ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1987, p. 870–875
0066-4804/87/060870-06$02.00/0

Vol. 31, No. 6

Assessment of Formulas for Calculating Critical Concentration by the Agar Diffusion Method

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Received 22 April 1986/Accepted 5 March 1987

The critical concentration of antibiotic was calculated by using the agar diffusion method with disks containing different charges of antibiotic. It is currently possible to use different calculation formulas (based on Fick's law) devised by Cooper and Woodman (the best known) and by Vesterdal. The results obtained with the formulas were compared with the MIC results (obtained by the agar dilution method). A total of 91 strains and two cephalosporins (cefotaxime and ceftriaxone) were studied. The formula of Cooper and Woodman led to critical concentrations that were higher than the MIC, but concentrations obtained with the Vesterdal formula were closer to the MIC. The critical concentration was independent of method parameters (dilution, for example).

Three methods may be used to determine bacterial susceptibility (3). Two are dilution techniques, which are used to calculate the MIC, and the third is an agar diffusion method, which is used in the present study to calculate the critical concentration (C_{cr}). This last method involves the use of a central antibiotic source (a disk) to establish a concentration gradient, which evolves over time and is subject to diffusion laws, i.e., Fick's law (5). This antibiotic gradient inhibits bacterial growth, creating an inhibition zone around the disk. A relationship between the diameter of this zone and the MIC determined by the dilution methods can then be established (3–7).

Various authors have studied the mathematical aspect of the theory of inhibition zones (5) and solved Fick's differential equation by formulas which differ because of their choice of methodological approach. The best-known and most often used formula is that of Cooper and Woodman, who worked with a constantly renewed antibiotic source. Since the antibiotic concentration in the source remained indefinitely constant and was equal in the formula to C_0 (in milligrams per liter), the diffusion was linear.

Vesterdal (5) had studied the radial diffusion found when the charge at the source is well defined and then gradually exhausted as the gradient is established, which is the case with the agar diffusion method (Fig. 1).

The two formulas represented in Table 1 differ only by a factor designated F.

Regardless of the formula used, the measured width of the inhibition zone depends on the disk charge and the critical concentration (C'). It is thus possible, with these formulas, to calculate C_{cr} from the known disk charge (M [in micrograms]) and width of the inhibition zone (x or r; $x = r - r_d$). Nevertheless, two other influential parameters should be determined: the coefficient of antibiotic diffusion in agar (D [millimeters per hour]) and the critical time (T [hours]) of the strain. By using several antibiotic charges in the same agar dish (at the same D and T), it is then possible to relate measured width, disk charge, and C_{cr}.

The aim of this study was thus to compare the results obtained by using the formula of Cooper and Woodman and that of Vesterdal. The former is more often used (1–4), but the latter seemed to us to correspond better to the method used. Since determining the MIC by the dilution technique remains the reference method, the result obtained by this method was compared with the two results obtained for C_{cr}.

MATERIALS AND METHODS

The activity of two antibiotics, cefotaxime (Roussel-Uclaf) and ceftriaxone (Roche), was studied relative to 91 bacteria strains: *Escherichia coli* (11 strains), *Klebsiella* species (11 strains), *Enterobacter* species (10 strains), *Citrobacter* species (9 strains), *Serratia* species (10 strains), *Proteus* species (10 strains), *Providencia* species (1 strain), *Staphylococcus aureus* (10 strains), *Acinetobacter* species (11 strains), and *Pseudomonas aeruginosa* (9 strains).

MICs were determined by a dilution method as specified by the International Collaborative Study (7); Mueller-Hinton agar (Difco Laboratories) was used. The final dilutions ranged between 0.00375 and 128 mg/liter, following a geometrical progression with a ratio of 2.

