New In Vitro Model to Study the Effect of Antibiotic Concentration and Rate of Elimination on Antibacterial Activity

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A new apparatus is described which serves to investigate the in vitro antibacterial activity of antibiotics as a function of different concentration time curves. The apparatus can be adjusted to simulate the biexponential serum level curves observed in vivo after oral or intramuscular administration. Preliminary studies were carried out with a cephalosporin derivative, cefazolin, against Escherichia coli and Klebsiella sp. strains simulating initial concentrations of 5, 10, and 20 µg/ml that decreased exponentially with half-lives of 30, 60, and 120 min. Surviving cells were counted at 1-h intervals for 10 h. In all the situations tested there was an initial phase of rapid bactericidal activity followed by a phase of bacteriostatic activity, whose length depended on the drug elimination rate but was relatively independent of the initial concentrations. Bacterial regrowth occurred when the antibiotic concentration fell below the minimum inhibitory concentration of the drug against the strains tested. The antibacterial activity of cefazolin, cephalotin, cephaloridine against E. coli and Klebsiella strains was also investigated, in a medium containing 4% human albumin, simulating the serum level curves observed in humans after an intramuscular dose of 1 g. The results obtained suggest that, for cephalosporins, a longer half-life might be more useful than higher peak levels.

Use of the minimum inhibitory concentration (MIC) is the most popular technique for assessing the potential therapeutic efficacy of antibiotics, even though it is generally agreed that the experimental procedure for MIC determination does not exactly reproduce the in vivo situation. In addition to problems linked to inoculum size and absence of host defense mechanisms, the MIC is usually assessed in static conditions, in which the antibiotic at constant concentration is in contact with the microorganism for a relatively long time. The in vivo situation is clearly different, since the antibiotic concentration usually changes with time and, after a single administration, the actual time of contact is relatively short, particularly with antibiotics that are rapidly eliminated from the body, such as cephalosporins, penicillins, and aminoglycosides.

The present paper describes a simple in vitro apparatus for investigating the antibacterial activity of antibiotics whose concentrations change with time, as usually happens in fluids and tissues after in vivo administration.

The apparatus was used to investigate the relationship between the antibacterial activity and the rate of elimination of cephalosporins.

MATERIALS AND METHODS

General. The simple dilution analog computer for the simulation of drug absorption, distribution, and elimination processes, described by Rowe and Morowitz (6), was adapted to study the antibacterial activity of antibiotics.

The apparatus works on the dilution technique consisting of addition of diluent at a constant rate to a stirred antibiotic solution in an Erlenmeyer flask fitted with a two-hole rubber stopper and connected to the rest of the apparatus with glass and Tygon tubing. The diluent is pumped into the flask by a peristaltic pump, and since the flask is tightly stoppered and the air head is minimal, as the diluent is pumped in it forces out the fluid at an equal flow rate, while the volume of fluid is maintained constant.

Two models are described.

(i) Apparatus model I. The first model simulates the pharmacokinetic one-compartment open model with intravenous administration (6). Flask A (Fig. 1), containing antibiotic solution in a bacterial culture of the test strain (volume Vl), was connected through a two-hole rubber stopper with glass and Tygon tubing to a reservoir containing a sterile culture broth as diluent and to a vessel to collect the fluid from flask A. Diluent was pumped into flask A at a constant rate by a peristaltic pump.

Flasks and tubing were sterilized before use. The apparatus was set up in a thermostatic room (37°C),
and a magnetic stirrer ensured homogeneous mixing in flask A. From the moment the pump started (time $t = 0$), the antibiotic concentration in flask A, $C_{0A}$, decreased exponentially according to the equation $C_{0A} = C_{0A}^0 e^{-K_A t}$, where $C_{0A}^0$ is the antibiotic concentration at time $t = 0$, and $K_A$ is the elimination rate constant; $t_1$ and the corresponding half-life are functions of the flow rate, $F$, of the pump and of volume $V_A$ according to the equations $K_A = F/V_A$, and $t_1 = 0.693/K_A = (0.693/F) \times V_A$. It was thus possible to simulate different concentration time curves by changing $C_{0A}^0$, the volume $V_A$, and/or the pump flow rate $F$.

