Kinetic Studies on Cytosine Arabinoside Permeation through the Egg Phosphatidylcholine Liposomal Membrane

Noriyasu FUKUOKA,*[,a] Toyohisa TSUKAMOTO,[a] Misao NAKANO,[a] Akira SHIBATA,[b] and Takuya YAMASHITA[b]

Department of Hospital Pharmacy, Kagawa Medical School,[a] Ikenobe, Miki-cho, Kita-gun, Kagawa 761-07, Japan and Faculty of Pharmaceutical Sciences, University of Tokushima,[b] Sho-machi 1, Tokushima 770, Japan.

Received November 8, 1995; accepted February 3, 1996

We analyzed the permeability of low-molecular weight drugs through the lipid bilayer membrane using a new model. This model (model 3) was compared with two conventional models (models 1 and 2) in the permeation behavior of cytosine arabinoside (ara-C) through mixed cholesterol-phosphatidylcholine liposomes using a dialysis bag.

In models 1 and 2 the ara-C permeation rate is calculated based on the following premise:

1) The rate is proportional to the first degree of the difference between the equilibrium concentration in the external medium of the dialysis bag at time $t=\infty$ and the concentration at each time.

2) The rate is proportional to the first degree of the concentration difference between the inside and outside of the liposomes.

Analysis using model 3 was performed on the premise that the ara-C permeation rate is proportional to the first degree of the concentration difference between the inside and outside of the liposomes and that between the inside and outside of the dialysis bag.

The ara-C permeation coefficients through the liposomal membrane obtained using models 1, 2, and 3 were 2.9×10^{-5}, 1.7×10^{-6}, and 1.7×10^{-9} (cm/min), respectively. The models were evaluated according to Akaike's information criteria (*AIC*) and the sum of squares (*SS*). The best results were obtained using model 3 ($AIC=-29.7$, $SS=1.2\times10^{-1}$). These results suggest that the film resistance inside the dialysis bag should be considered one of the rate determining steps of drug permeation in an experimental system in which the outside but not the inside of the dialysis bag is agitated.

Key words permeation; kinetic analysis; film resistance; liposome; dialysis

Determination of the dosage of an anti-cancer drug is the most important factor to minimize its adverse reaction and to demonstrate its anti-cancer action most effectively. In continuous release dosage forms, once an adverse reaction occurs, a patient can be fatally affected. We therefore studied the permeation behavior of the anti-cancer drug cytosine arabinoside (ara-C) to obtain information helpful in determining optimal of drug dosage in a targeting therapy.

The permeation of low molecular weight drugs through liposomes or microcapsules has been evaluated by measuring the time course of permeation of drug-trapped capsules placed in a dialysis bag.[1-3] In this method, assuming that the resistance of the dialysis bag is much lower than that of the liposomal membrane,[4,5] the resistance of the membrane alone is viewed as the resistance to drug permeation. Analysis was thus performed using an approximate expression that the permeation rate is proportional to the first degree of the difference between the equilibrium drug concentration in the external medium at time $t=\infty$ and the drug concentration in the external medium at time t.[6]

The dialysis bag is a selective permeation membrane showing differences in the permeation rate according to the molecular size and chemical properties of the substrate.[4a,7,8] This suggests that the resistance of this should not be ignored in any evaluation of drug permeation.

In the present study, therefore, we analyzed the permeation rate of ara-C through the liposomal membrane using a compartment model[9] in which the resistance of the dialysis membrane to the drug was taken into consideration. The resistance of the liposomal membrane and dialysis bag to the drug permeation was included in our model. The transfer rate of the drug was calculated using a material balance equation in which the drug concentration difference on the interface is the driving force according to Fick's law.

When the permeation behavior of a drug across the liposomal membrane mediated by closed system membranes such as the dialysis bag is analyzed, the membrane resistance of the bag cannot be ignored.

