DETERMINATION OF THE ID_{50} VALUES OF ANTIBACTERIAL AGENTS IN AGAR

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Microorganisms were plated on agar plates containing various concentrations of an antimicrobial drug and inhibition of growth was determined at each drug concentration. The ID_{50} value and the gradient of the line were calculated by the least square method. When 200~800 bacterial cells were inoculated on an agar plate, growth inhibition corresponded linearly with the log concentration of a drug within the range of 5~95% inhibition. The ID_{50} value and the gradient obtained were reproducible and reliable using microorganisms at stationary phase of growth with all tested bacterial species and all tested antimicrobial agents. It was found that the ID_{50} values of drugs were more reproducible and may be more reliable than the MIC (minimum inhibitory concentration) values of the drugs.

This paper deals with the determination of the concentration of a drug required to inhibit the growth of 50% of bacterial cells (ID_{50}) and the use of this number in the evaluation of the antimicrobial activity of the drugs.

Materials and Methods

Bacterial Strains

Escherichia coli ML4707, *Klebsiella pneumoniae* GN5703, *Serratia marcescens* GN7641 and *Pseudomonas aeruginosa* GN3315 were used during these experiments.

Drugs

Gentamicin C sulfate, sisomicin[1,2], piperacillin[3], carbenicillin, apalcillin[4] and nalidixic acid were used and they are the working standards for the assay of antibacterial activity.

Media

Peptone water consisting of 0.5% of NaCl and 1% of peptone (Daigo Eiyo Kagaku Co., Tokyo) was used for liquid cultures. Heart-infusion (HI) agar (Eiken Kagaku Co., Tokyo) was used for the determination of antibacterial activity.

Determination of ID_{50} in Agar

A bacterial culture in peptone water was diluted to $2\sim8\times10^3$ cells/ml with fresh peptone water. HI agar plates containing various concentrations of a drug were prepared and a 0.1-ml sample of diluted bacterial suspension was spread on each plate. After overnight incubation at 37°C, the number of colonies which had grown on the plate were counted. The mean growth inhibition was calculated from a mean number of colonies on five plates at each drug concentration and of five drug-free agar plates. The concentration of drug (ID_{50}) required to inhibit the growth of 50% of the total number of bacterial cells was calculated by the method of the least square indicated by the following formula;

$$Y=a\cdot X-b$$

$$a=\sum_{i=1}^{n}(x_i-\bar{x})(y_i-\bar{y})/\sum_{i=1}^{n}(x_i-\bar{x})^2$$

$$b=\bar{y}-a\cdot\bar{x}$$

where Y and X are rate of inhibition and logarithmic concentration of the drug, respectively, and $\bar{x}$ and $\bar{y}$ are mean of logarithmic concentration of the drug (x_i) and mean value of the inhibitory rate (y_i), respectively. An ID_{50} value was calculated as the drug concentration where growth inhibition rate was 0.5.

Determination of MIC

Microorganisms were inoculated in peptone water for 18 hours at 37°C. The number of bacterial cells was determined photometrically at 560 nm. The culture was diluted to 10^6 and 10^4 cells/ml with fresh peptone water and a loopful of each diluted sample was spotted on a series of HI agar plates containing serial two-fold dilutions of drugs. The minimum concentration of a drug which inhibited the growth of bacteria was scored as MIC[5].

