ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1980, p. 813-823
0066-4804/80/05-0813/11$02.00/0

Vol. 17, No. 5

# Rapid Determination of Minimum Inhibitory Concentrations of Antimicrobial Agents by Regression Analysis of Light Scattering Data

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A novel, rapid, automated method for determining the minimum inhibitory concentrations of antimicrobial drugs within ranges acceptable for therapeutic application is described and validated in this report. By employing a simple modification of the Autobac photometer currently in use and an optically clear, modified Mueller-Hinton broth, the method utilizes forward light scattering data measured in the presence of two concentrations of the antimicrobial agent to compute the minimum inhibitory concentrations. Empirically derived regression equations which simultaneously use scattering data from two drug concentrations are employed in this computation, rather than simple breakpoint analysis in which the light scattering measured in the presence of each drug concentration is referred to a threshold level. The minimum inhibitory concentrations obtained with this new method were highly reproducible and, as shown by side by side comparisons were in excellent agreement with the minimum inhibitory concentrations obtained with the International Collaborative Study broth dilution method.

The proliferation of new antimicrobial drugs with subtle but nonetheless important differences in activity spectra has heightened the need for rapid measurements of bacterial susceptibilities in terms of the minimum inhibitory concentrations (MICs) of these agents. The original Autobac antimicrobial susceptibility testing system (9) was designed to provide rapid categorization (within 3 to 5 h of colony isolation) of a bacterial isolate as susceptible, intermediate/equivocal, or resistant to an antimicrobial agent. This system was shown (5, 8, 12) to provide results comparable to those of the 18- to 24-h Bauer-Kirby agar diffusion method (1). This basic Autobac system was modified recently to provide rapid measurements of the MICs of a variety of the antimicrobials in current use. This newly developed method is similar to several previously reported methods (7, 13, 14) in that a minimum number of concentrations of the antimicrobial agents are used to provide MICs within a range maintainable during therapy.

This report provides a detailed description of the new Autobac method and gives special attention to the novel mathematical treatment of data required to establish the MICs. It also presents the results of an application of the method to a sizeable group of clinical isolates of 16 bacterial species maintained in stock culture, with documentation of the accuracy of the MIC measurements for a variety of antibacterial drugs in current use. The MICs measured by the rapid method compare favorably with results obtained with the International Collaborative Study (ICS) broth dilution procedure.

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## MATERIALS AND METHODS

**Antimicrobial agents.** The agents used in these studies were ampicillin trihydrate (Bristol Laboratories, Syracuse, N.Y.), disodium carbenicillin (Pfizer Inc., New York, N.Y.), sodium cephalothin (Eli Lilly & Co., Indianapolis, Ind.), chloramphenicol (Parke, Davis & Co., Detroit, Mich.), clindamycin hydrochloride (The Upjohn Co., Kalamazoo, Mich.), gentamicin sulfate (Schering Corp., Bloomfield, N.J.), kanamycin sulfate (Bristol Laboratories), sodium methicillin (Bristol Laboratories), sodium penicillin G (Pfizer Inc.), and tetracycline hydrochloride (Pfizer Inc.). Although supplied as assayed powders, the agents were checked for biopotency by using the assay procedures recommended in the *Code of Federal Regulations*. These bulk drugs were used to prepare the stock solutions required both for the reference broth dilution method and for impregnating the absorbent paper disks used in the Autobac MIC procedure.

**Organisms.** The 415 bacterial strains used in these studies were clinical isolates acquired by various microbiology laboratories and included 9 strains of *Citrobacter diversus*, 7 strains of *Citrobacter freundii*, 7 strains of *Enterobacter aerogenes*, 12 strains of *Enterobacter cloacae*, 2 strains of *Enterobacter hafniae*, 57 strains of *Escherichia coli*, 32 strains of *Klebsiella* sp., 1 strain of *Klebsiella pneumoniae*, 29 strains of

*Proteus mirabilis*, 11 strains of *Proteus morganii*, 2 strains of *Proteus rettgeri*, 5 strains of *Proteus vulgaris*, 15 strains of *Serratia marcescens*, 46 strains of *Pseudomonas aeruginosa*, 76 strains of *Staphylococcus aureus*, 56 strains of *Staphylococcus epidermidis*, and 48 strains of *Enterococcus*. Upon receipt, the organisms were subcultured to blood agar plates and reidentified. Freshly isolated colonies were harvested and suspended in sterile, defibrinated sheep blood; 0.1-ml samples of the suspensions were stored in sealed vials at −70°C. Control strains (*E. coli* ATCC 25922 and 29194, *S. aureus* ATCC 25923, and *P. aeruginosa* ATCC 27853 and CDC F-12) were stored in the same manner.

**Inoculum preparation.** Vials of the frozen blood cultures were thawed and subcultured onto either tryptic soy blood agar or Columbia base blood agar 18 to 24 h before MIC testing. The standardized inocula for the Autobac MIC method were prepared by transferring isolated colonies of each bacterial strain from an 18-h blood agar plate into approximately 6 ml of phosphate-buffered saline in a screw-capped optical tube (16 by 125 mm). After the saline suspension in the capped tube was blended with a Vortex mixer, the number of bacterial units per milliliter was checked with an Autobac photometer. Two standardization meter ranges were used; mode 1 was approximately $0.9 \times 10^8$ to $1.2 \times 10^8$ colony-forming units (CFU) per ml of saline suspension, and mode 2 was approximately $0.9 \times 10^7$ to $1.2 \times 10^7$ CFU/ml of saline suspension. Depending upon colony size, from 1 to 20 colonies were required to obtain a photometer reading near the mode 1 meter range. If necessary, final adjustment to within this meter range was then performed either by back dilution with buffered saline or by suspension of additional bacteria. By using buffered saline, a 10:1 dilution of a sample of the mode 1 standardized inoculum was made in order to prepare the mode 2 inoculum. This inoculum was subsequently checked with the photometer set on the mode 2 range. The final broth inocula were prepared from the mode 1 and mode 2 standardized saline inocula by pipetting 2 ml of the saline inoculum into 18 ml of Autobac MIC broth that was contained in a screw-capped tube (18 by 150 mm). This dilution step resulted in standardized broth inocula of approximately $10^7$ CFU/ml (from mode 1) and approximately $10^6$ CFU/ml (from mode 2).

