A Novel *In Vitro* Pharmacokinetic/Pharmacodynamic Model Based on Two-Compartment Open Model Used to Simulate Serum Drug Concentration-Time Profiles

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Abbreviations: CFU, colony forming unit; i. v., intravenous; MIC, minimum inhibitory concentration; T_d, the time point when infusion finished.

Abstract

An *in vitro* pharmacokinetic/pharmacodynamic perfusion model that simulates a two-compartment open model of serum drug concentration-time profiles following intravenous bolus injection and infusion was developed and mathematically described. In the present apparatus model, flow was kept in a one-way mode to avoid liquid traffic, and the washout effect seen in dilution models was overcome by embedding the tested bacteria in low melting point agarose gel. The validity of the equations and the reproducibility of the apparatus model were ascertained by simulating the concentration-time profiles of cefazolin and fosfomycin by substitution of their pharmacokinetic parameters obtained from humans for the equations. An empirical regimen 1X(q24h) of 1 g with cefazolin administered by intravenous infusion effectively killed a *Staphylococcus aureus* strain. The same regimen with fosfomycin produced a marked kill-curve with a fosfomycin-susceptible enterohaemorrhagic *Escherichia coli* O157:H7, whereas considerable regrowth was observed with a resistant strain. These results indicated that the present model was able to provide a convenient and reliable method for evaluating the efficacy of antimicrobial agents administered by intravenous infusion.

Introduction

In vitro pharmacokinetic models used to simulate *in vivo* antibiotic concentration-time profiles have been shown to be useful to explore antibacterial pharmacodynamics. These models are used to assess the optimal dosage and regimens that provide efficient clinical outcomes, as well as to minimize the potential for emergence of resistance (reviewed by 5, 14, 19). The pharmacokinetics of serum drug concentration following an intravenous (i. v.) injection is described using a one-compartment model which is particularly useful for the pharmacokinetic analysis of drugs that distribute relatively rapidly throughout the body (8). The first kinetic model that reproduced plasma levels of a drug was developed in 1968 (22) as a dilution basis model. Thereafter, Grasso et al. (10) has presented a one-compartment *in vitro* model that exposes bacteria to exponentially decreasing concentrations following i. v. bolus injection and an oral or intramuscular administration. The one-compartment model is the simplest one and is particularly useful for simulate drugs that distribute rapidly throughout the body. Therefore, the model has been used to study pharmacodynamic effects and examination conditions of several antibiotics with various technical modifications (1, 6, 7, 15, 16).

The elimination of drugs is described as a first-order process by the one-compartment model, however, most drugs practically require a finite time to distribute fully throughout the body, and these pharmacokinetics are described by two- and multi-compartment models (8). Serum levels observed in humans following i. v. injection is usually shown bi-exponential, and described using a two-compartment model consisting of the α (distribution) and β (elimination) phases. A kinetic apparatus model has been introduced by Murakawa and colleagues to simulate the bi-exponential serum concentration time curve that utilizes bidirectional flow between a central and peripheral compartments (18). In this model, the simulation was made by continuous dilution with drug free medium, and, as a result of the dilution, the elimination of bacterial cells from the main compartment was accompanied. This event is termed the "washout" effect and could probably falsify the resulting kill-curves. With improvements, dynamic *in vitro* models which do not allow bacteria to be washed out have been developed as the capillary model (3, 4). Alternatively, the antibiotic solution was removed from the main

compartment by a membrane (20, 24) or using centrifugation-ultrafiltration modules (2) to avoid the elimination of bacterial cells from the main compartment.

In the present study, we describe approximate equations for the two-compartment open model and a corresponding *in vitro* pharmacokinetic/pharmacodynamic perfusion model. Our *in vitro* model was maintained with a single pump to keep one-way flow and to avoid liquid traffic. Furthermore, tested bacteria were embedded in low melting point agarose gel and exposed to antibiotics to overcome the washout effect. The validity and reproducibility of the model were examined by testing the bactericidal activities of cefazolin and fosfomycin against *Staphylococcus aureus* and *Escherichia coli*, respectively.

Materials and Methods

Bacteria. *S. aureus* RN2677, a cefazolin-susceptible (MIC<=0.25 μg/ml) clinical isolate (17), *E. coli* M96 (O157:H7), a fosfomycin-susceptible (MIC = 4 μg/ml), Stx 1 and Stx 2 positive clinical isolate, and *E. coli* No. 44 (O157:H7), a fosfomycin-resistant (MIC = 64 μg/ml), Stx 1 and Stx 2 positive clinical isolate, were used. *S. aureus* and *E. coli* were aerobically cultured at 37˚C in cation-adjusted Mueller-Hinton broth and nutrient broth (Difco Laboratories, Detroit, Mich., U. S. A.), respectively. MICs for the strains were established using a micro-dilution method according to the National Committee for Clinical Laboratory Standard (21).