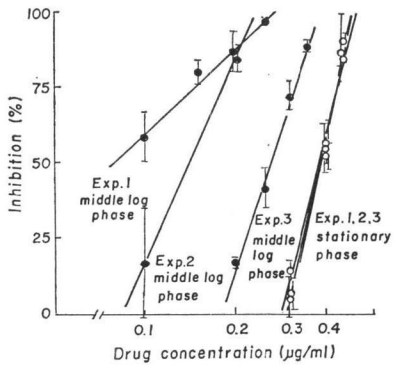
Results

Effect of Bacterial Growth Phase

The inhibition of *E. coli* ML4707 by gentamicin was determined using inocula at two different growth phases, *i. e.*, middle log phase and stationary phase. A linear correlation was obtained between inhibition of growth and logarithmic concentration of the drug at both growth phases. A representative result is shown in Fig. 1. Linearity was observed in the drug concentration range which inhibited 5~95% of the bacterial growth. When the growth inhibition was determined using bacteria in the stationary phase as the inocula, the results of three experiments obtained at different times were almost the same. The ID_{50} obtained from three experiments was 0.383±0.005 μg/ml with standard error of 1.3% and the gradient 4.15± 0.25 with standard error of 6.0%. However, the results obtained from three different experiments using middle log phase bacteria as inocula were quite different from each other; the ID_{50} being 0.16±0.094 μg/ml and the gradient 2.04± 1.04. The variation in ID_{50} and gradient were more than 50%. These results indicated that more reliable and reproducible values could be obtained with the stationary phase culture than with log phase culture. Moreover, the inhibition test using 200~800 cells/agar plate as inocula was found to give us the ID_{50} value with the lowest standard deviation.

Fig. 1. Effect of the growth phase of *E. coli* on the growth inhibition by gentamicin.

Antimicrobial activity of gentamicin against *E. coli* ML4707 at middle log phase and stationary phase was determined as described in Materials and Methods. The results of three different experiments are shown.



Inhibitory Effect of Various Drugs on *E. coli* ML4707

The determination of ID_{50} with other drugs besides gentamicin was carried out against *E. coli* ML4707. Fig. 2 shows the inhibitory patterns of 5 drugs, indicating a good relationship between growth inhibition and drug concentration. Table 1 shows the summarized results of MIC, ID_{50} and the gradient line obtained with degree of inhibition versus drug concentration for each drug. The results indicated that the ID_{50} determination method could be applied to gentamicin, sisomicin, piperacillin, carbenicillin, apalcillin and nalidixic acid. Furthermore, the antimicrobial activity of the drug could be evaluated more quantitatively by its ID_{50} value and by the degree of the gradient than by its MIC value. A low

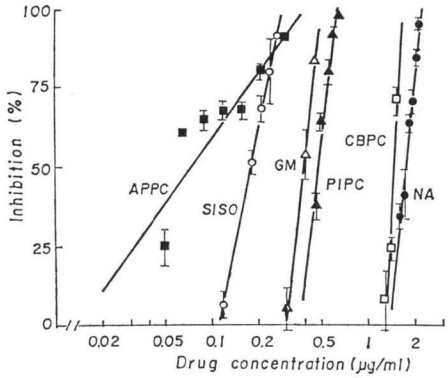
Table 1. Comparison of *in vitro* antibacterial activities of drugs

Drug	ID_{50} (μg/ml)	Gradient	MIC	
			A*	B*
Gentamicin	0.38	4.2	0.4	0.8
Sisomicin	0.18	2.4	0.4	0.4
Carbenicillin	1.44	10.0	3.1	3.1
Piperacillin	0.47	4.3	0.8	1.6
Apalcillin	0.07	0.7	0.4	0.8
Nalidixic acid	1.73	5.3	3.1	6.3

* A, a loopful of about 10^4 cells/ml; B, a loopful of 10^8 cells/ml was inoculated on a plate. Details of the determination of ID_{50} and MIC, see Materials and Methods.

ID_{50} value and high gradient show high antimicrobial activity and the "sharpness" of the antibacterial activity of the drug, respectively.

Fig. 2. Growth inhibition of *E. coli* with various antimicrobial agents. *E. coli* ML4707 at stationary phase was used. The antimicrobial activities of apalcillin (APPC), sisomicin (SISO), gentamicin (GM), piperacillin (PIPC), carbenicillin (CBPC) and nalidixic acid (NA) were assayed as described in Materials and Methods.