The same 18-h blood agar plates used to prepare the standardized inocula for the Autobac MIC method were used to prepare the standardized inocula for the reference MIC method. This was accomplished by suspending bacteria from four or five morphologically similar colonies from each agar plate into 6 ml of tryptic soy broth. The broth inocula were incubated at 35 to 37°C until the cultures visually achieved or exceeded the turbidity of a 0.5 McFarland standard. If turbidity exceeded the standard, the broth culture was back titrated with sterile tryptic soy broth. The resulting standardized suspensions containing approximately $10^8$ CFU/ml were diluted with Mueller-Hinton broth so that the inoculum level in the final tube dilution series was approximately $10^6$ CFU/ml for gram-positive bacteria and approximately $10^5$ CFU/ml for gram-negative bacteria.

**Media and reagents.** All MIC determinations by the reference broth dilution method were conducted in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) that was assayed for total calcium (spectrophotometric method; American Monitor Corp., Indianapolis, Ind., or Hycel, Inc., Houston, Tex.) and total magnesium (spectrophotometric method; Pierce Chemicals, Rockford, Ill.). When necessary, this broth was supplemented with $CaCl_2$ (anhydrous) and/or $MgCl_2 \cdot 6H_2O$ so that the final calcium and magnesium concentrations in the broth were approximately 0.06 and 0.03 g/liter, respectively.

To optimize the potential for achieving coincidence in the absolute value of the MIC derived from a rapid method with the MIC derived from a conventional method, such as ICS broth dilution, a broth having a composition similar to the composition of Mueller-Hinton medium was selected. This medium was modified by replacing the soluble starch component with 0.2% dextrose to allow better growth support of enterococcal isolates within a 3- to 5-h incubation time. In keeping with the recommendations of Reller et al. (10), the concentrations of calcium and magnesium in the broth were controlled by adding $CaCl_2$ (anhydrous) and $MgCl_2 \cdot 6H_2O$. The final composition of the broth used in the Autobac studies is given in Table 1. The broth was optically clarified by membrane filtration (pore size, 0.22 μm) and was terminally sterilized at 121°C for 15 min.

The standardized inocula intended for use in the Autobac method were prepared in phosphate-buffered saline containing 4.2 g of sodium chloride per liter, 3.2 g of dibasic potassium phosphate per liter, and 1.6 g of monobasic potassium phosphate per liter. This buffered saline was sterile and optically clear and was the same suspending solution currently in use in the Autobac interpretive susceptibility method.

**Instrumentation and devices.** Figure 1 shows a standard Autobac 1 photometer modified to enable convenient selection of either the $10^8$-CFU/ml range (MIC mode 1) or the $10^7$-CFU/ml range (MIC mode 2) on the inoculum standardization meter. The photometer was also modified to enable automatic calculation of the mean background constant for each MIC mode. The two background constants could be stored at accessible memory locations and could be recalled and changed by manual entry on a keypad built into the photometer. After incubation, the MIC mode 1 and MIC mode 2 background constants were used by the photometer to automatically calculate the light scattering index (LSI) for each sample chamber of the cuvette when it was read by using the appropriate photometer mode. Calculation f the LSIs was performed automatically by the photometer by using the following algorithm: $LSI = (G_c - G_x)/(G_c - G_k)$, where $G_c$ is the logarithm of the 35° angle light scat-

TABLE 1. *Composition of Autobac MIC broth*[a]

| Component | Concn (g/liter) |
|---|---|
| Acid hydrolysate of casein | 17.5 |
| Brain heart infusion | 4.5 |
| Dextrose | 2.0 |
| Total calcium | 0.06 |
| Total magnesium | 0.03 |

[a] The pH of the broth was 7.3.

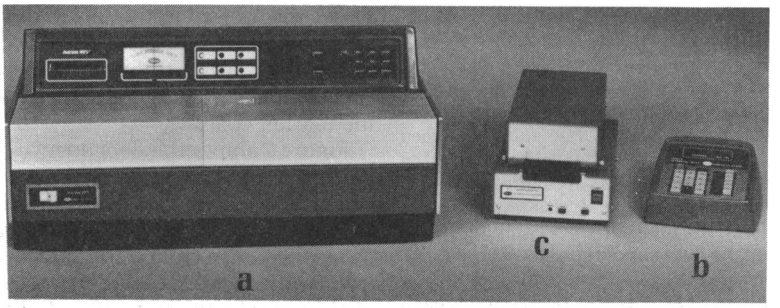


FIG. 1. *Autobac multitest system components. (a) Photometer. (b) Cuvette information device. (c) Alpha-numeric printer.*

tering intensity for the uninhibited control after incubation; $G_k$ is the logarithm of the 35° angle light scattering intensity for the uninhibited control before incubation (i.e., background); and $G_x$ is the logarithm of the 35° angle light scattering intensity for the antimicrobial-challenged sample after incubation. The photometer was modified to allow sample information (e.g., accession number, isolate number, and antimicrobial panel number) to be entered. This sample information was combined with the LSI results by the cuvette information device and was printed out as a report by an alpha-numeric printer. These two peripheral devices (Fig. 1) were interfaced with the photometer.

A standard Autobac incubator-shaker was also employed throughout these studies and was operated at 36 ± 1°C and 220 rpm.

A standard Autobac optical device (known as the cuvette) was also used throughout these studies, along with an optical glass wedge to ensure that the photometer was always properly calibrated.

The antimicrobial elution disks were prepared by impregnating each paper disk (no. 225; Eaton and Dikeman) with 10 μl of antimicrobial agent solution, using a Hamilton digital dispenser calibrated to deliver 10 ± 0.1 μl (mean ± standard deviation). Incorporation of $^{131}I$ enabled immediate verification of the accuracy and precision of the impregnated disks. Chloramphenicol, clindamycin, gentamicin, kanamycin, and tetracycline were dissolved in methanol–water (1:1). For carbenicillin, cephalothin, methicillin, and penicillin G the same solvent system was used, except that it contained 0.5 g of trisodium citrate per 100 ml. Finally, for ampicillin the solvent system contained water, methanol, and acetone (1:0.5:0.5). This solvent also contained 0.5 g of trisodium citrate per 100 ml. All disks were dried at 60°C for 5 min in an air tunnel and were immediately placed into glass cartridges. After each cartridge was sealed, it was stored at approximately −25°C until use. A Bausch & Lomb model Spectronic 20 spectrophotometer was used for the spectrophotometric assays of calcium and magnesium.

