Mathematical Corrections for Bacterial Loss In Pharmacodynamic In Vitro Dilution Models

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In vitro dilution models are used to simulate in vivo drug concentration-time profiles and thus to study the effects of various antibiotic concentrations on the bacteria investigated. The major disadvantage of these models is permanent dilution of the bacterial culture, which falsifies the resulting kill curves. Known equations, which usually correct bacterial loss by simple first-order kinetics, do not take into account special test conditions, such as variable elimination rate constants, exceptionally long periods of investigation, or formation of biofilms. In the present investigation, we examined the validity of these equations with regard to the test conditions mentioned. We simulated the concentration-time curves resulting from continuous infusion of 1,000 mg of meropenem with steady-state levels of 2.5, 5.0, and 7.5 µg/ml in an in vitro dilution model. The resulting kill curves were compared with the kill curves obtained from incubation of bacteria in an undiluted system with meropenem at constant concentrations corresponding to the above-mentioned steady-state levels. Comparison of the matching kill curves showed that the common corrections, which do not consider the formation of biofilms in the compartments, partly underestimated the effect of bacterial dilution. We defined a factor, f, as an extension to the known equations which compensates for the effect of biofilms. Another extension was developed to allow the investigation of variable elimination rate constants. With the help of these extended mathematical corrections, we were able to fit the kill curves resulting from the in vitro dilution model exactly to the kill curves given by an undiluted system.

Several in vitro kinetic models which simulate in vivo drug concentration-time profiles use continuous dilution to fit drug concentrations to the corresponding serum pharmacokinetics (4). The major disadvantage of these models is inevitable dilution of the bacterial culture, which falsifies the resulting kill curves. Several attempts have been made to compensate for this effect. (i) The antimicrobial activity of constant concentrations of antibiotics may be examined in a static system in which the bacterial culture is not diluted during the investigation (12). (ii) Dynamic in vitro models which do not allow bacteria to be washed out have been developed (1, 2). (iii) The loss of bacteria in an in vitro dilution model may be compensated for by mathematical correction.

In the present report, we have summarized the previously existing equations (5, 8, 11) and investigated their validity with regard to different test conditions by simulating continuous infusion of meropenem with steady-state levels of 2.5, 5.0, and 7.5 µg/ml in an in vitro dilution model. The resulting time-kill curves were compared with the kill curves resulting from incubation of test bacteria with meropenem at constant concentrations of 2.5 to 7.5 µg/ml in a static system (batch culture). We assumed that bacterial growth in the batch culture is not limited by the supply of nutrients as long as there is no regrowth beyond the initial inoculum. Under this prerequisite, all test conditions, except dilution, are identical in the corresponding experiments. Thus, the difference between the kill curves obtained under flowing and nonflowing conditions during the steady-state period gives the factor by which the kill curve obtained under flowing conditions has to be corrected for the bacterial loss due to dilution.

MATERIALS AND METHODS

Test bacteria. For the in vitro simulation, we used Pseudomonas aeruginosa III-3-1, which had originally been isolated from a human blood culture, and Escherichia coli K-12 strain W3110. The meropenem-MICs for the two strains were 0.25 and 0.06 µg/ml, respectively. The strains were kept on China blue lactose agar (Merck, Darmstadt, Federal Republic of Germany [FRG]). All studies were performed in Iso-sensitest broth (Oxoid, Wesel, FRG). Xanthomonas maltophilia IV-4-16 was used for the preparation of β-lactamase. The strain was a gift from Bayer AG, Leverkusen, FRG.

Antibiotics. Meropenem was kindly provided by Zeneca GmbH, Heidelberg, FRG. Stock solutions were freshly prepared in 5% K₂HPO₄. Cefoxitin was kindly provided by MSD Sharp & Dohme GmbH, Munich, FRG.

In vitro simulation. The data on a single 30-min infusion of 1,000 mg of meropenem were kindly provided by Zeneca. According to the rule given by Dost (3), the area under the concentration-time curve is in proportion to the total dose of an active substance and independent of the route of application as long as there is complete absorption and no first pass. From the pharmacokinetic data on the 30-min infusion, which has an area under the concentration-time curve of 62.60 µg·h/ml, we calculated the concentration-time courses resulting from continuous infusions of 1,000 mg of meropenem with steady-state levels ranging from 2.5 to 7.5 µg/ml. The theoretical serum concentrations of the simulated infusions are given in Fig. 1; measured concentration-time curves were accepted when the mean deviation from the expected values was less than 10%.

As a preparation for the experiments, the bacteria were grown overnight in Iso-sensitest broth at 37°C, diluted 100-fold, and precultured in flask B (Fig. 2) for 1 h at 37°C. This provided an initial inoculum of approximately 10⁵ CFU/ml.