Description of the apparatus model. The *in vitro* pharmacodynamic perfusion model (circuit C in Fig. 2) was devised, as a combination of circuits A and B (Fig. 2A and 2B, respectively). Flask A was connected with airtight silicon tubing to the initial flow path of flask B_1, and to the second flow path of flask B_2. Flask A, flask B_1 and flask B_2 were equipped with magnetic stirrers. At $t = T_d$ (the time point when infusion finished), the flow path from flask B_1 to flask A was changed to path from flask B_2 to flask A with a T-shaped stopcock. A peristaltic pump was used to provide a constant flow rate. The entire apparatus

was covered with a heat insulator box (60 x 40 x 50 cm) to be kept at 37˚C on a clean bench. Perfusion parameters for the equations, i. e., the initial drug concentrations, C_{b_1}, C_{b_2}, and C_c for flasks B_1, B_2, and C, respectively, constant medium volumes, V_a for flask A, V_b for flasks B_1 and B_2, and constant flow rate, Q, were calculated by substitution of the pharmacokinetic parameters from *in vivo* serum concentration analyses in human (11, 12). All calculations and drawings of the theoretical concentration-time curves were executed with the equations using Excel X software (Microsoft Corp., Calif., U. S. A.).

Dosage regimen. Our experimental design was based on the common empirical dosing of 1 g for cefazolin (Astellas Pharma Inc., Tokyo, Japan) and for fosfomycin (Meiji Seika Kaisha Ltd., Tokyo, Japan) with a following regimen: a 30-min i. v. infusion every 24 h [1X(q24h)].

Determination of antibiotic concentrations. The concentrations of cefazolin in the medium and agarose gel blocks were determined using a microbiological assay with *S. aureus* RN2677 as the test organism.

Quantification of bacterial growth and killing. Overnight cultures were diluted with the fresh medium of cation-adjusted Mueller-Hinton broth for *S. aureus* and nutrient broth for *E. coli* to yield the desired inocula and aliquots were mixed with an equal volume of 1.5% (w/v) NuSieve GTG agarose (BioWhittaker Molecular Applications, Rockland, Me., U. S. A.) solution prewarmed to 55˚C. Aliquots (20 μl) of the bacterial suspensions were solidified on a sterile nylon mesh and then placed in flask A for 3 h without antimicrobial agents at 37˚C. After the start of dosing, agarose gel-blocks were drawn at respective times, rinsed 5 times with 1 ml of sterile phosphate-buffered saline. The blocks were homogenized and digested by 1 unit of β-agarase (Cambrex Bio Science Wokingham, Berkshire, U.K.) at 37˚C for 15 min. After serial dilutions, the samples were plated on Mueller-Hinton agar plates for determining the colony forming unit (CFU). All drug regimens were tested at least in duplicate in three separate experiments. For the traditional model, the same numbers of bacterial cells were

directly inoculated to flask A. Aliquots were then removed, and the CFU was counted after serial dilutions.

MIC determination in the in-gel culture. An agarose-gel block containing 1 x 10^5 CFU/ml was placed in a well of a 24-well plate containing 1 ml of the broth without or with the serially diluted antibiotics. Plates were shaken lightly and incubated at 37˚C under aerobic condition. After 18 h, bacterial growth was microscopically recorded and MIC was defined as the lowest antibiotic concentration that inhibited visible colony.

Theoretical

In vitro *pharmacokinetic model of i.v. bolus injection*

Serum concentrations of most drugs following an i.v. bolus injection are bi-exponential and described using the two-compartment open model. $C(t)$, the concentration of a drug in serum at a given time, t, after the injection, is given by

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t} \quad [1]$$

where α is the first-order elimination rate constant of the distribution phase (α phase), β is the first-order elimination rate constant of the elimination phase (β phase), and A and B are the zero-time intercepts for the α and β phases, respectively. To simulate the drug concentration-time profile described by equation [1], circuit A (Fig. 2) was devised by being based on the elementary circuits (i) and (ii) (Fig. 1). $C_a(t)$, a drug concentration in flask A at a given time, t, in the elementary circuits, are described as follows:

in circuit (i) $$C_a(t) = C_a \cdot e^{-\lambda_a t} \quad [2]$$

in circuit (ii) $$C_a(t) = C_b \cdot \lambda_a/(\lambda_b - \lambda_a) \cdot (e^{-\lambda_a t} - e^{-\lambda_b t}) \quad [3]$$

where C_a and C_b are the initial drug concentrations in flask A and flask B, respectively, and λ_a

and λ_b are the first-order elimination rate constants of flask A and flask B, respectively. When medium flows at the constant rate of Q and the constant medium volumes in flasks A and B are V_a and V_b, respectively, the first-order elimination rate constants are written as $\lambda_a = Q/V_a$ and $\lambda_b = Q/V_b$.