**Reference MIC method.** The ICS broth dilution method of Ericsson and Sherris (4) was used as the reference MIC method for these studies. Visually standardized inocula were challenged with twofold dilutions of each antibiotic in Mueller-Hinton broth adjusted in total calcium and magnesium content. Each tube contained a final volume of 2 ml. Tubes were incubated at 35°C for approximately 18 h. After incubation, the series of tubes was visually examined in a well-lighted area to determine the minimum concentration of antibiotic that completely inhibited the growth of the organisms. Both positive (growth) and negative (sterility) control tubes were also run in parallel.

**Autobac MIC method.** The basic Autobac approach (5, 8, 9, 12) of challenging a photometrically standardized broth inoculum with a variety of antibiotics in a multichambered cuvette was utilized in the Autobac MIC procedure. The MIC modification of that basic technique involved two inoculum modes. MIC mode 2 of the photometer was used when gram-negative organisms were tested against beta-lactam antimicrobial agents. MIC mode 1 was used for testing all other antimicrobial agent-organism combinations. Three disk masses (low, middle, and high) of each antibiotic were used. The antibiotic contents of the three disks (each of which was placed into 1.5 ml of broth inoculum in a cuvette chamber) were graduated in two- or fourfold intervals to span the achievable range of blood levels for the various drugs. The cuvettes were incubated in the Autobac incubator-shaker at 36°C and 220 rpm (0.75-inch [ca. 1.9-cm] circle) for 4 to 5 h. By using the appropriate MIC modes, the cuvettes were read in the Autobac photometer after incubation, and the resulting LSIs were used to obtain MICs by either the breakpoint or the regression method.

The breakpoint or threshold method utilized a specific LSI (called the LSI breakpoint) to determine the MIC. The breakpoint varied depending upon the antibiotic-organism combination. Four organism groups were used for this purpose; these were the staphylococci, the enterococci, the *Enterobacteriaceae*, and the pseudomonads. The breakpoint method compared the three LSIs obtained from testing with three antimicrobial disk masses with the breakpoint. The MIC was defined as the lowest concentration (i.e., disk mass per 1.5 ml of broth inoculum in the cuvette chamber) giving an LSI which was equal to or greater than the LSI breakpoint.

The regression method utilized equations to calculate the MICs (dependent variable) from the LSIs (independent variables). The equations were derived from the LSI data and the reference MICs that were collected in parallel on populations of strains. The equations were formed by both linear and nonlinear stepwise regression analyses of the data, as described in Draper and Smith (3) and as implemented by using

the computer programs BMDP2R and BMDP3R described by Dixon and Brown (2). This approach generated two basic types of equations, those that were linear in the regression coefficients (although not necessarily linear in the LSIs) and those that were nonlinear in the regression coefficients. The equations used only two independent variables (the two LSIs resulting from challenges with two of the three antibiotic disk masses). As in the breakpoint analysis approach, separate equations were formed for each combination of antibiotic and organism group (staphylococci, enterococci, *Enterobacteriaceae*, pseudomonads).

The same data set was used for determining each breakpoint and each equation in the above methods and to evaluate the accuracy of the MICs derived from such breakpoints and equations. Since a small number of parameters were being estimated from a large body of data, no problem of overfitting the data should have resulted. However, a larger data base than the one associated with the feasibility study reported here was necessary to validate the robustness of these procedures for deriving breakpoints and empirical equations.

## RESULTS

**Test conditions.** To develop standardized MIC methods for the existing Autobac system, a number of test conditions had to be defined. These included the broth composition, the initial inoculum concentration, the incubation time, and the antibiotic concentrations.

**(i) Initial inoculum level and incubation time.** After the development of a suitable broth for the Autobac MIC method, we performed a series of experiments in which the inoculum levels of many different bacterial species and the periods of agitated incubation were varied. Broth inocula of $10^6$ to $10^7$ CFU/ml were challenged with a series of concentrations of a particular antibiotic in the 12 chambers of a standard Autobac cuvette. An additional chamber received no antibiotic and functioned as the control. By using the photometer, the LSIs of the samples were monitored as a function of incubation time.

Figure 2 shows the dependence of the LSIs on antibiotic concentration, inoculum level, and incubation time for two bacterial strains. Figure 2A shows the LSI of an *E. coli* strain subjected to a series of twofold dilutions of chloramphenicol. The average 18-h ICS broth dilution MIC of chloramphenicol was 16 μg/ml for this *E. coli* strain. When growth inhibition was defined as an LSI of ≥0.65 (Fig. 2, horizontal dashed line), the MIC determined by the Autobac method was only 8 μg/ml when the inoculum size was $10^6$ CFU/ml and incubation was for 4 h. However, when the incubation time was increased to 5 h, the points within the transition region of the curve shifted to lower LSIs (more growth). Increasing the period of incubation, therefore, decreased the apparent inhibition. If the period of incubation was held constant at 4 h and the initial inoculum was increased 10-fold to approximately $10^7$ CFU/ml, a similar decrease in apparent inhibition occurred. On average, the increase in inoculum size had a greater effect than the 1-h increase in incubation time. If both parameters were increased, the largest decrease in the LSIs was observed. Therefore, the MIC could be maximized by increasing both incubation time and inoculum size. As Fig. 2 shows, when six chloramphenicol concentrations were used, the minimum concentration required to inhibit the growth of this *E. coli* strain increased from 8 to 16 μg/ml as the inoculum size and incubation time were increased. Under these conditions (inoculum size, $10^7$ CFU/ml; and in-