For C_{cr} determination, we used antibiotic disks (Difco) (r_d = 3 mm, and a reservoir area A [in square millimeters]) loaded with 3, 10, 30, 100, 300, and 1,000 μg of antibiotics (1). A micropipette was used to dispense 10 μl of a suitably diluted solution of the antibiotic onto the disks. The disks were dried and then kept at −80°C. Five disks with different charges were used for each strain. Mueller-Hinton agar was dispensed in a 4-mm layer (4). The inoculum was adjusted to 10^6 bacteria per ml with a nephelometer, and seeding was performed by the flooding technique (7). After incubation for

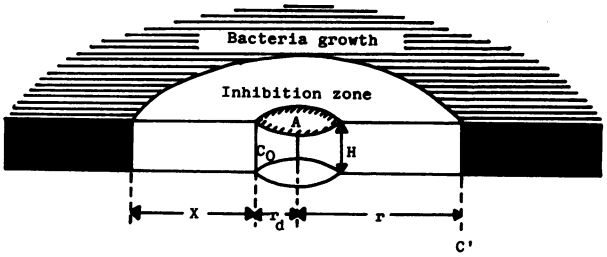

FIG. 1. Formation of the inhibition zone by radial diffusion.

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TABLE 1. Principal formulas for measuring C_{cr}

Formula	Measured width	Difference between formulas (F)[a]	Antibiotic quantity	Experimental system	Diffusion model
Cooper and Woodman	$x^2/4DT = \log C_o - \log C' + F$	0	Indefinite to $C_o = C_{Ste}$	Tubes, wells, or wide cups	Linear
Vesterdal	$r^2/4DT = \log C_o - \log C + F$	$-\log (4\ \mu DT/A)$	$C_oAH = M$	Small cups in agar	Radial

[a] Relative to the formula of Cooper and Woodman.

18 h at 37°C, the inhibition zones were measured with an image analyzer (S III; Système Analytique).

To calculate C_{cr} (1–5), we first determined the antibiotic concentration (in micrograms per milliliter) at the edge of the source (C_0', for which the inhibition zone was zero ($x = 0$ or $r = 0$). The least-squares method was used to calculate the line $\ln C_0 = f(x^2)$ for the formula of Cooper and Woodman and the line $C_0 = f(r^2)$ for the formula of Vesterdal. C_0' was easily extrapolated from the formulas for these lines.

When $C_0 = C_0'$, the formula of Cooper and Woodman then

TABLE 2. Comparison of C_{cr} values (calculated from the formula of Cooper and Woodman and the formula of Vesterdal) with MICs

Strains	Cefotaxime			Ceftriaxone		
	C_{cr} (Cooper) (mg/liter)	C_{cr} (Vesterdal) (mg/liter)	MIC (mg/liter)	C_{cr} (Cooper) (mg/liter)	C_{cr} (Vesterdal) (mg/liter)	MIC (mg/liter)
E. coli						
C1	0.55	0.35	0.06	0.52	0.036	0.03
C2	0.65	0.039	0.06	0.82	0.051	0.03
C4	0.57	0.039	0.03	0.59	0.04	0.015
C5	0.46	0.030	0.06	1.18	0.082	0.03
C6	0.40	0.025	0.03	0.17	0.01	0.03
C7	0.25	0.015	0.03	0.42	0.027	0.03
C8	0.76	0.052	0.06	0.99	0.071	0.03
C9	0.63	0.04	0.06	0.60	0.04	0.03
C11	0.17	0.01	0.03	0.22	0.013	0.015
C12	0.34	0.022	0.03	0.37	0.025	0.015
C13	1	0.067	0.03	0.37	0.024	0.03
Klebsiella spp.						
K1	0.56	0.031	0.03	1	0.061	0.03
K2	0.19	0.011	0.03	0.26	0.016	0.03
K3	0.51	0.032	0.06	1.86	0.127	0.06
K4	0.91	0.056	0.06	0.46	0.03	0.06
K6	0.47	0.027	0.03	0.21	0.012	0.03
K7	0.18	0.01	0.015	0.45	0.028	0.03
K8	0.18	0.01	0.015	0.30	0.017	0.015
K9	0.39	0.024	0.06	0.53	0.036	0.06
K10	5.10	0.41	0.50	21.03	2.44	4
K12	0.41	0.025	0.06	0.59	0.039	0.06
K13	0.94	0.063	0.06	1.23	0.09	0.125
Enterobacter spp.						
E1	1.13	0.069	0.06	0.57	0.035	0.25
E2	0.79	0.055	0.125	0.82	0.057	0.125
E3	138.40	15.30	64	46.10	5.33	16
E4	0.77	0.051	0.125	0.76	0.052	0.06
E5	1.38	0.10	0.25	1.21	0.89	0.25
E6	1.30	0.092	0.25	0.82	0.058	0.125
E7	1.07	0.076	0.06	1.70	0.13	0.125
E10	0.90	0.064	0.25	1.90	0.14	0.25
E12	0.76	0.054	0.25	1.17	0.089	0.25
E13	0.69	0.047	0.06	1.14	0.083	0.03
Citrobacter spp.						
CT1	1.94	0.13	0.25	1.16	0.82	0.25
CT2	0.93	0.059	0.125	0.82	0.053	0.25
CT3	0.94	0.056	0.125	0.61	0.038	0.06
CT5	0.80	0.05	0.125	0.83	0.054	0.125
CT10	1.10	0.07	0.06	0.64	0.04	0.06
CT11	0.76	0.049	0.125	0.57	0.039	0.125
CT12	58.33	6.90	16	48.9	7.36	32
CT13	0.80	0.054	0.125	1.50	0.10	0.06
CT14	0.89	0.062	0.06	0.96	0.066	0.06