Experimental

Materials and Reagents Liposomes were prepared using egg yolk phosphatidylcholine (EPC: Nichiyu Liposome), cholesterol (Wako Pure Chemical Industries, Ltd.), phosphatidylserine (Wako), and diethyl ether (reagent grade, Dojin Laboratories). Ara-C was supplied by Nippon Shinyaku Co., Ltd. NaCl, KCl, Na_2HPO_4, and KH_2PO_4 (Wako Pure Chemical Industries, Ltd.) were all of reagent grade. The dialysis bag (Visking tube: size, 27/32; pore size, 2.7×10^{-8} cm; width, 32 mm; Viscase Corp., U.S.A.) was boiled and stored in distilled water at 4 °C until the experiment.

Preparation of ara-C-Incorporated Liposomes ara-C-Incorporated liposomes were prepared by the reverse-phase evaporation vesicle (REV) method.[10] EPC (62.9 mg), cholesterol (31.3 mg), and phosphatidylserine (1.94 mg) were mixed with 6 ml of a mixed solvent (dichlorethane–diethyl ether, 1:5), and the solvent was eliminated *in vacuo* in a desiccator. Subsequently, 6 ml of the mixed solvent (dichlorethane–diethyl ether, 1:5) was again added, and 2 ml of 7.8 mM phosphate buffered saline (PBS) (pH, 7.5) containing ara-C (20 mg/ml) was also added slowly. This mixture was ultrasonicated for 3 min, ice-cooled for 1 min, and ultrasonicated again for 3 min. This suspension was placed in a round-bottom flask, and the solvent was eliminated *in vacuo* in a rotary evaporator to obtain liposomes. The residue was washed with 2 ml of PBS, and ara-C-incorporated liposomes were obtained. The liposomes were centrifuged at 5 °C and $200000\times g$. Free ara-C in the supernatant was recovered and measured using a spectrophotometer (Hitachi 220A) at 272 nm.

* To whom correspondence should be addressed.

Table 1. Parameters for the Analysis

	Particle diameter of liposomes 250.1 ± 44.0 nm Number of liposomes $(2.4 \pm 0.1) \times 10^{13}$ numbers/cm^3
Entire surface area of liposome[a] (s)	$(4.7 \pm 0.3) \times 10^4$ cm^2
Total inside volume of liposome[b] (V_1)	$(1.8 \pm 0.4) \times 10^{-1}$ cm^3
Inside volume of dialysis bag (V_2)	0.8 cm^3
Outside volume of dialysis bag (V_3)	4.0×10 cm^3
Surface area of dialysis bag	1.3×10 cm^2

The liposomes were assumed to be globular for determination of each parameter. *a*) This value was calculated by multiplying the surface area of one liposome by the number of liposomes. *b*) This value was calculated by multiplying the volume of a globularity, the radius of which was obtained by subtracting the thickness of the lipid bilayer membrane from the liposome radius, by number of liposomes.

The amount of incorporated ara-C was calculated by subtracting the amount of the free ara-C from the amount of the ara-C used. This amount of incorporated ara-C was used as the initial drug amount in liposomes in the following calculations.

Dialysis Experiment An aliquot of the ara-C incorporated liposome suspension was mixed with PBS to obtain a total volume of 1 ml (lipid concentration, 33 μmol/ml). This solution was placed in a dialysis bag (effective length, 20 mm). As the external medium, 40 ml of PBS was used. This medium was agitated using a magnetic stirrer at 25 ± 0.5 °C at 500 rpm.

The drug concentration was determined by spectrophotometry at 272 nm in 2 ml of the external medium obtained at 1-min intervals for the first 10 min, 10-min intervals after 10—60 min, and at a 60-min interval after 60—120 min. Each time the external medium was collected, 2 ml of PBS was added.

Measurement of the Mean Particle Size and Volume of ara-C- Incorporated Liposomes The mean particle diameter and the number of ara-C-incorporated liposomes were measured at 25 °C using a laser particle analyzer system LPA-3000/3100 (Otsuka Electronics) and a Coulter counter MODEL ZBI, respectively. The thickness of the lipid bilayer membrane was measured as 3.7 nm,[11] and the parameters of the liposomes were calculated. Each parameter obtained from the measurement values is shown in Table 1.