The viable cells and the antibiotic concentration in flask A can be determined on samples of fluid flowing from flask A, which constitutes a continuous sample of the bacterial culture.

A peristaltic pump with several channels permits simultaneous investigation of the effect of different concentrations and/or different rates of elimination of antibiotics against bacterial culture. In the studies described below, two to three experiments were carried out at the same time.

The general experimental procedure was as follows: a $10^{-3}$ dilution of an overnight culture of the test strain in nutrient broth (Oxoid no. 2) was introduced into flask A, setting the volume $V_A$ and/or the flow rate $F$ to obtain the selected $t_1$. After 1 h of incubation ($t = 0$), the dose, $D$, of antibiotic to obtain the initial concentration, $C_{0A}^0$, selected was added to the bacterial culture of volume $V_A$ [$C_{0A} = D/V_A$], and the peristaltic pump was started. Samples of fluid flowing out from flask A were collected at 1-h intervals.

(ii) Apparatus model II. This apparatus was modified as shown in Fig. 2 to obtain two consecutive first-order processes and then to simulate the pharmacokinetic one-compartment open model with first-order absorption.

Flask A containing the bacterial culture of the test strain at volume $V_A$ was connected with flask B containing dose $D$ of antibiotic dissolved in sterile culture broth at volume $V_B$. Flask B was connected through a peristaltic pump to a reservoir of diluent.

When the peristaltic pump was working with flow rate $F$, the antibiotic concentration in flask B decreased exponentially, whereas in flask A it increased to a maximum and then decreased according to the equation $C_{0B} = (D/V_B) \times \left[ K_B/(K_B - K_A) \right] \times (e^{-K_A t} - e^{-K_B t})$, where $K_A$ is the absorption rate constant ($F/V_B$) and $K_B$ is the elimination rate constant ($F/V_A$).

The experimental procedure with this model was as follows: a $10^{-3}$ dilution of an overnight culture of the test strain in nutrient broth (Oxoid no. 2) was introduced into flask A. After 1 h of incubation, the dose $D$ of antibiotic was introduced into flask B. The dose $D$, volumes $V_A$ and $V_B$, and flow rate $F$ were set to obtain the concentration time curve selected. The peristaltic pump was started, and samples of the fluid flowing out from flask A were collected at 1-h intervals.

Effects of dilution on bacterial growth curve. Using the apparatus described, as the antibiotic is diluted, the bacterial culture is diluted to a quantitatively similar extent.

To assess the effects of the rate of dilution on the growth curve of the bacterial culture, a preliminary control experiment was carried out without antibiotic.

Using model I, we compared the growth curve of bacterial cultures of Escherichia coli N 4242 diluted exponentially with $t_1$'s of 30, 60, and 120 min (dynamic situation) with the normal growth curve without dilution (static situation). The general procedure previously described was followed, except that no antibiotic was added to flask A. For the static experiment, flask A was not connected to the peristaltic pump, and samples of culture were collected directly from the flask.

Effect of various concentration time curves on the antibacterial activity of cefazolin. Using model I, we investigated the time course of antibacterial activity of cefazolin against strains of E. coli N 4242 and Klebsiella SSG 30 recently isolated from infected urine. Nine different concentration time curves of cefazolin were obtained with 5-, 10-, and 20-µg/ml initial concentrations, each decreasing exponentially with $t_1$'s of 30, 60, and 120 min. The samples of fluid from flask A were collected at 1-h intervals.

In each sample viable cells and antibiotic concentrations were measured.

The antibacterial activity of cefazolin against a strain of E. coli N 4242 was also investigated, with two different dosage schedules: a single addition of 20 µg/ml decreasing with a $t_1$ of 120 min and two additions of 20 µg/ml at 6-h intervals, decreasing with a $t_1$ of 60 min. Viable cells and antibiotic concentrations were measured in samples collected at 1-h intervals for 12 h.