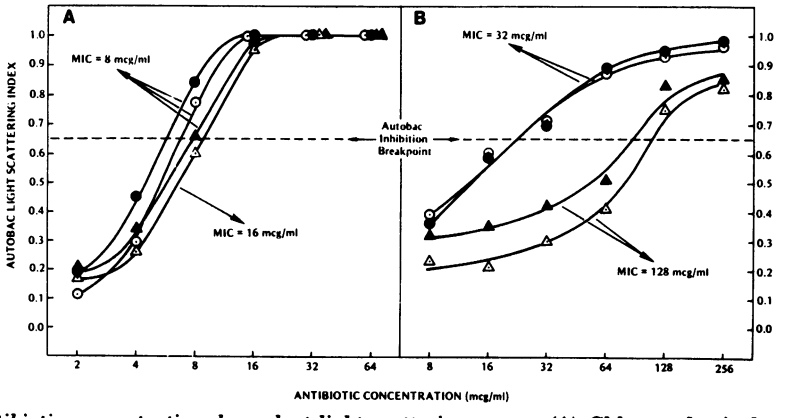


FIG. 2. *Antibiotic concentration-dependent light scattering curves. (A) Chloramphenicol and E. coli 286-51A: 18-h ICS broth dilution, MIC = 16 μg/ml. (B) Carbenicillin and P. vulgaris 351-57A: 18-h ICS broth dilution, MIC = 32 μg/ml. Symbols: ●, $10^6$-CFU/ml initial inoculum, 4 h of incubation (36°C); ⊙, $10^6$-CFU/ml initial inoculum, 5 h of incubation (36°C); ▲, $10^7$-CFU/ml initial inoculum, 4 h of incubation (36°C); △, $10^7$-CFU/ml initial inoculum, 5 h of incubation (36°C).*

cubation time, 5 h), the average value of the Autobac MIC coincided with the average value obtained with the reference method.

Figure 2B shows the results obtained when a strain of *P. vulgaris* was challenged with a series of twofold dilutions of carbenicillin. As in the case of chloramphenicol and *E. coli*, decreases in the LSI were again observed with increased incubation time and inoculum size, although the principal effect was caused by inoculum size. As Fig. 2B shows, a large increase in the Autobac MIC (from 32 to 128 μg/ml) was observed when the inoculum was increased 10-fold. Unlike the above-described chloramphenicol results, however, the increased inoculum size led to a 4- to 5-h carbenicillin MIC considerably higher than the average value (32 μg/ml) obtained with the reference method.

Analysis of data from experiments such as those presented in Fig. 2 for many antibiotic-species combinations revealed that a 5-h incubation for all organisms afforded the best coincidence between the MICs of the Autobac and reference methods. Further analysis showed that an inoculum size of $10^7$ CFU/ml was optimum for all antibiotic-species combinations except gram-negative organisms challenged by beta-lactam antibiotics. For these combinations an inoculum size of $10^6$ CFU/ml provided 5-h MIC values that correlated better with the reference method.

**(ii) Antibiotic concentrations.** The antibiotics and concentrations tested were selected in order to focus on developing therapeutic guidance for treatment of systemic infections. We chose 10 antibiotics for which MICs are most commonly used in the therapy of systemic infections. These drugs are listed in Table 2 along with the concentrations of each used in the Autobac MIC method. Initially, we selected three concentrations of each antibiotic, spanning the achievable range of serum levels in two- to fourfold dilutions.

**Conversion of LSIs into MICs. (i) Breakpoint approach.** The light scattering intensities of bacteria grown in a series of concentrations of an antimicrobial drug are normally translated into an MIC by a simple breakpoint method. Table 3 shows an example of this traditional approach, as embodied in the basic Autobac system, for testing *Enterobacteriaceae* strains with chloramphenicol. The LSIs measured after 5 h of incubation in three concentrations of chloramphenicol are each compared with some breakpoint LSI. In the example shown in Table 3 an LSI breakpoint of 0.90 was selected. Inhibition of bacterial growth is considered to have occurred when the LSI is equal to or greater than the breakpoint LSI. Therefore, in Table 3 if the LSIs computed by the photometer for the organism in the presence of all three disk masses of chloramphenicol are ≥0.90, then the MIC must be less than or equal to 8 μg/ml (12 μg eluted into 1.5 ml of broth inoculum), the minimum concentration of chloramphenicol em-

TABLE 3. *Autobac MIC chart: chloramphenicol*[a]

| LSI for: | | | MIC (μg/ml) |
|---|---|---|---|
| Disk 1 (12 μg) | Disk 2 (24 μg) | Disk 3 (48 μg) | |
| ≥0.90 | ≥0.90 | ≥0.90 | ≤8 |
| <0.90 | ≥0.90 | ≥0.90 | 16 |
| <0.90 | <0.90 | ≥0.90 | 32 |
| <0.90 | <0.90 | <0.90 | >32 |

[a] For *Enterobacteriaceae*, the initial inoculum size was $10^7$ CFU/ml, and incubation was for 5 h at 36°C and 220 rpm.

TABLE 2. *Antibiotics and their concentrations used in the Autobac and reference methods*

| Antibiotic | Mass per disk[a] | Concn[b] |
|---|---|---|
| Ampicillin | 3,[c] 12, 48[c] | 2,[c] 8, 32[c] |
| Carbenicillin | 12,[c] 48, 192[c] | 8,[c] 32, 128[c] |
| Cephalothin | 3,[c] 12, 48[c] | 2,[c] 8, 32[c] |
| Chloramphenicol | 12,[c] 24, 48[c] | 8,[c] 16, 32[c] |
| Clindamycin | 1.5,[c] 3, 6[c] | 1,[c] 2, 4[c] |
| Gentamicin | 3,[c] 6, 12[c] | 2,[c] 4, 8[c] |
| Kanamycin | 6,[c] 12, 24[c] | 4,[c] 8, 16[c] |
| Methicillin | 3,[c] 6, 12[c] | 2,[c] 4, 8[c] |
| Penicillin G | | |
| For staphylococci | 0.188,[c] 3, 48[c] | 0.125,[c] 2, 32[c] |
| For other organisms | 3, 12,[c] 48[c] | 2, 8[c] 32[c] |
| Tetracycline | 3,[c] 6, 12[c] | 2,[c] 4, 8[c] |

[a] Units for all antibiotics except penicillin G, micrograms; units for penicillin G, international units.

[b] Units for all antibiotics except penicillin G, micrograms per milliliter; units for penicillin G, international units per milliliter.