Kinetic Model for Drug Permeation The ara-C permeation behavior from the liposomal membrane was analyzed using conventional models 1[4,5] and 2[8] and our new model 3.

Model 1: The resistance to the drug permeation across the membrane is present in the liposomal membrane alone. The permeation rate is proportional to the first degree of the difference between the equilibrium concentration in the external medium at time $t = \infty$ and the concentration in the external medium at time t. This is expressed using the following approximation equation[4,5]:

$$\ln(C_\infty - C_t) = \ln(C_\infty - C_0) - kt$$

From this,

$$C_t = C_\infty \times (1 - \exp(-kt)) + C_0 \times \exp(-kt) \quad (1)$$

is obtained.

C_0: initial ara-C concentration in the external medium (μg/ml)
C_∞: ara-C concentration at $t = \infty$ when all liposomes were destroyed (μg/ml)
C_t: ara-C concentration in the external medium at time t (μg/ml)
k: permeation rate constant in the liposomal membrane (min^{-1})

Model 2: The resistance to the drug permeation across the membrane is present in the liposome membrane alone. The permeation rate is proportional to the first degree of the difference in the concentration in the medium inside the liposomes and that in the medium outside the liposomes according to Fick's law as expressed using the following equation:

$$dC/dt = P(C_i - C_0) \cdot S/V_1$$

From this,

$$C(t) = B(t)/V_0$$
$$= A(0)/(V_1 + V_0)(1 - \exp(-k(1/V_1 + 1/V_0)t))$$
$$+ B(0)/(V_1 + V_0)(1 + V_1/V_0 \exp(-k(1/V_1 + 1/V_0)t)) \quad (2)$$

is obtained.

C_i: ara-C concentration in the medium inside the liposomes (μg/ml)
C_0: ara-C concentration in the medium outside the liposomes (μg/ml)
P: permeation coefficient (cm/min)
S: entire surface area of the liposomes (cm^2)
V_1: total volume of the medium inside the liposomes (cm^3)
$A(0)$: initial ara-C amount in the medium inside the liposomes (μg)
$B(0)$: initial ara-C amount in the medium outside the liposomes (μg)
k = constant: $P \cdot S = k$ (cm^3/min)

Model 3: In general, there is a boundary film in the membrane.[12] The resistance of this film[12] is to be due to the permeation resistance of the dialysis tube to the drug. Assuming that the drug permeates the dialysis tube by simple diffusion through pores,[13] this diffusion phase is thought to be the boundary film.

In the present experimental system, the boundary film of the dialysis tube is composed of internal and external films. However, since the external area of the dialysis tube was agitated using a stirrer, we determined film resistance to be present in the internal area of this tube, and this is the resistance to ara-C permeation. Therefore, we produced a model in which permeation resistance is present in both the liposomal membrane and the dialysis bag, and the drug permeation rate across each membrane is proportional to the first degree of the difference in the drug concentration between the medium inside and that outside the liposomal membrane and the concentration between the medium inside and that outside the dialysis bag according to Fick's law.

The experimental system was divided into three compartments, *i.e.*, medium inside the liposomes, medium inside the dialysis bag, and medium outside the dialysis bag, and the following equations were obtained for the material balance of ara-C in each compartment according to Fick's law:

$$dE(t)/dt = -k_1(E(t)/V_1 - F(t)/V_2) \quad (i)$$

$$dF(t)/dt = k_1(E(t)/V_1 - F(t)/V_2) - k_2(F(t)/V_2 - G(t)/V_3) \quad (ii)$$

$$dG(t)/dt = k_2(F(t)/V_2 - G(t)/V_3) \quad (iii)$$

$E(t)$: ara-C amount in the medium inside the liposomes
$F(t)$: ara-C amount inside the dialysis bag
$G(t)$: ara-C amount in the external medium
V_1: total volume of the medium inside the liposomes
V_2: volume of the medium inside the dialysis bag
V_3: volume of the external medium
k_1: permeation rate constant of ara-C through liposomal membrane (liposome surface area × permeation rate)
k_2: permeation rate constant of ara-C through dialysis bag (dialysis bag surface area × permeation rate)