Comparison of antibacterial activity of cefazolin, cephalotin, and cephradine. Using the second model, we compared the antibacterial activity of
cefaゾlin, cepacetrile, and cephradine against *E. coli* N 957 and *Klebsiella* SSG 30, simulating the mean serum level curves obtained in humans after intramuscular administration of 1 g (3-5).

*E. coli* N 4242 was not used in this experiment because preliminary studies indicated that the activity of cephradine was too low against this strain; its MIC against *E. coli* N 4242 (10 μg/ml) was slightly lower than maximum serum levels simulated.

We therefore used *E. coli* N 957, a strain that was more susceptible to cephradine. This study was carried out in nutrient broth containing 4% human albumin (Behringwerke AG) to take into account the effects of binding to serum proteins. Cephalosporins used were obtained as commercial preparations. Viable cell counts and total antibiotic concentrations were determined in samples collected at 1-h intervals for 10 h. Protein binding of the three cephalosporins was also assessed on samples collected 1 h after the start.

Conventional antibiotic susceptibility test. The conventional susceptibility of the above bacterial strains to cefazolin, cepacetrile, and cephradine was determined by a dilution method in nutrient broth (Oxoid no. 2). The antibiotics were diluted in 1:1.33 steps in tubes containing 0.5 ml of broth. Each tube was inoculated with 5 × 10^6 organisms per ml. Incubation was at 37°C for 18 h.

The MIC was defined as the lowest concentration that inhibited growth.

**Viable cell counts.** Viable cell counts were taken by streaking on the surface of nutrient agar (Oxoid no. 2) plates 0.1 ml of undiluted samples or appropriate dilutions thereof according to the concentration of bacteria. Colonies were counted after 24 h of incubation at 37°C. The antibiotic did not affect the viable cell count.

**Assay of the antibiotic concentration.** Total antibiotic concentrations in the samples were determined by the agar diffusion method, using *Bacillus cereus* var. *mycoides* ATCC 9694. Standard curves were made in the same medium as the samples: nutrient broth or nutrient broth containing 4% human albumin. The viable cells did not interfere with the antibiotic assay.

**Protein binding.** Protein binding was determined by an ultrafiltration technique, according to Buck et al. (1).

**RESULTS**

Figure 3 shows the growth curves of *E. coli* N 4242 without antibiotic in a static situation and in the model I apparatus at various dilution rates. In the range of dilution rates tested, minor differences were observed between the bacterial growth curves, indicating that when the rate constants of dilutions are lower than the cell growth rate, the growth curve does not differ substantially from that observed in a static situation. Therefore, the antibiotic effects on the viable cell count at different rates of elimination can be compared directly without corrections for the effects of different dilution rates on the bacterial culture.

Figure 4 shows the antibacterial activity of cefazolin against *E. coli* N 4242 and *Klebsiella* SSG 30 at nine different concentration time curves. The MIC of cefazolin against both strains was 1 μg/ml. In all the experiments there was a phase of rapid bactericidal activity followed by a phase of bacterial regrowth with or without an intervening period of bacteriostasis.

Comparing the curves obtained with the *E. coli* strain at equal initial concentrations and different t_i's, a similar maximum bactericidal activity was observed [except for C(0) = 5 μg/ml, t_i = 30 min], but the time of bacterial regrowth depended on the rate of elimination of the drug. Comparing the curves obtained at equal t_i and different concentrations, bacterial regrowth was not substantially delayed even with quadruplicate initial concentrations, particularly with t_i's of 30 and 60 min.

For *Klebsiella* the curves obtained with initial concentrations of 5 and 10 μg/ml resembled those for *E. coli*, differing only in that the maximum bactericidal activity was lower and the bacterial count was reduced only to about 10^2 cells per ml. At the initial concentration of 20 μg/ml with a t_i of 120 min, not only was the bacteriostatic effect prolonged, as expected, but also the peak bactericidal effect was stronger than that observed with t_i's of 30 and 60 min.

In the experiment carried out to compare the effect of two different dosage schedules (Fig. 5), a single dose of 20 μg of cefazolin per ml diluted with a t_i of 120 min produced an antibacterial effect, in terms of both bactericidal and bacte-
riostatic activities, at least equal to that obtained with two doses of 20 μg/ml, at 6-h intervals, decreasing with a $t_1$ of 60 min.