Kill kinetics under flowing conditions. Flasks A1 and A2 contained broth with different antibiotic concentrations, and flask R contained drug-free broth (Fig. 2). During the experiments, medium was pumped from these compartments to flask B, which was kept at 37°C and contained bacteria in a constant volume (83 ml) of Iso-sensitest broth. We were able to simulate the different stages (i.e., loading dose, steady state, and elimination) of the continuous infusions investigated in compartment B by exchange of the supply flask (A1, A2, or R) and the flow rate (Table 1). The flow rates employed, which ranged from 2 to 0.7 ml/min, were given by the product of the respective elimination rate constant, k₂, and the volume of broth in compartment B. By the administration of loading doses (flask A1), the steady-state levels were reached within the first 30 min in all of the experiments.

Kill kinetics under nonflowing conditions (batch culture). In a second series of experiments, the antibiotic was directly added to test compartment B to give a concentration corresponding to the steady-state situation. These preparations were not diluted during the course of the experiment.

In all of the experiments, test samples were taken at t = 0, 0.5, 1, 2, 3, 4, 5, 6,
7, 8, 12, and 24 h. Bacterial colony counts in each sample were determined as follows. A 0.5-ml portion was removed from each sample and serially diluted in sterile saline. A 0.1-ml volume of each dilution was subcultured onto China blue lactose agar. Colony counts were determined after 18 h of incubation at 37°C and again after additional incubation for about 12 h at 37°C. The minimal detectable level was 100 CFU/ml.

To eliminate the inhibiting effect of the antibiotic in the samples, two methods were used. (i) When the expected level was above 10,000 CFU/ml, the samples were diluted as described above. (ii) For levels below 10,000 CFU/ml, 0.05 ml of β-lactamase extract was added to 0.95 ml of each sample. The samples were then processed as usual. The resulting colony counts multiplied by a factor of 1.05 to correct for the dilution due to the addition of β-lactamase. The β-lactamase allowed unimpaired bacterial growth from samples containing up to 25 µg of meropenem per ml.

The difference between the measured log_{10} CFU per milliliter and the log_{10} CFU per milliliter of the initial inoculum was determined and plotted versus time of incubation.

Determination of MICs. MICs were determined with the broth dilution technique (9) in Iso-sensitest broth before and after each simulation. During the infusions investigated, no increase in the MIC occurred.

Determination of antibiotic concentrations. The concentrations of meropenem in the samples were determined by microbiological assay (6) in Iso-sensitest agar with E. coli H-1-32 as the test organism. Standards and samples (30 µl) were placed in wells 6 mm in diameter. The meropenem assay was linear over a range of 0.3 to 2.5 µg/ml; the coefficients of variation (standard deviation/100% mean diameter) were 5.94% at 0.3 µg/ml, 4.36% at 0.6 µg/ml, 4.50% at 1.25 µg/ml, and 4.22% at 2.5 µg/ml. The mean diameter of the zones of inhibition was 22.39 mm. Concentration-time curves were accepted when the mean difference between the measured and expected values was less than 10%.

Preparation of β-lactamase. For preparation of β-lactamase from X. malolactifluva IV-4-16, we used the method given by Saino et al. (10) with slight modifications. In contrast to Saino et al., we used Iso-sensitest broth as the bacterial growth medium and cefoxitin at a final concentration of 60 µg/ml as an inducer of β-lactamase production. After centrifugation, the cells were washed twice, resuspended in phosphate buffer at 0.05 times the original volume, and kept frozen overnight at −20°C. The next day, the suspension was warmed to 4°C, disrupted ultrasonically for 10 s in an ice bath, and centrifuged at 17,000 × g for 20 min at 4°C. The supernatant was filtered through a membrane filter (cellulose nitrate, 0.22-µm pore size) and kept frozen at −20°C.

The effect of β-lactamase was tested as follows. An overnight culture of P. aeruginosa III-1 was diluted in Iso-sensitest broth to a final inoculum concentration of approximately 400 CFU/ml. Samples of 0.5 ml were mixed either with 0.05 ml of β-lactamase or 0.05 ml of Iso-sensitest broth. Afterwards, 0.5 ml of meropenem diluted in Iso-sensitest broth was added to each sample to give final antibiotic concentrations ranging from 2.5 to 25 µg/ml. The growth control was mixed with 0.05 ml of β-lactamase and 0.5 ml of Iso-sensitest broth. A 0.1-ml volume of each sample was immediately subcultured onto China blue lactose agar and incubated at 37°C for 18 h. The logarithm of the resulting number of CFU was plotted versus the antibiotic concentration in the sample (Fig. 3).