From Eqs. i, ii, and iii the ara-C concentration in the external medium at time t was obtained using the following Eq. 3:

$$C(t) = G(t)/V_3 = \{(k_1k_2\{E(0) + F(0) + G(0)\}/\alpha \cdot \beta + \{(G(0)(\alpha^2 V_1 V_2 + k_1\alpha(V_1 + V_2) + k_1k_2 + k_2\alpha V_1) + k_2(\alpha V_1 + k_1)F(0) + k_1k_2E(0)\} \cdot \alpha(\alpha - \beta)/\exp(\alpha t) + \{G(0)(\beta^2 V_1 V_2 + k_1\beta(V_1 + V_2) + k_1k_2 + k_2\beta V_1) + k_2(\beta V_1 + k_1)F(0) + k_1k_2E(0)\} \cdot \beta(\beta - \alpha)/\exp(\beta t)\}/V_1V_2V_3 \quad (3)$$

Here, α and β have the following relationship:

$$\alpha + \beta = \{k_1V_3(V_1 + V_2) + k_2V_1(V_2 + V_3)\}/V_1V_2V_3$$
$$\alpha \cdot \beta = k_1k_2(V_1 + V_2 + V_3)/V_1V_2V_3$$

These analyses were performed using the program MULTI[14] based on the non-linear least squares method.

Results and Discussion

Analysis Using Model 1 Figure 1a shows serial changes in the concentration of ara-C from the liposomal membrane and results of fitting based on Eq. 1.

From Eq. 1, the permeation rate constant k was 2.34×10^{-2} (min^{-1}). The permeation coefficient of the liposomal membrane was calculated using the following equation[5a]:

