \( \geq 99\% \) serum.

For clinical pharmacological studies using healthy adult volunteers as test subjects, a normal human serum pool is used as necessary to dilute the serum samples to concentrations within the range of this assay. Control studies employing a particular differentially diluted experimental sample show that extrapolation back to the original standard concentration is not affected by the degree of dilution with the normal human serum pool within the concentrations employed in these studies.

This conclusion may not apply when patients with serious metabolic diseases are subjects of study, and it may be advisable to use a patient’s own antibiotic-free serum as a control to establish a standard curve and for any dilutions of specimens obtained after drug administration. Bennett et al. (3) describe a dilution scheme in which a standard curve is established with the final concentrations containing 90% of the patient’s control serum and 10% of the serum pool. A similar scheme can be devised requiring one-half the volume of patient’s serum which is required for the Bennett technique. For example, the penicillin G standard curve dilution pattern mentioned earlier can be employed, but the key dilution \( i/v \) would be 4.0 units/ml, 10 times the normal final concentration. Succeeding standard curve concentrations would therefore also be 10 times the final concentration in the serum pool diluent. The modified dilution scheme would be as follows: (i) 0.05 ml of 4.0 units/ml in serum pool plus 0.45 ml patient serum = 0.4 units/ml; (ii) 0.05 ml of 3.0 units/ml in serum pool plus 0.45 ml patient serum = 0.3 units/ml; (iii) 0.1 ml of 2.0 units/ml in serum pool plus 0.9 ml patient serum = 0.2 units/ml; (iv) 0.05 ml of 1.5 units/ml in serum pool plus 0.45 ml patient serum = 0.15 units/ml; (v) 0.05 ml of 1.0 units/ml in serum pool plus 0.45 ml of patient serum = 0.1 units/ml. Establishment of a five-point standard curve in 90% patient’s control serum would thus require only 2.7 ml of the patient’s control serum.

Analysis of data. In the past, time-consuming and tedious calculations commonly performed manually were often necessary for estimating a regression line from sample data. Because the sample data for the
Fig. 1. Standard buffer curves for penicillins, cephalosporins, and aminoglycosides.

Microbioassay standard curves in these studies show a linear correlation with ±5% range of error, the Olivetti Underwood Programma 101 programs 2.14 and 2.15 (7) are used for rapid analysis of sample data.

Observed values are plotted on semilogarithm graph paper, with concentrations on the logarithmic scale and zone diameter on the linear scale. Concentration values are converted into four-place logarithms and X and Y paired values are entered in
Fig. 2. Standard buffer curves for polypeptides, tetracyclines, chloramphenicol, rifamycin AMP, and erythromycin.

the appropriately programmed Programma 101. The $X$, $Y$, slope of the regression line of $Y$ on $X$, standard deviation, error variance, and 95% confidence limits of the mean for any particular set of data are calculated and printed, in addition to other statistical information, such as $N$, $\sum X$, $\sum X^2$, $\sum Y$, $\sum Y^2$, $\sum XY$, $\sum x^3$, $\sum y^3$, $\sum xy$ coefficient, variance of sample, coefficient of variation, unbiased estimate of population variance, unbiased estimate of standard deviation of population, standard error of the mean, $Y$ intercept of the regression line, explained variation in $Y$, unexplained variation in $Y$, standard error of estimate of $Y$ on $X$, and unbiased estimate of $Y$ on $X$. Reference is made to the Statistical Analysis
### Table 4. Range of microbioassay

| Drug                | Predicted minimal concn in buffer a | Predicted minimal concn in serum a | Predicted per cent drug serum-bound at mean conc ±5% |
|---------------------|-------------------------------------|-----------------------------------|-----------------------------------------------|
| Penicillin G        | 0.12                                | 0.16                              | 20                                            |
| Ampicillin          | 0.022                               | 0.022                             | ≤5                                            |
| Methicillin         | 0.595                               | 0.564                             | ≤5                                            |
| Oxacillin           | 0.125                               | 0.627                             | 73                                            |
| Cloxacillin         | 0.113                               | 2.400                             | 96                                            |
| Dicloxacillin       | 0.536                               | 2.030                             | 76                                            |
| Neomycin            | 0.170                               | 0.193                             | 10                                            |
| Kanamycin           | 0.063                               | 0.070                             | ≤5                                            |
| Streptomycin        | 0.120                               | 0.141                             | 9                                             |
| Tetracycline        | 0.090                               | 0.162                             | 40                                            |
| Doxycycline         | 0.026                               | 0.039                             | 40                                            |
| Oxytetracycline     | 0.087                               | 0.162                             | 54                                            |
| Minocycline         | 0.028                               | 0.062                             | 50                                            |
| Cephalothin         | 0.627                               | 1.240                             | 46                                            |
| Cephaloridine       | 0.264                               | 0.383                             | 26                                            |
| Cephaloglycin       | 0.044                               | 0.065                             | 38                                            |
| Cephalexin          | 0.138                               | 0.229                             | 37                                            |
| Colistin            | 0.165                               | 0.204                             | 15                                            |
| Polymixin B         | 1.010                               | 2.050                             | 66                                            |
| Erythromycin        | 0.024                               | 0.026                             | ≤5                                            |
| Chloramphenicol     | 9.0                                 | 10.9                              | 6                                             |

a Values expressed in micrograms per milliliter except for penicillin G, which is expressed in Oxford units per milliliter.

manual (7) for details on the equations used in the program.

The serum half-life of a drug undergoing clinical trials is calculated in a similar fashion by using the slope of the regression line of drug concentration versus hours elapsed after drug administration, and by applying the usual formula,

\[ T_{1/2} = \log_2 \frac{2}{\beta} \]

where \( T_{1/2} \) is the serum half-life and \( \beta \) is the rate constant for decrease in serum concentrations.