Circuit (ii) was previously reported by Grasso *et al.* (10) to simulate the one-compartment open model with first-order absorption as described by $C = (D/V_a)\cdot\{(k_a/(k_a - K)\}\cdot(e^{-Kt} - e^{-k_a t})$, where the absorption rate constant, $k_a = Q/V_b$ (= λ_b), the elimination rate constant, $K = Q/V_a$ (= λ_a). The dose, D, is given by $C_b V_b$,, and then rearrangement yields $D/V_a = C_b V_b/V_a = C_b\lambda_a/\lambda_b$.

Since circuit A is equivalent to the additional circumstance of circuits (i) and (ii), $C_a(t)$ in circuit A is given by the sum of equations [2] and [3], resulting in the following:

$$C_a(t) = C_a \cdot e^{-\lambda_a t} + C_b \cdot \lambda_a/(\lambda_b - \lambda_a) \cdot (e^{-\lambda_a t} - e^{-\lambda_b t})$$

$$= \{C_a + C_b\lambda_a/(\lambda_b - \lambda_a)\}e^{-\lambda_a t} + \{-C_b\lambda_a/(\lambda_b - \lambda_a)\}e^{-\lambda_b t} \quad [4]$$

Substitution α and β for λ_a and λ_b, respectively, yields

$$C_a(t) = \{C_a + C_b\alpha/(\beta - \alpha)\}e^{-\alpha t} + \{-C_b\alpha/(\beta - \alpha)\}e^{-\beta t} \quad [5]$$

Comparison of constant terms between equation [1] and [5] and rearrangement gives

$$C_a = A + B$$

$$C_b = (\alpha - \beta)B/\alpha$$

In vitro *pharmacokinetic model of i.v. infusion*

When a drug is administered by an i.v. drip infusion for the time period of T_d, the serum drug concentration-time equation is described by the two-compartment open model (8, 11) as follows:

when $0 <= t <= T_d$ (i. e., during a period of drip infusion),

$$C(t) = A/\alpha\cdot T_d \cdot (1 - e^{-\alpha t}) + B/\beta\cdot T_d \cdot (1 - e^{-\beta t})$$

$$= A/\alpha\cdot T_d + B/\beta\cdot T_d - A/\alpha\cdot T_d\cdot e^{-\alpha t} - B/\beta\cdot T_d\cdot e^{-\beta t} \quad [6]$$

when $t > T_d$ (i. e., after the drip infusion),

$$C(t) = \{A/\alpha \cdot T_d(1- e^{-\alpha T_d})\}\cdot e^{-\alpha (t - T_d)} + \{B/\beta \cdot T_d (1- e^{-\beta T_d})\}e^{-\beta (t - T_d)}$$

[7]

where the above parameters are the same as in equation [1], except that dosing is finished at T_d.

To reproduce the increasing drug concentration phase given by equation [6], circuit B (Fig. 2) was devised. An equation giving $C_a(t)$ in circuit B was obtained by considering equations of circuit (iii), in which flask B and the reservoir contained the drug at C_c, and of circuit (iv), in which the reservoir contained the drug at C_c at $t = 0$. $C_a(t)$ in circuit (iii) was described as follows:

$$C_a(t) = C_c (1 - e^{-\lambda_a t})$$

[8]

$C_a(t)$ in circuit (iv) was acquired by subtracting equation [3] describing circuit (ii) with $C_b = C_c$, from equation [8] describing circuit (iii), resulting in the following:

$$C_a(t) = C_c (1 - e^{-\lambda_a t}) - C_c\lambda_a/(\lambda_b - \lambda_a) \cdot (e^{-\lambda_a t} - e^{-\lambda_b t}) \qquad [9]$$

Since circuit B was equivalent to the additional circumstance of circuits (ii) and (iv), $C_a(t)$ in circuit B was described as the sum of equation [3], where $C_b = C_{b1}$, and equation [9], resulting in the following:

$$\begin{aligned} C_a(t) &= C_{b_1}\cdot\lambda_a/(\lambda_b - \lambda_a) \cdot (e^{-\lambda_a t} - e^{-\lambda_b t}) + C_c(1 - e^{-\lambda_a t}) - C_c\cdot\lambda_a/(\lambda_b - \lambda_a) \cdot (e^{-\lambda_a t} - e^{-\lambda_b t}) \\ &= C_c (1 - \lambda_b/(\lambda_b - \lambda_a)e^{-\lambda_a t} + \lambda_a /(\lambda_b - \lambda_a)e^{-\lambda_b t}) + C_{b_1}\cdot\lambda_a /(\lambda_b - \lambda_a) \cdot (e^{-\lambda_a t} - e^{-\lambda_b t}) \\ &= C_c + \{(C_{b_1}\cdot\lambda_a - C_c \cdot\lambda_b) /(\lambda_b - \lambda_a)\}e^{-\lambda_a t} + \{(C_c\cdot\lambda_a - C_{b_1}\cdot\lambda_a)/(\lambda_b - \lambda_a)\}e^{-\lambda_b t} \end{aligned} \qquad [10]$$