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TABLE 2—*Continued*

Strains	Cefotaxime			Ceftriaxone		
	C_{cr} (Cooper) (mg/liter)	C_{cr} (Vesterdal) (mg/liter)	MIC (mg/liter)	C_{cr} (Cooper) (mg/liter)	C_{cr} (Vesterdal) (mg/liter)	MIC (mg/liter)
Serratia spp.						
S1	3.84	0.27	4	5.20	0.40	4
S2	1.01	0.07	0.25	0.53	0.036	0.125
S3	1.11	0.078	0.25	1.08	0.078	0.25
S4	1.48	0.10	0.25	1.62	0.11	0.25
S5	3.34	0.21	1	1.84	0.12	1
S6	1.48	0.10	0.50	1.37	0.094	0.50
S7	1.57	0.10	0.25	1.59	0.11	0.25
S8	2.90	0.22	0.50	1.38	0.11	0.25
S9	4.70	0.33	0.50	2.21	0.15	0.25
S10	1.03	0.063	0.50	0.49	0.032	0.25
Proteus spp.						
P1	0.057	0.0028	0.00375	0.83	0.0044	0.00375
P2	0.031	0.0016	0.00375	0.035	0.0018	0.00375
P3	0.18	0.011	0.06	0.084	0.0048	0.00375
P4	0.12	0.0065	0.0075	0.075	0.0041	0.00375
P5	0.41	0.024	0.015	0.023	0.0011	0.00375
P6	0.15	0.0086	0.015	0.067	0.0035	0.00375
P7	0.30	0.017	0.06	0.096	0.0054	0.015
P8	0.037	0.0018	0.0075	0.018	0.0008	0.00375
P10	0.42	0.024	0.06	0.29	0.017	0.015
P11	0.10	0.0052	0.03	0.045	0.0022	0.00375
S. aureus						
ST1	9.74	0.78	0.50	14.8	1.39	2
ST2	18.90	1.53	2	19.5	1.90	8
ST3	17.50	1.49	2	20.60	2.08	8
ST4	19.70	1.70	2	20.10	2.16	4
ST5	17.60	1.49	2	20.80	2.17	4
ST6	18	1.44	2	20.30	1.94	4
ST7	15.60	1.32	2	19.40	1.88	2
ST8	19.20	1.70	2	20.30	2.12	4
ST9	19.50	1.50	2	20.50	1.94	4
ST10	21.40	1.87	2	22.50	2.34	4
P. aeruginosa						
PS1	45.20	4.26	8	42.90	3.91	8
PS4	49.20	4.24	8	38.40	3.47	8
PS5	69.40	6.50	32	61.60	5.54	16
PS6	67.20	6.64	32	87.50	8.42	32
PS7	77.70	5.68	32	120.60	11.50	16
PS8	60.50	5.57	32	61.60	4.30	32
PS9	42.90	3.50	32	32.10	2.56	32
PS10	155.10	11.80	32	102.70	9.53	16
PS11	47	3.91	8	34.40	2.64	4
Acinetobacter spp.						
A1	71.90	7.79	16	74.70	8.82	16
A2	44	3.94	4	41.20	3.96	4
A3	82.50	10.19	16	86.20	11.50	16
A4	58.80	5.79	16	45.20	5.10	8
A5	53.30	5.69	8	45.10	5.50	8
A6	62.90	7.71	16	59.80	7.70	16
A7	31.30	2.82	8	37.50	3.80	16
A8	28.50	2.12	4	37.10	3	4
A9	72.30	8.67	32	70.50	8.80	16
A10	64.50	7.28	16	74.60	8.90	16
A11	62.60	6.82	16	60.80	7.10	16