Mathematical formulation. In 1986, Haag et al. (5) developed the following differential equations to describe changes in the number of cells (CFU per milliliter) in compartment B in the course of time. $N(t)$ represents the number of CFU per milliliter in the free medium (liquid compartment) at a given time, $t$, and $N_B(t)$ stands for the number of cells in the biofilm (solid compartment) at the same time. $dN(t)/dt$ and $dN_B(t)/dt$ represent the first derivatives of $N(t)$ and $N_B(t)$, respectively.

$k_a$ is the apparent bacterial growth rate constant ($k_a$ is the difference between the real growth rate constant and the rate constant of bacterial death; it is assumed that $k_a$ is the same under flowing and nonflowing conditions), $k_d$ is the rate constant for absorption of cells from the broth into the biofilm, $k_b$ is the rate constant for desorption of cells from the solid compartment into the free medium, $k_e$ is the elimination rate constant.

$$dN(t)/dt = (k_a - k_d - k_b) \cdot N(t) + k_e \cdot N_B(t) \quad (1a)$$

$$dN_B(t)/dt = (k_a - k_d) \cdot N_B(t) + k_d \cdot N(t) \quad (1b)$$

These equations have been developed under the assumptions that (i) $k_a$ is the same in broth and biofilm, (ii) $k_a$, $k_d$, and $k_e$ are constant over the course investigated, and (iii) $N_B(t)$ is far below the maximum bacterial density under nonflowing conditions ($N_{max}$).

When $k_a$ equals zero (i.e., the culture in compartment B is not diluted), equation 1a is reduced to the following:

$$dN_B(t)/dt = (k_a - k_d) \cdot N_B(t) + k_e \cdot N(t) \quad (2)$$

where $dN_B(t)/dt$ describes the change in the number of CFU per milliliter in an undiluted compartment in the course of time.

For a description of the total time course of $N(t)$, Haag et al. (5) transformed equations 1a and 1b into the following second-order differential equation, in which $[dN(t)/dt]^2$ represents the second derivative of $N(t)$:

$$[dN(t)/dt]^2 - 2k_a k_d - k_a k_b + k_d k_b \cdot dN(t)/dt + [k_a - k_b] k_d (k_a - k_d - k_b) \cdot N(t) = 0 \quad (3)$$
According to Haag et al., solving equation 3 results in the following:

\[ N(t) = A \cdot e^{kt} + B \cdot e^{rt} \]  
(4)

where \( m_1 > 0, m_2 < 0, m_1 = 1/2 \cdot (2k_e - k_d - k_r + \sqrt{(k_e + k_r - k_d)^2 + 4k_e k_d}) \), and \( m_2 = 1/2 \cdot (2k_e - k_d - k_r - \sqrt{(k_e + k_r - k_d)^2 + 4k_e k_d}) \). \( N_0 \) is the initial number of CFU per milliliter in the flask. By definition, \( N_0 = N(0) \) while \( N(t = 0) = 0 \). Equation 10 simplifies to zero for high dilution rates and for \( t \rightarrow \infty \). According to equation 4, \( N(t) \) consists of the number of CFU per milliliter, which comes from the liquid compartment, given by \( B \cdot e^{rt} \), plus the number of CFU per milliliter which comes from the biofilm, given by \( A \cdot e^{kt} \).

Starting from equations 4 and 5, we defined two hypothetical cases. In the first case, during the course of the investigation the development of a biofilm may be neglected (i.e., \( k_r \gg k_d \) and \( k_d \approx k_d \)). Under this condition, square terms of \( k_r \) can be neglected. Taking into consideration the fact that \( A + B = N_0 \) equations 4 and 5 reduce into the following:

\[ N(t) = N_0 \cdot e^{k_e t} \]  
(6a)

\[ N(t) = N_0 \cdot e^{r t} \]  
(6b)

Division of equations 6a and b gives the following:

\[ N(t) = N(t) \cdot e^{(k_e - r) t} \]  
(7)

This equation, which compensates for the loss of bacteria according to simple first-order kinetics, has been known for a long time (8).