where $C_{b_1} < C_c$ was assumed and $0 <= t <= T_d$. Equation [10] is equal to [6], where $\lambda_a = \alpha$, $\lambda_b = \beta$, $C_c = A/\alpha T_d + B/\beta T_d$, and $C_{b_1} = A/\alpha T_d + B/\alpha T_d$.

Results

In vitro *pharmacokinetic/pharmacodynamic models of i. v. bolus injection and drip infusion*

To simulate the drug concentration-time profile described bi-exponentially, circuit A,

based on the elementary circuits (i) and (ii), was devised (Fig. 2). Drug concentration in Flask A at a given time t, $C_a(t)$, was described as follows (see Theoretical):

$$C_a(t) = \{C_a + C_b\lambda_a/(\lambda_b - \lambda_a)\}e^{-\lambda_a t} + \{-C_b\lambda_a/(\lambda_b - \lambda_a)\}e^{-\lambda_b t} \quad [4]$$

where C_a and C_b are the initial drug concentrations in flask A and flask B, and λ_a and λ_b are the first-order elimination rate constants of flask A and flask B, respectively. When the constant medium volumes in flasks A and B are V_a and V_b, respectively, and medium flows at the constant rate of Q, the first-order elimination rate constants are given by $\lambda_a = Q/V_a$ and $\lambda_b = Q/V_b$. Since these parameters can be determined, changes of drug concentrations corresponding to the serum concentration-time profile following an i. v. bolus infusion were reproduced in flask A in circuit A.

Our new *in vitro* model circuit C (Fig. 2) was devised to simulate the serum drug concentration-time profiles following an i. v. drip infusion for the time period of T_d. Circuit C consisted of circuit B which was designed to simulate the increasing phase during a infusion (i. e., $0 <= t <= T_d$) and circuit A representing the elimination phase after infusion (i. e., $t > T_d$). The flow path from flasks C and B_1 was changed from reservoir and flask B_2 to flask A at T_d. In circuit C, the following parameters are introduced: Q is the constant flow rate, C_{b1}, C_{b2}, and C_c are the initial drug concentrations in flasks B_1, B_2, and C, respectively, V_a is the constant medium volume in flask A, V_b is the constant medium volume in flasks B_1 and B_2, and $\lambda_b = Q/V_b$ is the first-order elimination rate constant of flasks B_1 and B_2.

When $0 <= t <= T_d$, $C_a(t)$ in circuit C was given as the following (see Theoretical):

$$C_a(t) = C_c + \{(C_{b1}\cdot\lambda_a - C_c\cdot\lambda_b)/(\lambda_b - \lambda_a)\}e^{-\lambda_a t} + \{(C_c\cdot\lambda_a - C_{b1}\cdot\lambda_a)/(\lambda_b - \lambda_a)\}e^{-\lambda_b t} \quad [10]$$

where $C_{b1} < C_c$ was assumed, and $\lambda_a = \alpha$, $\lambda_b = \beta$, $C_c = A/\alpha T_d + B/\beta T_d$, and $C_{b1} = A/\alpha T_d + B/\alpha T_d$.

When $t = T_d$ (i. e., the time point when drip infusion finished), equation [10] became as follows:

$$C_a(T_d) = C_c(1 - \lambda_b/(\lambda_b - \lambda_a)e^{-\lambda_a T_d} + \lambda_a/(\lambda_b - \lambda_a)e^{-\lambda_b T_d}) + C_{b1}\lambda_a/(\lambda_b - \lambda_a)\cdot(e^{-\lambda_a T_d} - e^{-\lambda_b T_d})$$

[11]

When $t > T_d$ (i. e., after infusion), the flow path was equivalent to circuit A, where C_a was

$C_a(T_d)$ as described by equation [11]. Thus, substitution of equation [11] for C_a in equation [4] and combination of the exponential terms resulted in the following:

$$C_a(t) = \{C_c(1 - \lambda_b/(\lambda_b - \lambda_a)e^{-\lambda aTd} + \lambda_a/(\lambda_b - \lambda_a)e^{-\lambda bTd}) + C_{b1}\lambda_a/(\lambda_b - \lambda_a) \cdot (e^{-\lambda aTd} - e^{-\lambda bTd})\}e^{-\lambda a\,(t - Td)}$$

$$+ C_{b2}\lambda_a/(\lambda_b - \lambda_a) \cdot (e^{-\lambda a\,(t - Td)} - e^{-\lambda b(t - Td)}) \qquad [12]$$

where $C_{b_2} = \{(\alpha - \beta)/\alpha\} \cdot \{B/(\beta T_d) \cdot (1 - e^{-\beta T_d})\}$. Since these dosing parameters were determined, changes in drug concentrations corresponding to the serum concentration-time profile after an i.v. drip infusion were reproduced in flask A in the *in vitro* model C.