becomes $x^2/4DT = 0 = \ln C_0' - \ln C'$, and thus $\ln C' = \ln C_0'$, so that under these conditions, the critical concentration $C' = C'_0$. The formula of Vesterdal is then $r^2/4DT = \ln C_0' - \ln C' - \ln (4DT/r_d^2) = 0$, and thus $\ln C' = \ln C_0' - \ln (4DT/r_d^2)$. Since r_d is the disk radius (3 mm), $r_d^2 = 9$ mm^2; however, $4DT$ must be calculated.

According to our way of calculating the formula, line $\ln C_0 = f(r^2)$ has the form $y = ax + b$; when obtained from the formula of Vesterdal, it can be expressed as $\ln C_0 = (1/4DT)r^2 - (\ln C' + \ln 4DT/r_d^2)$. $1/4DT$ is thus equal to the slope (a) of line $\ln C_0 = f(r^2)$, so that $4DT = 1/a$ and $4DT/r_d^2 = 1/9a$.

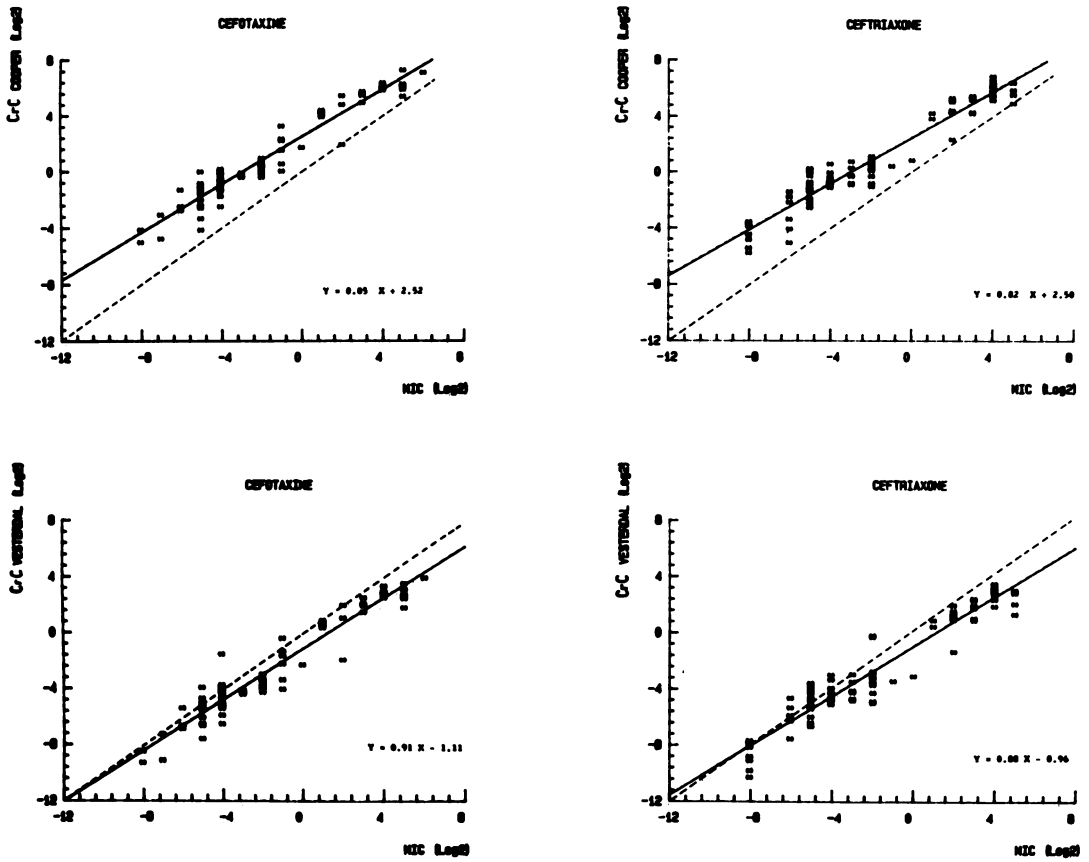

FIG. 2. Correlation between C_{cr} values and MICs for cefotaxime and ceftriaxone (-----, $Y = x$). CrC, Critical concentration (C_{cr}).

Ln C_0 = f(r^2) was calculated by a regression program including r^2 and ln C_0. Hence, ln C' = ln C_0' − ln 1/9a and so $C' = C_0' \times 9 \times a$.

The linear and parabolic regression programs were used to compare the results obtained from these two formulas with the reference MIC. The same programs were also used to correlate the diameters and the square of the inhibition zone radii with C_{cr} values (Vesterdal) and the reference MIC.

RESULTS

The MIC, Cooper C_{cr}, and Vesterdal C_{cr} results are given in Table 2. Regardless of antibiotic and bacterial species, the Vesterdal C_{cr} was closer to the MIC than the Cooper C_{cr} was. For both antibiotics, C_{cr}-MIC correlations were better when the Vesterdal formula was used (Fig. 2). The slopes were, respectively, 0.91 and 0.88, indicating that the C_{cr} and MIC variations were very close. The coefficient b, respectively −1.11 and −0.96, indicates that the $\log_2$ of the mean Vesterdal C_{cr} was lower than the MIC by ca. 1. This difference decreased with the weakest MIC and increased slightly with the most resistant strains. For a MIC of 64 mg/liter, the $\log_2$ difference was then −1.65 for cefotaxime and −1.68 for ceftriaxone. With the formula of Cooper, the differences were much greater. The C_{cr} values were then higher than the MIC, by about 2.5 on the $\log_2$ scale (which is six times the MIC).