Figure 6 shows the results obtained comparing the activities of cefazolin, cephradine, and cephalotriile in conditions simulating the mean serum level curves observed in humans after a single 1-g intramuscular administration. The
FIG. 5. Antibacterial activity of cefazolin against E. coli N 4242 after a single addition of 20 \( \mu \)g/ml decreasing with a \( t_1 \) of 120 min, and after two additions, at 6-h intervals, of 20 \( \mu \)g/ml decreasing with a \( t_1 \) of 60 min. The upper panel shows the time course of drug concentrations for the two \( t_1 \)'s; the lower panel shows the antibacterial activity over the same period of time.

FIG. 6. Antibacterial activity of cefazolin, cephradine, and cephalothin in medium containing 4% human albumin against E. coli N 957 (left) and Klebsiella SSG 30 (right). The time courses of the antibiotic concentrations reproduce the mean serum level curves observed in humans after a 1-g dose intramuscularly.
main pharmacokinetic parameters and the MICs of the drugs in this experiment are shown in Table 1.

Cefazolin's antibacterial activity was longer lasting on both strains tested, and its bactericidal effect was also stronger on *Klebsiella*.

**DISCUSSION**

The apparatus described is useful for assessing the antibacterial activity of antibiotics whose concentrations change continuously over time, as happens in the body after drug administration.

The apparatus can be used to investigate the relationship between antibacterial activity in vitro and the pharmacokinetic behavior of antibiotics, simulating the continuous variations occurring after administration in humans.

For preliminary testing, cephalosporins were selected among the available antibiotics because of their distinct pharmacokinetic properties and different antibacterial activities.

The time course of antibiotic activity during the exponential decrease in antibiotic concentration was studied using cefazolin as a representative cephalosporin, so as to reproduce the serum level time course seen in the body after a single therapeutic dose of cephalosporins.

In these experiments we always observed a rapid decrease in viable cells followed by a variable period of bacteriostasis. Subsequently, bacterial regrowth occurred.

Complete sterilization was never achieved under the experimental conditions used (inocula, $10^6$ to $10^8$ cells per ml). This is in agreement with the report by Greenwood and O'Grady (2) that part of the bacterial population (the persisters) survives the bactericidal effect of $\beta$-lactam antibiotics. Our experiments confirm these results with the observation that bacterial regrowth always occurred when the antibiotic concentration fell below the MIC.

The interval required for regrowth depended essentially on the elimination rate of the antibiotic (Fig. 4). According to this property, the time course of antibacterial activity up to 12 h after a single addition of 20 $\mu$g of cefazolin per ml ($t_1$, 120 min) was quite similar to that observed after two separate additions of 20 $\mu$g/ml each ($t_1$, 60 min) at 6-h intervals. This behavior confirms that the dosage schedule of a cephalosporin should be chosen taking into account the rate of elimination of the drug.

Duration of antibacterial activity was not improved by increasing the initial antibiotic concentration, especially when the shortest $t_1$'s (30 and 60 min) were used. In a situation where the initial antibiotic concentration was 5 $\mu$g/ml, i.e., five times the MIC and a $t_1$ of 30 min, the antibacterial activity observed was very limited against the gram-negative organisms tested, particularly against *Klebsiella*. The success of therapy could be jeopardized when MICs of short-lasting cephalosporins against gram-negative organisms are of the same order of magnitude as the peak blood levels.

The antibacterial profiles of the three cephalosporins tested, each with different microbiological potency against the two strains tested and different pharmacokinetics and protein-binding properties, fully confirmed previous observations.

The short-lasting antibacterial activities of cephradine and cepacetril are in good agreement with their rates of elimination and with the maximum free antibiotic concentration, which was only two to four times higher than the MIC.

The strong, long-lasting antibacterial activity of cefazolin reflects its long half-life and its potency on the strains tested, resulting in maximum free antibiotic concentrations six to eight times higher than the MIC.

**LITERATURE CITED**