Application of ID_{50} Determination to Other Bacterial Species

The ID_{50} determination method was applied against other bacterial species, *i.e.*, *Klebsiella pneumoniae* GN5703, *Serratia marcescens* GN7641 and *Pseudomonas aeruginosa* GN3315. As shown in Figs. 3, 4 and 5 linear correlation was observed between growth inhibition rate and logarithmic concentration of each drug against *K. pneumoniae*, *S. marcescens* and *P. aeruginosa* as well as against *E. coli*. The ID_{50} values of piperacillin and carbenicillin against *K. pneumoniae* GN5703 were 2.34 and 114 μg/ml, respectively, indicating piperacillin is far more effective *in vitro* against *K. pneumoniae* GN5703 than carbenicillin. The ID_{50} values of sisomicin, gentamicin and piperacillin against *S. marcescens* GN7641

Fig. 3. Growth inhibition of *K. pneumoniae* with piperacillin and carbenicillin. Growth inhibitory activities of piperacillin (PIPC) and carbenicillin (CBPC) against *K. pneumoniae* GN5703 at stationary phase were determined as described in Materials and Methods.

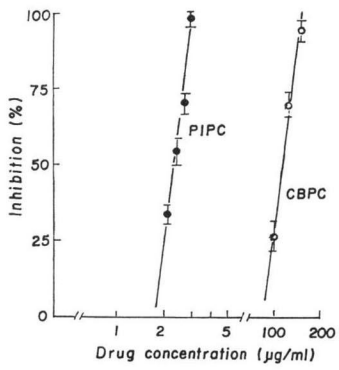
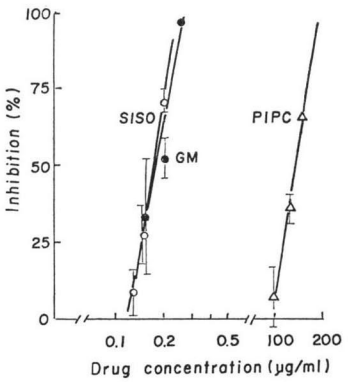


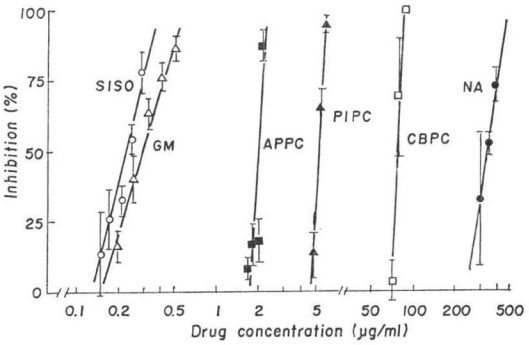
Fig. 4. Growth inhibition of *S. marcescens* with sisomicin, gentamicin and piperacillin.

Antimicrobial activities of sisomicin (SISO), gentamicin (GM) and piperacillin (PIPC) against *S. marcescens* GN7641 were determined as described in Materials and Methods.



were 0.174, 0.178 and 135 μg/ml, respectively, indicating that *in vitro* antimicrobial activity of sisomicin is almost the same as that of gentamicin and these two aminoglycoside antibiotics are more active than piperacillin toward *S. marcescens* GN7641. The ID_{50} values of gentamicin, sisomicin, carbenicillin, piperacillin, apalcillin and nalidixic acid against *P. aeruginosa* GN3315 were 0.30, 0.23, 79.6, 5.49, 2.05 and 340 μg/ml, respectively, indicating that piperacillin and apalcillin are far more effective against *P. aeruginosa* GN3315 *in vitro* than carbenicillin. These data suggest that ID_{50} values could be used for the quantitative evaluation of drugs against a wide range of bacterial species.

Fig. 5. Growth inhibition of *P. aeruginosa* with various antimicrobial agents. Antimicrobial activities of sisomicin (SISO), gentamicin (GM), apalcillin (APPC), piperacillin (PIPC), carbenicillin (CBPC) and nalidixic acid (NA) against *P. aeruginosa* GN3315 were determined as described in Materials and Methods.