In the second case, the desorption of biofilm cells into the liquid compartment supersedes the loss of bacteria due to dilution by far (i.e., \( k_d \ll k_r \)). Under this condition, square terms of \( k_d \) can be neglected. Taking into consideration the fact that \( A + B = N_0 \) equations 4 and 5 reduce into the following:

\[ N(t) = N_0 \cdot e^{k_d t} + B \cdot e^{k_d t} \]  
(8)

\[ \left[1 + e^{r t - k_d t}\right] \]

Accordingly, \( N(t) \) is given by the following:

\[ N(t) = N_0 \cdot e^{k_d t} + B \cdot e^{k_d t} \cdot \left[1 + e^{r t - k_d t}\right] \]  
(9)

Division of equations 8 and 9 results in the following:

\[ N(t) = N(t) \cdot e^{r t - k_d t} \]  
(10)

Comparison of equations 7 and 10 reveals that the effect of biofilms upon the loss of bacteria should be compensated for by a factor, \( f \), between 0.5 and 1 in the exponent of the correction. With an increasing influence of the biofilm, this factor will come closer to 0.5. Thus, equations 7 and 10 may be combined to form the following general solution:

\[ N(t) = N(t) \cdot e^{k_d t} \]  
(11)

where \( 1 \approx f \approx 0.5 \).

As mentioned before, equation 11 is valid only as long as \( k_r \) is constant during the complete course of the investigation. If the serum pharmacokinetics showed an additional phase of distribution and, accordingly, two different elimination rate constants for the \( \alpha \) and the \( \beta \) phases, the equations have to be modified. Prerequisite to the following is that \( k_r \) remain constant over defined periods \( \Delta t \).

Under the assumption that there is no biofilm in the flasks, the starting point is given as equations 6a and b. By definition these equations are valid within periods \( \Delta t \) as follows:

\[ N_1 = N_0 \cdot e^{k_e t - k_d t} \]  
(12a)

\[ N_2 = N_1 \cdot e^{k_d t} \]  
(12b)

\( \Delta t \) and \( \Delta t \) represent adjoining periods \( \Delta t \); the corresponding elimination rate constants are \( k_{d1} \) and \( k_{d2} \). \( N_1 \) and \( N_2 \) stand for the numbers of CFU per milliliter at the end of the respective periods.

Combination of equations 12a and b results in the following:

\[ N_2 = N_1 \cdot e^{k_d t - k_r t} \]  
(13)

With \( k_{d1} = k_{d2} = 0 \), the bacterial density in an undiluted compartment at the end of the second interval (\( N_2' \)) can be expressed as follows:

\[ N_2' = \left( N_0 \cdot e^{k_e t - k_d t}\right) \cdot \left(1 + k_{d2} \cdot t\right) \]  
(14)

Division of equations 13 and 14 results in the following:

\[ \frac{N_2'}{N_1} = \left(N_0 \cdot e^{k_e t - k_d t}\right) \cdot \left(1 + k_{d2} \cdot t\right) \]  
(15)

Equation 15 can be transformed into the following general solution:

\[ N(t) = N(0) \cdot e^{k_d t} \]  
(16)

\( \Delta t \) are \( k_r \) represent any number of periods, \( \Delta t \), with their corresponding elimination rate constants, \( k_r \). If there is a biofilm in the compartment, an identical transformation can be carried out with equations 8 and 9 as the starting point. This results in the following:

\[ N(t) = N(0) \cdot e^{k_d t} \]  
(17)

Combination of equations 16 and 17 gives the following general solution:

\[ N(t) = N(0) \cdot e^{k_d t} \]  
(18)

where \( 1 \approx f \approx 0.5 \).

Unlike a bacterial growth curve, \( N(t) \), as it is given by equation 18, does not reach the stationary phase. Correction of bacterial loss by unlimited first-order kinetics is therefore valid only as long as the cells are in the log phase (i.e., \( N(t) \) is much smaller than \( N_{\text{max}} \)). For cultures approaching the stationary phase, White et al. (11) developed the following equation, which comprises \( N_{\text{max}} \) as the maximum bacterial density:

\[ N(t) = N(0) \cdot \left[\frac{N(t)}{N(t)} + \left(N_{\text{max}} - N(t)\right) \cdot e^{-k_r t}\right] \]  
(19)

With bacterial densities beyond \( 10^6 \) KBE/ml, \( k_d \) is not the same under flowing and nonflowing conditions. Equation 19, therefore, compensates not only for bacterial loss due to dilution but also for the different growth rates due to the limited supply of nutrients in the bath culture. Nonetheless, equation 19 is valid only when there is no biofilm in the flasks and as long as \( k_r \) is constant. Considering the influence of biofilms and a variable \( k_r \), equation 19 is transformed into the following:

\[ N(t) = N_{\text{max}} \cdot \left[N(t)/N(0) + \left(N_{\text{max}} - N(t)\right) \cdot e^{-k_r t}\right] \]  
(20)

where \( 1 \approx f \approx 0.5 \).