When C_{cr} values of each antibiotic for each bacterial species were plotted, it could be noted that the scattering of C_{cr} for very sensitive strains (e.g., *Proteus* spp.) was as great as that of the MIC. With resistant strains (for which MICs were between 0.5 and 8 mg/liter), Vesterdal C_{cr} values seemed less scattered than MICs. In Table 2, for *S. aureus* and ceftriaxone, the C_{cr} is between 1.88 and 2.34 mg/liter and the MIC is between 2 and 8 mg/liter for strains 2 and 10. The mean (arithmetic) C_{cr} value is equal to 2.06 ± 0.15 mg/liter. The modal MIC is 4.31 ± 1.5 mg/liter.

To specify the possible relations between the MIC and the Vesterdal C_{cr}, these two values were correlated with each diameter and square of the inhibition zone radii. The results obtained with the cefotaxime disk at 10 μg are presented in Fig. 3. Linear and parabolic regressions were calculated for each concordance curve. When the concordance was a simple dependence ($y = ax + b$), the curves were identical; otherwise, the differences are immediately apparent in the figure. Thus it can be noted that the regression r^2-C_{cr} is linear and the regression D-C_{cr} is parabolic, as might be expected, since C_{cr} was calculated mathematically from r^2. The r^2-MIC and D-MIC regressions behaved similarly to the preceding ones, although with a better linear correlation between r^2 and the MIC.

DISCUSSION

The ideal parameter for expression of antibiotic activity on a bacterial strain should be independent of the experimental method used. The MIC is the reference value for defining this activity; however, it is dependent on certain experimental factors such as the progression of antibiotic dilutions, the quantity of the inoculum, the incubation time, and the