Evaluation of the in vitro *model*

In the present *in vitro* pharmacodynamic perfusion model, a constant flow was maintained with a single peristaltic pump placed before flask A (Fig. 2). The flow path was changed using a T-shaped stopcock, and then one-way flow was always preserved. The entire apparatus was simple enough and compact to be manipulated on a clean bench. Accordingly, bacterial contamination has scarcely occurred by the standard aseptic techniques. For model cases, the system was designed to fit to the mean time curves obtained from the in vivo studies of human with cefazolin (11) and fosfomycin (12). The MICs of cefazolin for *S. aureus* and fosfomycin for *E. coli* strains tested were determined using the in-gel culture method. These values with the strains were comparable to the values determined using the micro-dilution method, and the cefazolin MIC for *S. aureus* RN2677 was <= 0.25 μg/ml, the fosfomycin MICs for *E. coli* M96 was 4 μg/ml, and for *E. coli* No. 44 was 64 μg/ml. Substitution of the pharmacokinetic parameters to equations [10] and [12] gave perfusion parameters for the model, where the constant flow rate was preferentially calculated to give similar numbers of the constant medium volumes in flask A (approximately 20 ml) (Table 1).

When a relatively low inoculum of *S. aureus* was applied to the model, cell numbers continuously decreased in a liquid culture until 6 h after starting perfusion, due to the washout effect (Fig. 3). Although regrowth was observed, if no bacterial growth was assumed, cells were completely washed out within 3 h under the condition. To overcome a decrease in inoculum, *S. aureus* was embedded in agarose gel and the bacterial growth was examined in the

perfusion model. In contrast to the liquid culture under the perfusion condition, the growth of the organism was observed in in-gel culture, and it was obviously comparable with that of a static liquid culture. These results suggested that the effects of agarose-embedding and treatment with agarase performed prior to counting CFU were nearly negligible toward bacterial growth and viability of the *S. aureus* strain. Thus, the washout effect shown in the liquid culture in the perfusion model could be overcome by the in-gel culture method. As shown in Fig. 3, the concentrations of cefazolin in the agarose gel blocks were comparable to those in the medium in flask A at the time of measurement, indicating that a fast equilibration occurred between agarose gel blocks and the medium under the perfusion condition examined. Furthermore, these drug concentration values were obviously in good agreement with the serum drug concentration-time profile calculated from the pharmacokinetic data. When the bactericidal activity of the empirical regimen with cefazolin was explored with the *in vitro* perfusion model, cefazolin seemed to markedly kill the *S. aureus* strain in both in-gel and liquid culture methods (Fig. 3), and notably, the bactericidal activity was judged as to be more effective in the broth than in the in-gel cultures, because of the washout effect.

The present system was also applied to examine a bactericidal activity of the empirical regimen of fosfomycin against fosfomycin-susceptible and –resistant enterohaemorrhagic *E. coli* O157:H7 strains (Fig. 4). With an inoculum dose of 10^7 CFU/ml, bacterial growth was observed in the in-gel culture with a slightly lesser extent than in the static culture, for 8 hours incubation in the perfusion model. In contrast, the growth was not shown in the broth culture, due to a flow out of cells. Fosfomycin produced a marked kill-curve with the sensitive strain M96, and the level of bacteria was lowered to below the detection level. In accordance with the growth curve without antibiotics, decrease of cell numbers with the administration of the drug was steeper in the broth than in-gel culture. With the fosfomycin-resistant strain No. 44, moderate bactericidal activity was observed until 4 h after starting perfusion. However, complete kill was not achieved by this regiment and considerable regrowth occurred 4 hour after the incubation in the in-gel culture. Regrowth was also demonstrated in the broth culture. However, it obviously delayed because of the elimination of cell numbers caused by continuous dilution.

Discussion

A marked difference in bacterial exposure to antibiotics is generally recognized between *in vitro* static conditions and *in vivo* conditions. Therefore, *in vitro* models that simulate *in vivo* pharmacokinetics as closely as possible are required to provide useful data to evaluate the effectiveness of antibiotics while minimizing the potential for emergence of resistance (5, 9, 23, 25). In the present study, we attempted to devise an *in vitro* model that is simple enough for convenient manipulation and simulation of concentration-time profiles based on the two-compartment open model. We showed that the resulting equations [10] and [12] were able to simulate the serum drug-concentration profiles after an i. v. infusion described with the two-compartment open model. All parameters required for operating the *in vitro* model were calculated by substitution of the *in vivo* pharmacokinetic parameters for the equations. By applying these parameters, changes in drug concentrations in the increasing phase corresponding to the period of infusion and in the following bi-exponential decreasing phase were successfully reproduced in flask A in the model, in which a one-way flow was maintained.