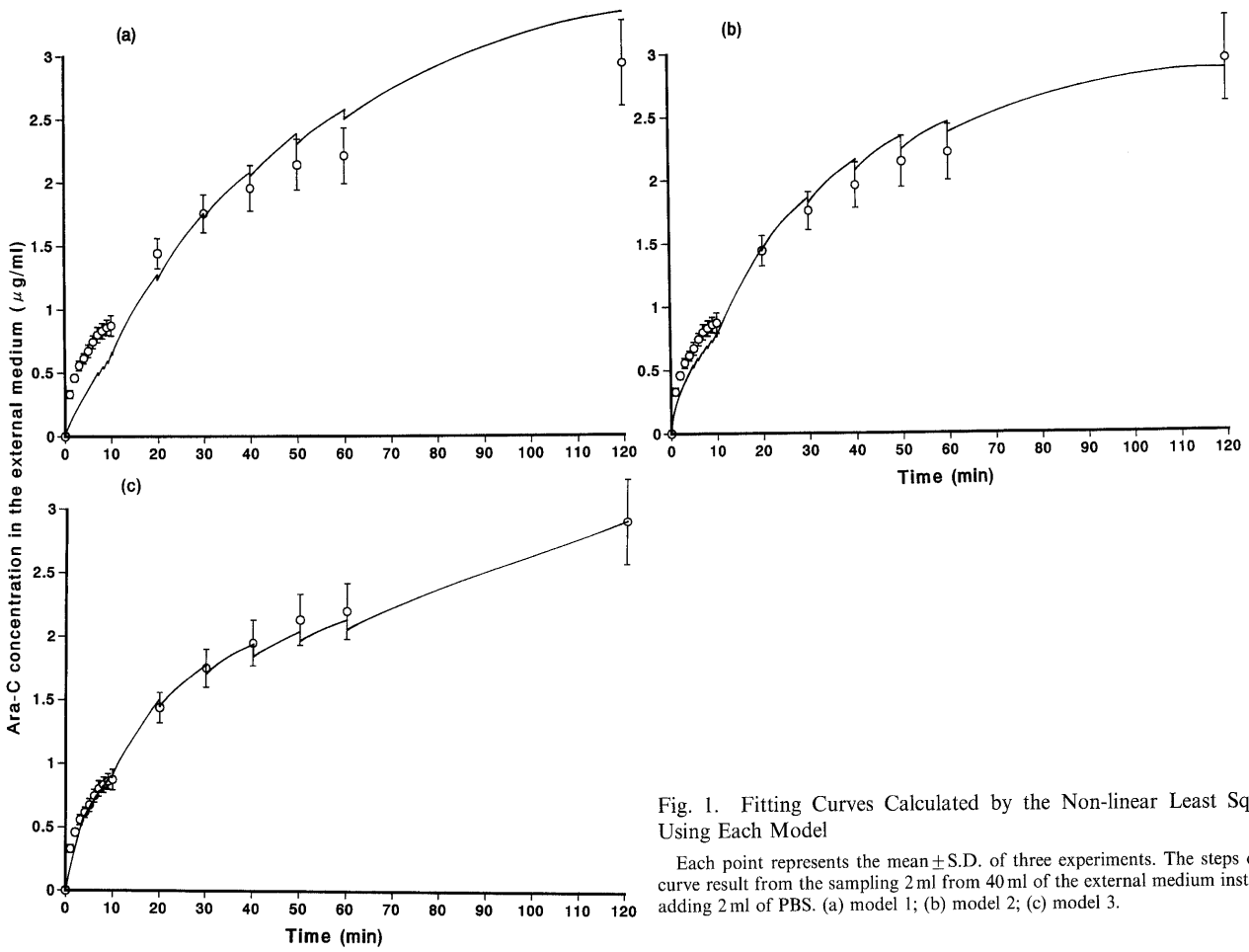

Fig. 1. Fitting Curves Calculated by the Non-linear Least Squares Using Each Model

Each point represents the mean ± S.D. of three experiments. The steps of this curve result from the sampling 2 ml from 40 ml of the external medium instead of adding 2 ml of PBS. (a) model 1; (b) model 2; (c) model 3.

$P = k \times r/3$ (P: permeation coefficient, r: liposome radius)

$P = 2.93 \times 10^{-5}$ (cm/min) was obtained.

Analysis Using Model 2 In Eq. 2 the initial amount of ara-C in the medium outside the liposomes was believed to be 0: $B(0) = 0$. The other parameters are shown in Table 1. Figure 1b shows the permeation behavior of ara-C through the liposomal membrane and the results of fitting using Eq. 2. Compared with model 1 in which the ara-C concentration in the medium inside the liposomes was ignored, fitting was markedly improved by considering the concentration difference at the liposomal membrane interface as the driving force of the drug transfer according to Fick's law. However, the data obtained by measurement after 1—10 min were higher than those obtained by analysis and this increase reached a plateau after 8 min. After 20 min or more, measurement data became slightly inconsistent with analysis data. These findings may represent biphasic permeation behavior because there was a difference in the ara-C concentration not only between the medium inside the liposomes and that inside the dialysis bag but also between the medium inside and that outside the dialysis bag. Therefore, the permeation rate seemed to be controlled by the resistance of the dialysis bag in the former phase and by the resistance of the liposomal membrane in the latter phase. The ara-C permeation coefficient across the liposomal membrane using model 2 was 1.7×10^{-6} (cm/min).

Analysis Using Model 3 The parameters for the analysis using Eq. 3 are shown in Table 1. In this method using a dialysis bag, ara-C permeation through liposomes is evaluated by measurement of ara-C from the bag. The biphasic nature of ara-C permeation of the liposomal membrane can be accurately analyzed by setting resistance in both the liposomal membrane and the dialysis bag.