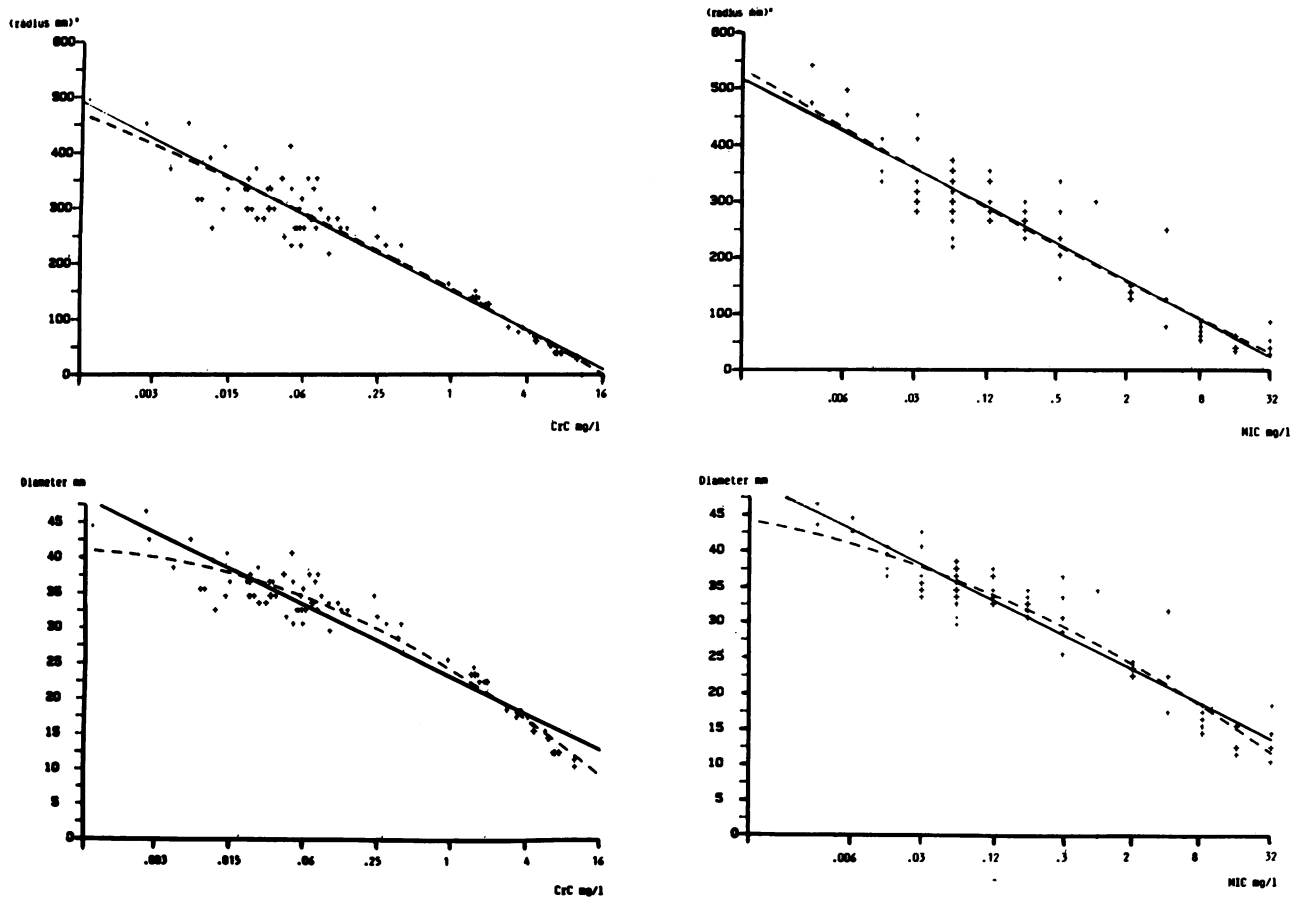

FIG. 3. Linear (——) and parabolic (-----) correlation between Vesterdal C_{cr}, MIC, and the diameters and squares of the radii of the inhibition zone (10-μg cefotaxime disk). CrC, Critical concentration (C_{cr}).

culture medium (liquid or solid). It is possible to express antibiotic activity by using another value, C_{cr}, which can be calculated by another method, the agar diffusion method, relative to the inhibition zone which develops around the antibiotic disk.