The washout effect is a disadvantage in the previously presented dilution models. When concentration-time profiles of antibiotics are simulated, especially those that possess high tissue transitional properties, quick elimination of the drug from the test vessel must be achieved by flow at high-speed. When a constant flow rate reproducing the faster α phase has to be employed in the present model, the dilution factors of the washout are no longer negligible (Figs. 3A and 4A). Mathematical formulas have been used to overcome the effect (13), however, they are not always easy to apply for examinations with low inocula levels or with bacteria significantly damaged by a fast flow. Thus, an alternative method in which the tested bacteria were immobilized in agarose gels was applied in the present study.

Disadvantageous effects against cell viability were expected, however, both of *S. aureus*

(Fig. 3) and *E. coli* O157:H7 (Fig. 4) clinical isolates exhibited quite similar growth curves in the in-gel cultures as compared to those in static liquid cultures. These results suggested that embedding bacteria in the agarose gel and treatment with agarase for determining CFU scarcely affected the resulting kill-curves under our experimental conditions. In addition to the isolates examined, we observed that the in-gel culture was applicable to other clinical isolates of gram-positive cocci, such as methicillin-resistant *S. aureus and Streptococcus pneumoniae,* and gram-negative bacilli, such as *Haemophilus influenzae* and *Pseudomonas aeruginosa* (T. Tomita, unpublished data).

The *in vitro* perfusion model apparently demonstrated that the empirical regimen of 1X(q24h) for 1 g of cefazolin exerted a strong bactericidal activity against the antibiotic-susceptible *S. aureus* RN2677, as well as that for fosfomycin on the target *E. coli* M96. On the other hand, the kill-curve obtained with the model apparently demonstrated insufficient activity of the regimen for fosfomycin against fosfomycin-resistant *E. coli* No. 44. Therefore, our results showed that the present new *in vitro* pharmacokinetic/pharmacodynamic perfusion model was able to provide a convenient and reliable method for evaluating the efficacy of antimicrobial agents administered by i. v. infusion. Efficacious doses and regimens of newly developing antimicrobial agents can be estimated using this *in vitro* model in a preclinical study, together with the results of animal models and human studies.

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Figure Legends

Fig. 1. Schematic illustrations of elementary circuits for the *in vitro* pharmacokinetic perfusion model and concentration-time curves. Elementary circuits (i) through (iv) are schematically illustrated. Flask A was a test flask, and flasks A and B were equipped with magnetic stirrers. R is a reservoir, and P is a peristaltic pump. C_a, C_b, and C_c represent the initial concentrations of drugs in flasks A, B, and a reservoir, respectively. Q shows the constant flow rate, and V_a and V_b, the constant medium volumes in flasks A and B, respectively. Concentration-time curves in flask A (solid lines on a linear scale) are drawn according to the equations. In circuit (iv), the solid line corresponding to equation [9] is obtained by subtraction of the dotted line to equation [3] from the thin line to equation [8]. Details are described in the text.

Fig. 2. Schematic illustration of the *in vitro* pharmacokinetic perfusion model to reproduce serum drug concentrations after i. v. infusion. Circuit C is composed of circuit A described by equation [12] and circuit B described by equation [10] where $C_{b1} = C_b$. Test flask A and flask B, B_1 and B_2 are equipped with magnetic stirrers. R is a reservoir and P is a peristaltic pump. The perfusion parameters written in italic characters are described in the text and Table 1. In circuit C, the flow path was from flask B_1 to flask A during $t < T_d$. At $t = T_d$, the flow path was changed using a T-shape stopcock (T) and maintained from flask B_2 to flask A. The entire system was kept under sterile at 37˚C. Concentration-time curves in flask A (solid lines on a linear scale) are drawn in the right side according to the equations. In circuits A and B, the solid lines correspond to the sum of the dashed lines.