Discussion

The antimicrobial activity of a drug is usually evaluated *in vitro* by the determination of MIC values[5] and of maximal drug concentration which allows bacterial growth (MAC)[6] to the same grade as that on a plate without drug. The *in vivo* antimicrobial activity of a drug is estimated by the determination of the concentration required to protect 50% of infected animals (ED_{50})[7,8].

The MIC value of a drug is estimated by the concentration of a drug required to inhibit the visible growth of bacteria and is affected by the number of inoculated bacterial cells, especially with β-lactam antibiotics. Values of MIC and MAC of a drug are restricted to the 2-fold dilution studies and can

Fig. 6. Growth inhibition of *E. coli* with gentamicin. The overnight culture of *E. coli* ML4707 was diluted to 10^5 cells/ml with penassay broth containing various concentrations of gentamicin in L-tubes and were cultivated at 37°C on a shaker according to the method described by TREFFERS[9]. The growth inhibition of drug was determined as the decrease in absorbance at 560 nm at 175, 205, 260 and 315 min.

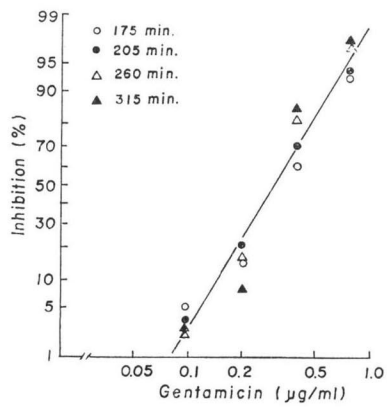
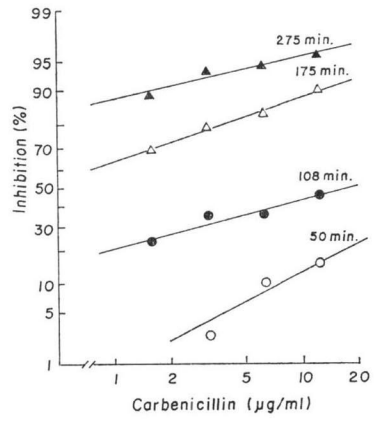


Fig. 7. Growth inhibition of *E. coli* with carbenicillin. Bacterial culture of *E. coli* ML4707 (10^5 cells/ml) containing various concentrations of carbenicillin was cultivated at 37°C on a shaker according to the method described by TREFFERS[9]. The growth inhibition of drug was determined as the decrease in absorbance at 560 nm at 50, 108, 175 and 275 min.



not easily be statistically compared with values for other drugs, whereas ED_{50} values obtained *in vivo* can be compared statistically.

The ID_{50} method employed in this paper is a quantitative method for *in vitro* evaluation of antimicrobial activity of drugs. The value obtained by the ID_{50} method is reproducible and reliable, when bacterial cells at stationary phase of growth are used and 200~800 cells are inoculated on an agar plate. Moreover, the gradient of the line obtained from plots of growth inhibition versus concentration of a drug is constant even when the gradient value is estimated with various strains having various ID_{50} values.

TREFFERS[9] proposed the ID_{50} method for evaluation of antimicrobial activity of drugs. He determined photometrically the growth inhibition of microorganisms after culturing in liquid media containing antimicrobial drug. His method can be used for the examination of correspondense of antimicrobial activity of a drug to the inoculating number of bacterial cells. According to the liquid culture method[9], a linearity was seen between growth inhibition by gentamicin (GM) and GM concentration (Fig. 6). However, a linearity was not obtained, when carbenicillin was used as an antibacterial drug (Fig. 7). This can be partly explained by the fact that antibacterial activity of β-lactam antibiotics is greatly affected by their inoculum size. By contrast, aminoglycoside antibiotics such as GM has a high bactericidal activity and their antibacterial activity is not greatly affected by inoculum size. The results obtained with agar plates are affected very little by media, culturing conditions or kinds of drug, and reproducible results can be obtained, suggesting that the ID_{50} method is useful for *in vitro* evaluation of exact antimicrobial activity of a drug.

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