[c] The two disk masses and concentrations for each antibiotic used in the Autobac MIC method when regression analysis was used to convert LSI values to MIC values.

ployed in the test. However, if inhibition was found only in the presence of the 24- and 48-μg disk masses, then the MIC would be 16 μg/ml. Likewise, if inhibition only occurred with the 48-μg disk, an MIC of 32 μg/ml would be reported. Finally, if the LSI values found with all three disk masses were less than 0.90, then the MIC would be reported as greater than 32 μg/ml. For those antibiotics for which more than twofold dilution intervals were used, MICs were expressed as ranges. For example, the fourfold dilution intervals used with ampicillin or cephalothin (Table 2) enabled possible MIC values of ≤2, 4 to 8, 16 to 32, and >32 μg/ml. The breakpoint LSI selected was that LSI enabling the maximum extent of symmetrical overlap of the 5-h MICs of the Autobac method with the MICs of the ICS reference broth dilution method. The breakpoint LSI was independently selected for each combination of antibiotic and organism group tested. In this regard, the major organism groups tested were staphylococci, enterococci, *Enterobacteriaceae*, and pseudomonads.

**(ii) Regression approach.** A breakpoint method has the disadvantage that the LSI measured in the presence of any one of the antibiotic concentrations is considered to be independent of the LSIs measured for the other concentrations. For example, LSIs were measured after 5 h of incubation for concencentrations of 2, 4, and 8 μg of gentamicin per ml. If two *Enterobacteriaceae* isolates (isolates A and B) were tested and gave the LSIs shown in Table 4, the breakpoint method would give an MIC of 8 μg of gentamicin per ml, for both isolates since the LSI breakpoint of 0.60 was met or exceeded only when a concentration of 8 μg/ml was reached. The two accompanying LSIs measured in the presence of gentamicin concentrations of 2 and 4 μg/ml are not utilized by the breakpoint method. In contrast, a regression method takes into account the fact that LSIs measured in the presence of all antibiotic concentrations are variables upon which the MIC depends. A regression analysis would, therefore, consider the additional information contained in the LSIs measured in the presence of either the 2-μg/ml concentration or the 4-μg/ml concentration or both. In the example shown in Table 4 the regression approach might find the gentamicin MIC for isolate A to be somewhat lower (e.g., 6 μg/ml) than that of isolate B (e.g., 8 μg/ml). Furthermore, the regression method would not be as sensitive as the breakpoint method to minor variations in the LSI for strains with an LSI near the breakpoint. In Table 4, isolate B is such an example since the LSI in the presence of the high disk mass is 0.61 and the breakpoint is 0.60. If this isolate had an LSI of 0.59 for the high disk, then the MIC would be >8 μg/ml. Finally, the regression approach has the advantage that it can provide continuous MICs between and slightly outside the range of the drug concentrations tested.

TABLE 4. *Dependence of Autobac LSI on gentamicin concentration for two Enterobacteriaceae isolates*[a]

| Gentamicin | | 5-h LSI value for: | |
|---|---|---|---|
| Mass per disk (μg) | Concn (μg/ml) | Isolate A | Isolate B |
| 3 | 2 | 0.28 | 0.02 |
| 6 | 4 | 0.48 | 0.15 |
| 12 | 8 | 0.85 | 0.61 |

[a] Inhibition breakpoint LSI, 0.60.

The regression approach attempts to fit an equation to the LSIs measured in the presence of different concentrations of an antimicrobial agent when such an agent has been used to challenge a population of different strains having known reference MICs of the agent. The dependent variable is the $\log_2$ MIC, and the independent variables are the LSIs. The first approach taken was to fit equations by using the LSIs, as well as their squared values and cross-products. In doing this, it was found that in general there was no additional statistical information in the data measured in the presence of the middle antibiotic concentration once the data of the upper and lower concentrations had been fitted. Thus, the LSI data resulting from two antibiotic concentrations were capable of providing as much information as was available from all three concentrations in the series. Table 2 shows the two disk masses selected for the various antibiotics.

The statistical approach used was stepwise linear regression analysis. The above approach is called linear regression, since the equation, although not necessarily linear in the LSIs, is linear in the regression coefficients. Figure 3A provides the equation for the chloramphenicol-*Enterobacteriaceae* combination, as well as an illustration of the nature of the equation. The equation describes the cross-hatched surface shown folded in three dimensions ($x = LSI_{12}$, $y = LSI_{48}$, $z = \log_2$ MIC, where $LSI_{12}$ and $LSI_{48}$ are the LSIs from the 12- and 48-μg disks of chloramphenicol, respectively). Hence, by measuring the LSIs for these two disk potencies, the log of the MIC is uniquely determined by vertically extrapolating to the cross-hatched surface.

An alternative nonlinear regression equation approach is to fit an equation of the following form:

$$\log_2 \text{MIC} = C_1\exp(C_2\text{LSI}_{\text{low}}) + C_3\exp(C_4\text{LSI}_{\text{high}}) \quad (1)$$

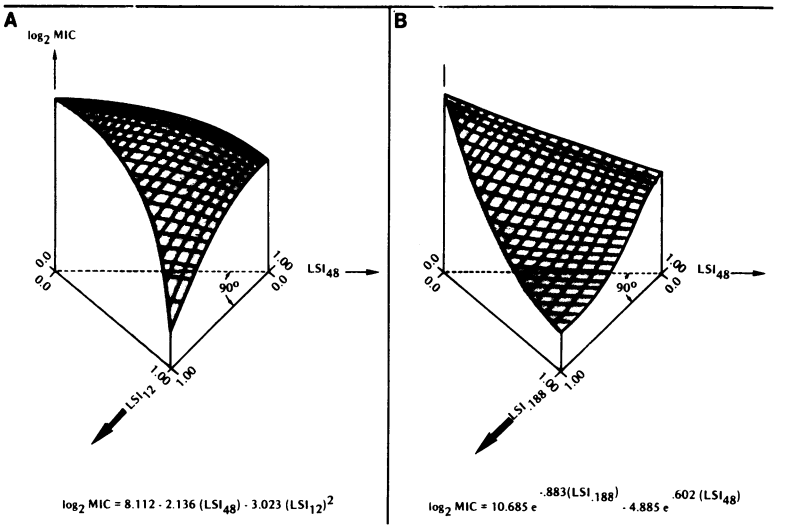


FIG. 3. *Graphic illustration of two forms of regression equations for calculation of MICs from Autobac LSIs. (A) Chloramphenicol-Enterobacteriaceae. (B) Penicillin G-staphylococci.*