Fig. 3. Evaluation of the *in vitro* pharmacokinetic perfusion model with cefazolin against *S. aureus.* (A) *S. aureus* was inoculated in agarose gel blocks (●) and the broth (○) in the *in vitro* perfusion model. Bacterial growth in the model was monitored as well as in a static culture (□). A constant flow rate (1.1 ml/min) was applied to give $\alpha = 0.0567$ min^{-1}. The dashed line represents the calculated cell numbers in a liquid culture where no bacterial growth

was assumed. (B) Cefazolin was administered with the perfusion parameters presented in Table 1. The concentrations of cefazolin in the agarose gel blocks (●) and the medium (○) in flask A were measured using a bioassay method. The solid line represents the serum drug concentration-time profile based on the pharmacokinetic data calculated with equations [10] and [12]. (C) Bactericidal activity of the drug regimen of 1X(q24h) with cefazolin against *S. aureus* embedded in agarose (●) and in the broth (○) was measured using the *in vitro* model with the perfusion parameters presented in Table 1. Error bars represent SD of 3 determinations and a broken line indicates the lower limit of detection.

Fig. 4. Bactericidal activity of the drug regimen of 1X(q24h) with fosfomycin against *E. coli* O157:H7 fosfomycin-susceptible and -resistant isolates. (A) *E. coli* M96 was inoculated in agarose gel (●) and the broth (○) in the *in vitro* perfusion model. Bacterial growth in the model was monitored as well as in a static culture (□). A constant flow rate (0.8 ml/min) was applied to give $\alpha = 0.04$ min^{-1}. The dashed line represents the calculated cell numbers in a liquid culture where no bacterial growth was assumed. The bactericidal activity of the drug regimen was measured with (B) strain M96 and (C) No. 44 inoculated in agarose gel (●) and in the broth (○) using the in vitro model. Perfusion parameters presented in Table 1 were applied to simulate the human serum level. Error bars represent SD of 3 determinations and a broken line indicates the lower limit of detection.

Table 1. Pharmacokinetic and perfusion parameters for an *in vitro* model simulating serum concentration-time profiles after i. v. infusion

Agent	Pharmacokinetic parameters[a]					Perfusion parameters[b]					
	A (μg/ml)	α (min^{-1})	B (μg/ml)	β (min^{-1})	C_{max} (μg/ml)	*Q* (ml/min)	V_a (ml)	V_b (ml)	C_{b1} (μg/ml)	C_{b2} (μg/ml)	C_c (μg/ml)
Cefazolin	118.3	0.0567	85.7	0.0068	134.4	1.1	19.4	160.9	120.0	68.2	487.4
Fosfomycin	87.1	0.0400	64.4	0.0060	87.2	0.8	20.0	132.7	63.1	45.9	214.2

[a]Data with the empirical dosing of 1 g for cefazolin (11) and fosfomycin (12).

[b]Calculated from the *in vivo* pharmacokinetic parameters with equations [10] and [12]. *Q*, the constant flow rate; V_a, the constant medium volume in flask A; V_b, the constant medium volume of flasks B_1 and B_2; C_{b1}, C_{b2}, and C_c, the initial drug concentrations of antibiotics in flasks B_1, B_2, and a reservoir, respectively. The constant flow rate *Q* was preferentially calculated to give the constant medium volume in flask A of approximately 20 ml.