The formation of this zone depends on bacterial density. In effect, once a certain value for the bacterial population, known as the critical population, is reached, bacterial growth is no longer affected by the antibiotic at any concentration. The time required for the critical population to be reached is called the critical time (T) and depends on the bacterial inoculum, the latency phase, the growth rate of the bacteria, and thus the culture medium. In general, for rapidly growing bacteria such as enterobacteria, T is approximately 6 h (4). Once T is reached, the diffusion of the antibiotic allows the concentration gradient to be established. The diameter of the inhibition zone will be influenced by the diffusion velocity (D) of the antibiotic and by bacterial susceptibility concentration. With reference to a point located at a distance from the disk equal to the width of the inhibition zone (i.e., just at the edge of, but still inside, this zone), and very shortly before T is reached, the antibiotic concentration found can be considered the minimal concentration inhibiting bacterial growth and preventing the bacterial population from reaching a critical point and continuing to develop. This minimal concentration corresponds to C_{cr}. Thus, this value represents a MIC obtained by a technique involving the use of a continuous antibiotic gradient and a constant inoculum (critical population), with a short incubation time (T) limiting the degradation of the antibiotic by the culture medium.

However, this value has rarely been used in recent years (1–4), mainly because of its ostensibly poor correlation with the MIC (reference values). In fact, the formula consistently used until now has been that of Cooper (5), presumably because of its simplicity. With this formula, C_{cr} are six times as high as the MIC, results which have already been observed or reported (1–4). Actually, this formula does not allow for the technical conditions of the agar diffusion method, for which diffusion is radial and not linear. With the formula of Vesterdal, $\log_2 C_{cr}$ values are lower by 1 than the MICs, which is to be expected. In fact, MICs measured by the dilution method are discontinuous values (2) and are thus overestimated with respect to continuous values. Nevertheless, in linear regressions, MICs have always been correlated with the diameter of the inhibition zone, whereas C_{cr} values are calculated from the square of the radius. Nor can the C_{cr}-diameter regression be linear, but, rather, it is parabolic. MICs behave in an identical way, providing indirect proof that, on the one hand, there is a very close correlation between MIC and C_{cr} and that, on the other hand, the diffusion of the antibiotic in the disk method is truly radial.

These results also confirm the D-MIC parabolic regression

curves reported for broad-spectrum cephalosporins (8). This type of regression was not clearly observed with the first antibiotic molecules, owing to the poorer range of the MIC (e.g., from 1 to 64 mg/liter for chloramphenicol) and the importance of the MIC variation coefficient. Under these conditions, it is normal for linear and parabolic regression to be confused. The C_{cr} variation coefficient, as determined by the formula of Vesterdal, was lower than that of the MIC, which was to be expected as well, since the gradient was continuous and the method is not influenced by the geometrical progression ratio of the antibiotic dilutions.

The greatest variations observed in susceptible strains must be attributed to excessive disk charges that resulted in inhibition zones which were too large. For calculations based on squares of radii, these variations increased quite rapidly with the enlargement of the inhibition zones.

What functional knowledge should the microbiologist retain to apply this method? This technique could be used to compute the parameters of the diffusion method by using statistical and mathematical calculations. For example, if the MIC breakpoints are known, it is easy to obtain the corresponding critical concentrations by a regression program and then compute the disk charge giving the minimum variations while perfectly differentiating the various classes (susceptible, intermediate, and resistant). It is also possible to calculate the zone diameter breakpoints (6).

Since the critical concentrations are obtained by extrapolation when $r = 0$, the factors normally affecting antibiotic activity (pH, salt concentrations, etc.) have little effect. A great difference between MIC and C_{cr} should suggest an inhibitor activity of the culture medium (ceftiolen).

By modifying the method to allow the calculation of the critical time (T), it is possible, with a single disk, to calculate the critical concentration without needing to take into consideration the importance of the initial inoculum and the bacterial growth rate. This method could thus be applied to bacteria with a low growth rate.

The use of the formula of Vesterdal makes it possible to calculate C_{cr} which correspond to inhibitory concentrations. These C_{cr} values are very closely related to MICs and have the advantage of being continuous values which are not very dependent on technical factors (dilution, inoculum, and incubation) and have a low variation coefficient.

ACKNOWLEDGMENT

We are grateful to M. Massart for preparing the manuscript.

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