**RESULTS**

Accurate and reproducible standard buffer and serum curves have been established for ampicillin, methicillin, penicillin G, oxacillin, cloxacillin, dicloxacillin, cephaloglycin, cephalaxin, cephaloridine, cephalothin, erythromycin, rifampicin amino methyl piperazine (AMP), kanamycin, streptomycin, neomycin, colistin, polymixin B, doxycycline, minocycline, oxytetracycline, tetracycline, and chloramphenicol. (Fig. 1 and 2). The figures are presented because they graphically show relative slopes of the buffer standard curves. These reproducible curves, together with the data presented in Table 4, will enable an investigator wishing to use this technique to assess his ability to use this technique and to determine whether experimental samples require dilution.

Slopes of buffer and serum standard curves for each antimicrobial were subjected to standard statistical analysis for parallelism. At \( P = 0.01 \), there was no significant difference between slopes of the buffer and serum standard curves in the concentration ranges covered by this microtechnique. On the assumption of parallelism, a serum-binding percentage for in vitro dilutions of antimicrobials in human serum has been calculated for the mean concentration of each standard curve (Table 4).

**DISCUSSION**

Since the filter-paper discs measure 6.5 mm in diameter, growth inhibition zones of less than 7.5 mm diameter cannot be read with accuracy. The 7.5-mm zone of inhibition thus determines the lowest concentration of drug that can be accurately measured with this assay (Table 4). The highest concentrations of the standards listed in Table 3 are arbitrary. Generally, an upper limit is determined by a zone diameter which stops just short of interfering with neighboring zones produced by the reference standard. Higher concentrations have been assayed either with use of a lower concentration for the reference standard or by increasing the dilution factor for the unknowns.

A microassay technique with filter-paper discs has been employed by Bristol Laboratories (personal communication) for studies with dicloxacillin. According to the description, a filter-paper disc is dipped into an antimicrobial-containing solution to saturation. Our experience with the "dipping" method was unsatisfactory. A disc may absorb from 0.03 to 0.05 ml of test material. Thus, actual concentrations on discs said to contain identical concentrations may actually differ widely from each other—evidenced by greatly differing zones of inhibition—because the subjective judgment of the individual performing the assay is used to determine a saturation point.

In this microassay system, the minimal detectable concentrations for chloramphenicol are 9.0 \( \mu \)g/ml in buffer and 10.9 \( \mu \)g/ml in serum. A more sensitive microassay for chloramphenicol could not be developed in this laboratory. Other test organisms including **Shigella sonnei** ATCC 11060, **Bordetella bronchiseptica** ATCC 4617, **Staphylococcus aureus** Bristol A9596, and a clinical isolate of **Streptococcus pyogenes** were tested without yielding significant improvements in the readability of the growth inhibition zones. However, this assay provides comparable degrees of...
sensitivity although requiring a smaller sample volume than other published techniques (4–6).

To our knowledge, no other microtechnique has been systematically standardized for the variety of antimicrobials covered in this presentation at comparable sensitivity levels, required sample volumes, and reproducibility of results.

Methods of dilution of the antimicrobial standards and preparation of the seeded plates are sufficiently reproducible so that this assay has been performed by four different technicians in this laboratory over the past 3 years without any appreciable change in assay parameters. The technique should fill a need for low-range antimicrobial quantitative analysis while also qualifying as a microtechnique.

ACKNOWLEDGMENTS

Powdered laboratory standards of the antimicrobials used in this study were obtained through courtesy of Abbott Laboratories (erythromycin), Bristol Laboratories (ampicillin, cloxacillin, dicloxacillin, kanamycin, methicillin, and oxacillin), CIBA Pharmaceutical Co. (rifampin AMP), Eli Lilly & Co. (cephalexin, cephaloglycin, cephalexin, and ceftazidime), Lederle Laboratories (minocycline), Parke, Davis & Co. (chloramphenicol), Chas. Pfizer & Co., Inc. (doxycycline, oxytetracycline, penicillin G, polymyxin B, streptomycin, and tetracycline), The Upjohn Company (neomycin), and Warner-Chilcott (colistin).

The excellent technical assistance of E. Howell and L. Walker in our laboratory, and the technical advice of James H. Lannon, Assistant Director of Control, Regulatory Affairs, Bristol Laboratories, is gratefully acknowledged.

This investigation was supported by Public Health Service research grant CC 00220, from the National Communicable Disease Center, Atlanta, Georgia, and by grants from Bristol Laboratories, Syracuse, N.Y., and CIBA Pharmaceutical Co., Summit, N.J.

LITERATURE CITED

1. Anderson, T. G., and A. Troyanosky. 1960. Antimicrobial testing by the disc method. Antibiot. Annu. 1959–1960, p. 387–395.
2. Axline, S. G., and H. J. Simon. 1965. Clinical pharmacology of antimicrobials in premature infants. I. Kanamycin, streptomycin and neomycin. Antimicrob. Agents Chemother. 1964, p. 135–141.
3. Bennett, J. V., J. L. Brodie, E. J. Benner, and W. M. M. Kirby. 1966. Simplified, accurate method for antibiotic assay of clinical specimens. Appl. Microbiol. 14:170–177.
4. Garrett, D. C. 1964. The quantitative analysis of drugs, p. 813–817. Chapman and Hall, Ltd., London.
5. Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics: a laboratory manual. Medical Encyclopedia, Inc., New York.
6. Kirshbaum, A., and B. Arret. 1967. Outline of details for official microbiological assays of antibiotics. J. Pharm. Sci. 56:511–515.
7. Williams, J. B. 1968. Statistical analysis. Olivetti Underwood Corp., New York.