In this equation $LSI_{low}$ and $LSI_{high}$ are the LSIs corresponding to the low and high antimicrobial disk masses, and the parameters $C_1$, $C_2$, $C_3$, and $C_4$ are fitted by the data. However, it is possible to tie down this equation when the LSIs are at their extremes. For example, data may indicate that when $LSI_{low} = LSI_{high} = 0$, then $\log_2$ MIC $= U$, and when $LSI_{low} = LSI_{high} = 1.0$, then $\log_2$ MIC $= D$. When this is done, equation 1 becomes,

$$\log_2 \text{MIC} = \frac{\{[U\exp(C_4) - D]\exp(C_2 LSI_{low})\} + \{[D - U\exp(C_2)]\exp(C_4 LSI_{high})\}}{\exp(C_4) - \exp(C_2)} \quad (2)$$

Note that equation 2 contains only two parameters; $C_2$ and $C_4$. Equation 2 may also be derived as a solution of the following differential equation:

$$\frac{1}{C_2}\frac{\partial Z}{\partial X} + \frac{1}{C_4}\frac{\partial Z}{\partial Y} - Z = 0, \quad (3)$$

where $Z$ is a dependent variable and $X$ and $Y$ are independent variables.

Figure 3B presents an example of a nonlinear equation of the form of equation 2, as well as its graphical representation for the combination of penicillin G and staphylococci.

By using the above-described procedures, equations were generated from the LSI data of two of the disk masses of each antibiotic and the accompanying ICS reference broth dilution data collected with populations of representative strains of the four principal organism groups tested (staphylococci, enterococci, *Enterobacteriaceae*, and pseudomonads).

**Comparative MIC data.** Figure 4 shows Autobac MICs generated by both breakpoint and regression analyses compared with the corresponding ICS broth dilution MICs. Data were collected for 95 strains of the family *Enterobacteriaceae* which were subjected to chloramphenicol broth dilution testing in parallel by both the Autobac and reference MIC methods. The 95 strains and their ICS MIC ranges were as follows: 30 strains of *E. coli*, 4 to >128 μg/ml; 10 strains of *Klebsiella* sp., 0.5 to 128 μg/ml; 10 strains of *Enterobacter* sp., 8 to 128 μg/ml; 15 strains of *P. mirabilis*, 4 to 16 μg/ml; 15 other strains of *Proteus* sp., 4 to >128 μg/ml; 10 strains of *S. marcescens*, 32 to >128 μg/ml; and 5 strains of *Citrobacter* sp., 8 to 64 μg/ml. For purposes of comparing the two Autobac analysis methods, the continuous MICs that resulted from the regression method were rounded to the closest of the four possible MICs given by the breakpoint method.

Figure 4A shows that when breakpoint analysis was used, 91 (95.8%) of the 95 strains had Autobac MICs that were within ±1 twofold dilution of the reference MIC. In contrast, 93 (97.9%) of the 95 strains had Autobac values within ±1 twofold dilution of the 18-h reference values when regression analysis was used. In addition, a more symmetrical distribution of the MICs about the diagnoal of perfect agreement was possible when the regression analysis rapid method was used to generate the MICs.

Table 5 shows the agreement between the MICs of the rapid method when both breakpoint and regression analyses were used and the reference method for all 17 antibiotic-organism

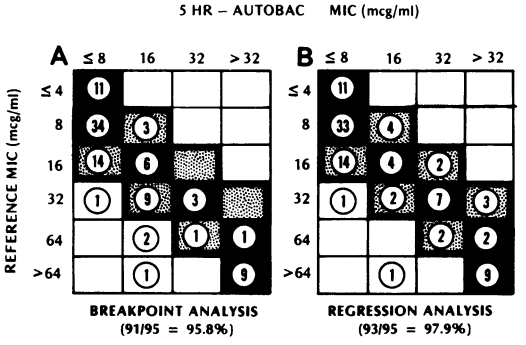


FIG. 4. *Distribution of Autobac and ICS broth dilution chloramphenicol MICs determined for 95 strains of Enterobacteriaceae. The numbers in the circles represent the numbers of strains at the MIC coordinates indicated; the black squares represent agreement between the absolute values of the MICs measured by the reference and Autobac methods; the stippled squares represent ±1 twofold dilution differences between the MICs determined by the reference and Autobac methods; and the white squares represent ≥2 twofold dilution differences between the MICs determined by the reference and Autobac methods.*

groups tested. The strains of each species tested in parallel by both MIC methods were specifically selected for each antibiotic so as to represent the widest range of MICs possible. The overall agreement betwen the rapid Autobac method in which the traditional breakpoint analysis was used and the ICS reference method for the 1,094 tests conducted was 92.7%. In contrast, a 94.7% agreement between the Autobac and reference methods was found when regression analysis was employed to convert the LSIs to MICs. In all but two antibiotic-organism combinations, agreement was 90% or greater with breakpoint analysis, but only one group showed less than a 91% agreement with regression analysis.

Table 6 shows the differences between the MICs obtained by the 5-h method and the corresponding MICs obtained by the ICS broth dilution method. MIC differences between the two methods were symmetrically distributed about the center point for most antibiotics, regardless of which analysis procedure was used in the rapid method; 2.1% of the organisms were found to have MIC differences of ≥3 twofold dilutions when breakpoint analysis was used, whereas only 1.2% were found to have such differences when regression analysis was used. A similar comparison of the incidence of twofold dilution differences showed that 5.1% of all strains tested had a ±2 dilution difference (i.e., 2.3% having a −2 dilution difference and 2.8% having a +2 dilution difference) when breakpoint analysis was used, compared with 4.1% having a ±2 dilution difference (i.e., 2.3% having a −2 dilution difference and 1.8% having a +2 dilution difference) when regression analysis was used. The most asymmetrically distributed MIC differences were observed with chloramphenicol, where a number of enterococci, *E. coli*, *Klebsiella* sp., *Enterobacter* sp., and *P. mirabilis* strains were found to have reference MICs of 16 μg/ml, compared with MICs of ≤8 μg/ml as determined by the Autobac method when either the breakpoint or regression method was used.