RESULTS

Evaluation of equation 18. We recorded the kill curves resulting from the continuous infusions shown in Fig. 1 under flowing and nonflowing conditions with \( P. aeruginosa \) II-3-1 as the test organism. For each corresponding pair of curves, we calculated the correction factor, \( f \), which was necessary in equation 18 to transform the kill curve obtained under flowing conditions during the steady-state period into the corresponding curve resulting from nonflowing conditions. As we expected the value of \( f \) to be dependent on the formation of adherent bacterial layers in the flasks, the existence of biofilms at the end of the investigation (i.e., after 24 h) was recorded. The data obtained are shown in Table 2. The kill curve resulting from 2.5 mg of meropenem per ml under nonflowing conditions showed a biphasic course (Fig. 4). Separating the two
phases and their corresponding correction factors, we found that the average values of $f \pm$ the standard deviation were 0.87 ± 0.07 for the first 24 h of the investigation and 0.62 ± 0.10 from $t = 4$ h to the end of the experiment. We suppose that the number of biofilm cells increases continuously from the start of the experiment, which is reflected by a continuous decrease in $f$. As we have described $f$ by two average constants, this time dependency ought to be one of the factors influencing the observed standard deviation of $f$.

**Evaluation of equation 20.** To verify the validity of equation 20, we chose two different sets of test conditions. (i) The growth curves of E. coli K-12 strain W3110 uninfluenced by antibiotics were recorded under flowing and nonflowing conditions (Fig. 5). In agreement with White et al. (11), we found that correction for bacterial loss by an unlimited first-order correction gives false results when the observed bacterial density under nonflowing conditions exceeds $10^6$ CFU/ml. For the curves depicted, we used an $f$ value of 1 without regard to the formation of biofilms. The $N_{\text{max}}$ value of E. coli W3110 was determined to be $1.1 \cdot 10^9$ CFU/ml in the nonflowing system.

(ii) On the basis of our investigations, we expected the unlimited correction also to yield false results when the experiments exceeded a certain period of time. We therefore continued some of our simulations (for test conditions, refer to Table 1) for more than 24 h with elimination rate constants ranging from 0.53 to 1.44 h$^{-1}$ (data not shown). It became evident that the results given by equation 18 gradually deviated from the data obtained from an undiluted system when the time of incubation exceeded 12 h. Although the deviation was not significant until the experiment exceeded 24 h, provided there was no bacterial regrowth beyond $10^9$ CFU/ml, we decided to use equation 20, which showed no or less deviation, from 12 h onward as a correction in all our investigations. The $N_{\text{max}}$ value of P. aeruginosa III-3-1 is $1.3 \cdot 10^7$ CFU/ml. As the bacterial densities at this time were usually rather low (less than $10^4$ CFU/ml), the influence of correction factor $f$ had to be considered; its values correspond to the data given in Table 2.

**DISCUSSION**

In vitro models using first-order dilution represent a simple and generally applicable method to simulate serum pharmacokinetics (4). Their major disadvantage is continuous loss of bacteria by dilution, which interferes with the reduction in the number of CFU caused by the antimicrobial agent investigated. Neglecting this loss, as can be seen in some investigations (7), may be justified as long as the underlying concentration-time curve of the antibiotic is not altered. Thus, the comparison of the resulting kill kinetics is acceptable because the correction factor is a constant depending on the time of investigation and the resulting kill kinetics at least all bear the same mistake. The effect of bacterial dilution may also be neglected with antibiotics which reduce the number of CFU quickly almost to the limit of detection and do not allow bacterial regrowth during the period of investigation.

Correction for bacterial loss by dilution becomes necessary when kill curves resulting from the investigation of antibiotics with different elimination half-lives or strengths are to be compared. Corresponding investigations have used mainly simple first-order corrections (8). These corrections correspond to equation 7 and may be accepted for most experiments, especially as long as the period of investigation does not exceed 8 h. According to our investigation, equation 19 should be used from 12 h onward. As the onset of formation and the activity of biofilms can only be roughly estimated by the data obtained from the in vitro model alone, the use of a constant correction factor, $f = 1$, in both equations might be acceptable for the qualitative comparison of different antibiotics. With $f = 1$, the resulting kill curves represent the worst case (i.e., the least reduction of bacterial density); thus, the effects of the anti-
otics cannot be overestimated by choosing the wrong factor. Nonetheless, the results presented here show that certain test conditions considerably influence the resulting kill curves and require individually fitted corrections. Biofilm formation is specific for each species and may, in addition, be specific for each apparatus used for pharmacokinetic simulation. Therefore, for quantitative comparison of antibiotics, the required correction should be evaluated for each individual setting by the method demonstrated here.

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