Fig. 1

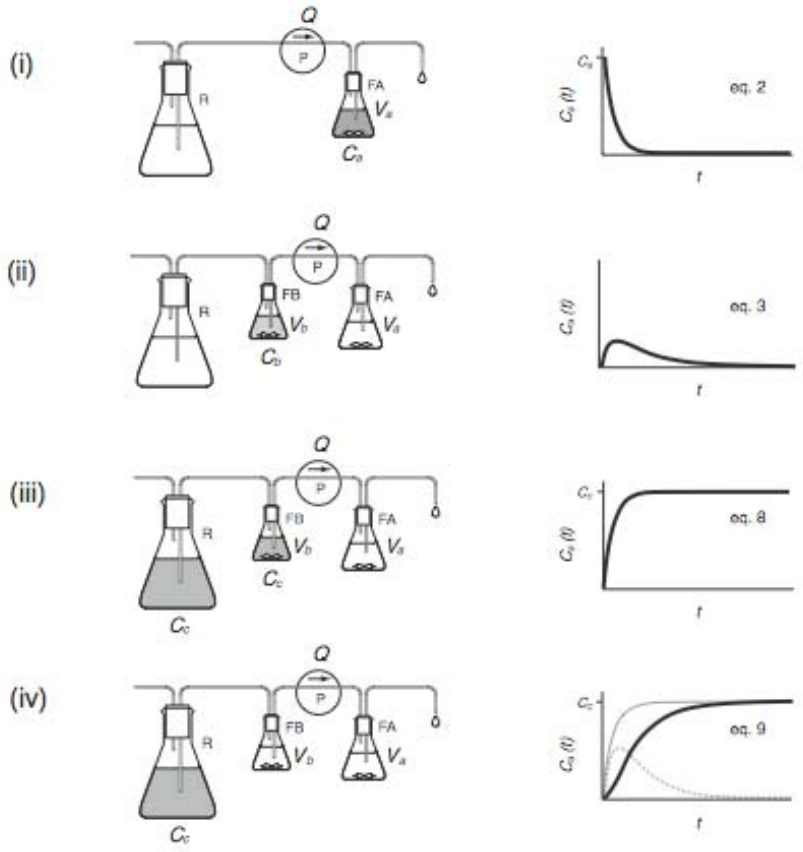

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Fig. 2

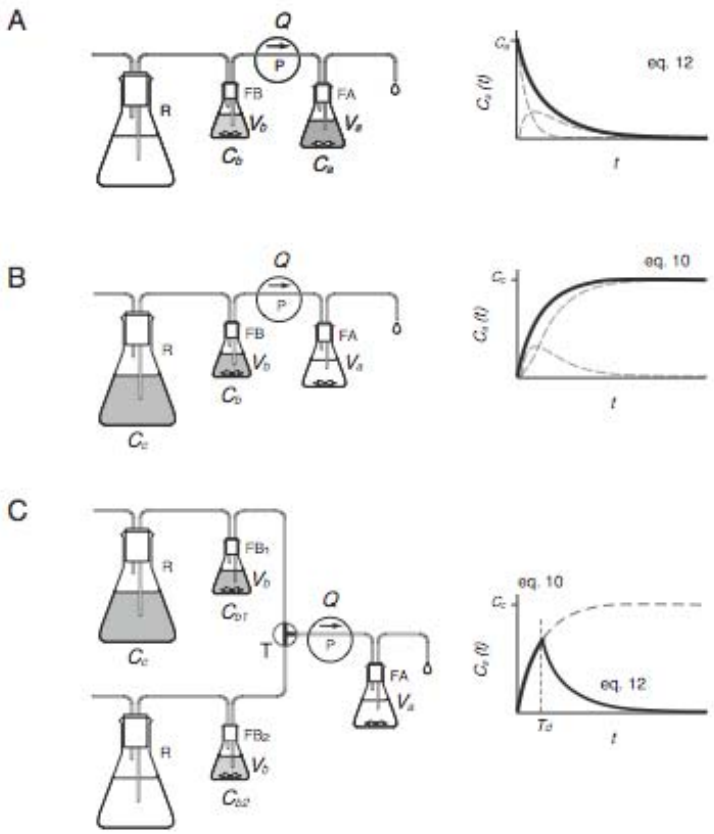

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Fig. 3

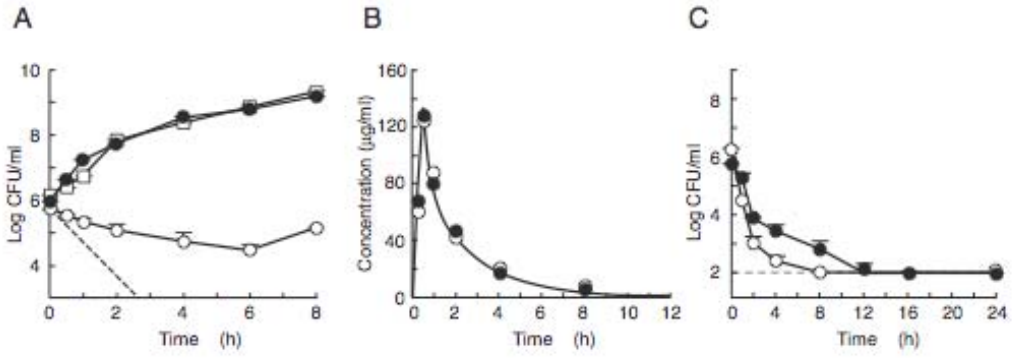

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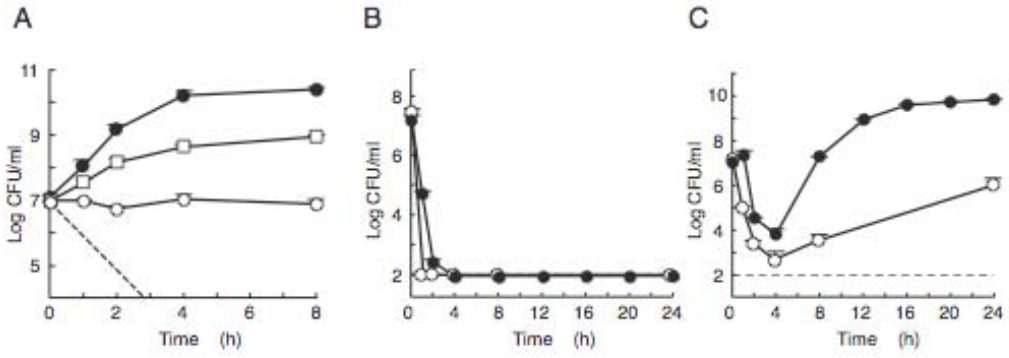
Fig. 4



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