One final aspect of the feasibility studies on this 5-h method was the reproducibility of the MIC. Figure 5 shows the results of a series of replicate MIC studies conducted over a 3-month period. This study examined the repeatability of measuring the chloramphenicol MIC of *E. coli* 420-51A. LSI data collected in the presence of the 12- and 48-μg disks were converted into an MIC by using the regression equation given in Fig. 3. In total, 512 replicate determinations of the MIC were conducted over the 3-month period in four independent lots of the Autobac MIC broth. The results, presented (Fig. 5) as the number of replicates per 2-μg/ml increments of the MIC, show a high degree of reproducibility; 96% of all MICs occurred between 44 and 62 μg/ml. This translates to a long-term repeatability of this 5-h chloramphenicol mean MIC to within approximately ±⅛ of a twofold dilution for 96% of all determinations.

## DISCUSSION

Measurements of the MICs of antimicrobial drugs can provide valuable quantitative information for therapeutic management of a number of infectious disease states. MIC methods have, however, been underemployed in clinical laboratories primarily because of their labor intensiveness compared with the less quantitative disk diffusion methods. Furthermore, traditional MIC procedures require overnight incubation before results are available, and such procedures also involve considerable subjectivity in both inoculum preparation and endpoint determination.

Previous studies by Lampe et al. (6) showed that the incubation time required to estimate MICs accurately might be substantially reduced relative to the overnight incubation period required for traditional MIC procedures. By increasing the initial inoculum level, these workers demonstrated that MIC values in good agreement with the 18-h ICS broth dilution method could be measured for a number of bacterial strains after an incubation period as short as 3 h.

TABLE 5. *Percent agreement between the Autobac and ICS broth dilution methods*

| Antibiotic | % Agreement (within ±1 twofold dilution) between the Autobac and reference MIC methods | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Breakpoint analysis[a] | | | | | Regression analysis[a] | | | | |
| | Staphylococci[b] | Enterococci | *Enterobacteriaceae*[c] | *P. aeruginosa* | Avg | Staphylococci | Enterococci | *Enterobacteriaceae* | *P. aeruginosa* | Avg |
| Ampicillin | | | 86.9 ($n$ = 84)[d] | | 86.9 ($n$ = 84) | | | 81.0 ($n$ = 84) | | 81.0 ($n$ = 84) |
| Carbenicillin | | | 95.5 ($n$ = 89) | 85.7 ($n$ = 35) | 92.7 ($n$ = 124) | | | 96.6 ($n$ = 89) | 97.1 ($n$ = 35) | 96.8 ($n$ = 124) |
| Cephalothin | 94.3 ($n$ = 35) | | 92.9 ($n$ = 84) | | 93.3 ($n$ = 119) | 91.4 ($n$ = 35) | | 91.7 ($n$ = 84) | | 91.6 ($n$ = 119) |
| Chloramphenicol | | 93.3 ($n$ = 30) | 95.8 ($n$ = 95) | | 95.2 ($n$ = 125) | | 100.0 ($n$ = 30) | 97.9 ($n$ = 95) | | 98.4 ($n$ = 125) |
| Clindamycin | 97.6 ($n$ = 85) | | | | 97.6 ($n$ = 85) | 97.6 ($n$ = 85) | | | | 97.6 ($n$ = 85) |
| Gentamicin | | | 98.8 ($n$ = 85) | 95.8 ($n$ = 24) | 98.2 ($n$ = 109) | | | 100.0 ($n$ = 85) | 100.0 ($n$ = 24) | 100.0 ($n$ = 109) |
| Kanamycin | | | 82.4 ($n$ = 85) | 100.0 ($n$ = 24) | 86.2 ($n$ = 109) | | | 97.6 ($n$ = 85) | 100.0 ($n$ = 24) | 98.2 ($n$ = 109) |
| Methicillin | 90.0 ($n$ = 80) | | | | 90.0 ($n$ = 80) | 93.8 ($n$ = 80) | | | | 93.8 ($n$ = 80) |
| Penicillin G | 90.0[e] ($n$ = 80) | 96.7 ($n$ = 30) | 90.0 ($n$ = 60) | | 91.2 ($n$ = 170) | 96.2[e] ($n$ = 80) | 93.3 ($n$ = 30) | 91.7 ($n$ = 60) | | 94.1 ($n$ = 170) |
| Tetracycline | | | 95.5 ($n$ = 89) | | 95.5 ($n$ = 89) | | | 92.1 ($n$ = 89) | | 92.1 ($n$ = 89) |
| Average | 92.9 ($n$ = 280) | 95.0 ($n$ = 60) | 92.4 ($n$ = 671) | 92.8 ($n$ = 83) | 92.7 ($n$ = 1,094) | 95.4 ($n$ = 280) | 96.7 ($n$ = 60) | 93.7 ($n$ = 671) | 98.8 ($n$ = 83) | 94.7 ($n$ = 1,094) |

[a] Type of analysis used in the Autobac method to convert the LSI data into MICs.
[b] *S. aureus* and *S. epidermidis*.
[c] *E. coli*, *Klebsiella* sp., *Proteus* sp., *Enterobacter* sp., *Serratia* sp., and *Citrobacter* sp.
[d] $n$, Number of bacterial strains tested.
[e] Agreement was within ±1 fourfold dilution since a fourfold dilution series was used in the reference method.

TABLE 6. *Distribution of MIC differences between the Autobac and ICS broth dilution methods*

| Antibiotic | Type of Autobac analysis | % Incidence of twofold MIC differences between the Autobac and reference MIC methods[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ≥−4 | −3 | −2 | −1 | 0 | +1 | +2 | +3 | ≥+4 |
| Ampicillin | Breakpoint | 2.4 | 1.2 | 5.9 | 9.5 | 67.9 | 9.5 | 3.6 | 0.0 | 0.0 |
| (*n* = 84)[b] | Regression | 2.4 | 1.2 | 9.5 | 10.7 | 59.5 | 10.7 | 5.9 | 0.0 | 0.0 |
| Carbenicillin | Breakpoint | 0.0 | 1.6 | 0.8 | 13.7 | 73.4 | 5.6 | 3.2 | 0.8 | 0.8 |
| (*n* = 124) | Regression | 0.0 | 0.0 | 0.8 | 14.5 | 72.6 | 9.7 | 2.4 | 0.0 | 0.0 |
| Cephalothin | Breakpoint | 0.0 | 0.0 | 2.5 | 6.7 | 77.3 | 9.2 | 3.4 | 0.8 | 0.0 |
| (*n* = 119) | Regression | 0.0 | 1.7 | 5.0 | 9.2 | 72.3 | 10.1 | 1.7 | 0.0 | 0.0 |
| Chloramphenicol | Breakpoint | 0.0 | 0.8 | 2.4 | 29.6 | 63.2 | 2.4 | 0.0 | 0.8 | 0.8 |
| (*n* = 125) | Regression | 0.0 | 0.8 | 0.8 | 24.0 | 67.2 | 7.2 | 0.0 | 0.0 | 0.0 |
| Clindamycin | Breakpoint | 1.2 | 0.0 | 0.0 | 0.0 | 97.6 | 0.0 | 0.0 | 0.0 | 1.2 |
| (*n* = 85) | Regression | 1.2 | 0.0 | 0.0 | 0.0 | 97.6 | 0.0 | 0.0 | 0.0 | 1.2 |
| Gentamicin | Breakpoint | 0.0 | 0.0 | 0.9 | 10.1 | 78.9 | 9.2 | 0.0 | 0.9 | 0.0 |
| (*n* = 109) | Regression | 0.0 | 0.0 | 0.0 | 3.7 | 89.0 | 7.3 | 0.0 | 0.0 | 0.0 |
| Kanamycin | Breakpoint | 0.9 | 0.0 | 0.9 | 3.7 | 68.8 | 13.8 | 11.9 | 0.0 | 0.0 |
| (*n* = 109) | Regression | 0.9 | 0.0 | 0.9 | 7.3 | 89.0 | 1.8 | 0.0 | 0.0 | 0.0 |
| Methicillin | Breakpoint | 2.5 | 0.0 | 3.8 | 8.8 | 81.3 | 0.0 | 1.2 | 1.2 | 1.2 |
| (*n* = 80) | Regression | 2.5 | 0.0 | 1.2 | 10.0 | 81.3 | 2.5 | 2.5 | 0.0 | 0.0 |
| Penicillin G | Breakpoint | 0.0 | 0.0 | 2.3 | 6.5 | 74.1 | 10.6 | 3.5 | 1.8 | 1.2 |
| (*n* = 170) | Regression | 0.0 | 0.0 | 2.3 | 10.0 | 74.7 | 9.4 | 2.9 | 0.6 | 0.0 |
| Tetracycline | Breakpoint | 0.0 | 0.0 | 4.5 | 15.7 | 75.3 | 4.5 | 0.0 | 0.0 | 0.0 |
| (*n* = 89) | Regression | 0.0 | 1.2 | 3.4 | 9.0 | 71.9 | 11.2 | 3.4 | 0.0 | 0.0 |
| All antibiotics | Breakpoint | 0.5 | 0.4 | 2.3 | 10.7 | 75.0 | 7.0 | 2.8 | 0.7 | 0.5 |
| (*n* = 1094) | Regression | 0.5 | 0.5 | 2.3 | 10.3 | 77.1 | 7.3 | 1.8 | 0.1 | 0.1 |

[a] A negative differential occurred when the Autobac MIC was less than the reference MIC (e.g., 5.0 in the −2 column means that 5.0% of all strains tested with that antibiotic had Autobac MIC values 2 twofold dilutions lower than the reference MIC values).

[b] *n*, Number of bacterial strains tested.

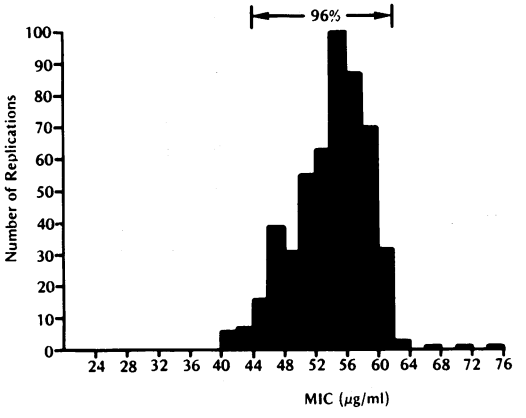


FIG. 5. *Histogram of 512 replicate determinations of the MIC of chloramphenicol for E. coli 420-51A.*

In view of the potential feasibility of early MIC measurements and the need for reduction in both the labor and subjectivity associated with manual methods, it was decided to undertake a series of studies designed to extend the interpretive (i.e., susceptible, intermediate, and resistant) capability of the present Autobac system to include the capability of rapid MIC measurements. The results of these developmental studies demonstrate that, by adjusting the initial inoculum level, medium, incubation period, and scattering breakpoints, it is possible to devise a rapid, standardized MIC method for the Autobac system that enables accurate MIC measurements for the vast majority of clinically important organism-antibiotic combinations. In this regard, an overall accuracy level of 92.7% (within ±1 twofold dilution) was found for the 10 antibiotics tested (range, 86.2 to 98.2%) when the 5-h Autobac MICs obtained by breakpoint analysis were compared with the 18-h ICS broth dilution MICs.

In the course of this investigation it was also discovered that equations could be formed that would enable MICs to be obtained from light scattering data. Furthermore, such MICs were generally more accurate and reproducible than MICs obtained by application of a light scattering breakpoint. When this equation approach was used, the overall accuracy increased from 92.7 to 94.7%. This increase in accuracy was encouraging, especially since regression analysis required only two disk masses of each antibiotic, whereas breakpoint analysis required three.

It should be emphasized that, in contrast to the traditional breakpoint procedure, the use of an equation to derive MICs from growth inhibition data provides the potential of generating

a continuum of MICs between and somewhat beyond the actual antibiotic concentrations used in the test. As such, the equation approach could provide a more accurate assessment of the MICs than is possible when an endpoint technique is used. The interpolation and extrapolation aspects of the equation approach could, therefore, provide more finely tuned MICs, which could have added predictive value especially helpful in the case of toxic antimicrobials. Naturally, the extent to which such interpolation and extrapolation is possible will depend upon the reproducibility of the MIC for a particular antibiotic-organism combination. In this regard, the preliminary studies conducted to date with this rapid method indicate considerable improvement over the twofold dilution (±2 standard deviations) repeatability typically encountered with manual dilution methods.

To assess the full potential of this rapid, automated MIC procedure, an extensive clinical trial has been conducted and is the subject of another report (11).

## ACKNOWLEDGMENTS

We are indebted to S. Dassin, G. Golden, D. Lord, and C. Tyler for technical assistance.

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