Figure 1c shows measurement values of the permeation behavior of ara-C through the liposomal membrane and results of curve fitting using Eq. 3. The results of analysis using Eq. 3 were in good agreement with the biphasic nature of efflux of free ara-C from the dialysis bag and subsequent continuous ara-C efflux from the liposomes. This is because the film resistance was set in both the liposomal membrane and inside of the dialysis bag. The obtained permeation coefficient of ara-C through the liposomal membrane was 1.7×10^{-9} (cm/min).

Comparison of Analytical Results among Models 1, 2, and 3 The results of analysis by the 3 models are shown in Table 2.

The permeation coefficient of ara-C through the lipid bilayer membrane was similar to that of glucose or tryptophan using model 1.[15]

Comparison of models **1** and **2** showed that fitting was better in the latter (Figs. 1a and b). This may be because model 2 better reflects the experimental condition, *i.e.*, the concentration difference between the inside and outside of the liposomal membrane as the driving force for the drug permeation rate. Akaike's information criteria (*AIC*) and the sum of squares (*SS*),[16] which were used to

Table 2. Comparison of Parameters Calculated by Each Model

	Model 1	Model 2	Model 3
Permeation rate constant through liposomal membrane (cm^3/min)	—	$(1.6\pm0.4)\times10^{-2}$	$(1.6\pm0.3)\times10^{-5}$
Permeation rate coefficient through liposomal membrane (cm/min)	$(2.9\pm0.5)\times10^{-5}$	$(1.7\pm0.4)\times10^{-6}$	$(1.7\pm0.3)\times10^{-9}$
Permeation rate constant through dialysis membrane (cm^3/min)	—	—	$(5.0\pm0.2)\times10^{-2}$
Permeation rate coefficient through dialysis membrane (cm/min)	—	—	$(3.9\pm0.2)\times10^{-3}$
Correlation coefficient[a]	0.991	0.994	0.999
Akaike's information criteria (*AIC*)	6.8 ± 0.2	-1.8 ± 0.2	-29.7 ± 0.4
Sum of squares (*SS*)	1.3 ± 0.4	$(8.0\pm0.3)\times10^{-1}$	$(1.2\pm0.3)\times10^{-1}$

Data are the mean $\pm$ S.D. of three experiments. *a*) Correlation coefficient is mean value of three experiments.

evaluate models, were also better using model 2 than model 1 (Table 2).

Analysis using model 3 revealed a permeation coefficient through the dialysis bag about 10^6 times that through the liposomal membrane. This value was obtained because the film resistance in the dialysis bag was taken into consideration in this model. The appropriateness of this model (Fig. 1c) was shown not only by the results of curve fitting but also by *AIC* and *SS* values (Table 2).

These results appear to indicate that film resistance should be taken into consideration in kinetic analysis of the permeation behavior of low molecules (drugs) from the liposomal membrane using a closed system membrane such as the dialysis bag.

References

1) Yamaoka K., Moriyama H., Saito Y., Sato T., *Yakuzaigaku*, **48**, 310—312 (1988).
2) Higaki K., Takechi N., Hashida M., Sezaki H., *Chem. Pharm. Bull.*, **36**, 1214—1217 (1988).
3) Saito K., Hosoi F., Makuuchi K., Koishi M., *Yakuzaigaku*, **47**, 154—160 (1987).
4) *a*) Mayer M. C., Guttman D. E., *J. Pharm. Sci.*, **59**, 33—38 (1970); *b*) *Idem*, *ibid.*, **57**, 1627—1629 (1968).
5) Disalvo E. A., *Biochim. Biophys. Acta*, **905**, 9—16 (1987); *b*) Shinoda K., Adachi I., Ueno M., Horikoshi I., *Yakugaku Zasshi*, **110**, 186—190 (1990).
6) Takamura K., Koishi M., *Kolloid-Z. u. Z. Polymere.*, **248**, 929—933 (1971).
7) Nakagawa T., "Maku No Hataraki," Kyoritsu Shuppan, Tokyo, 1985, p. 79.
8) Klein R. K., Moore M. J., Smith M. W., *Biochim. Biophys. Acta*, **233**, 420—433 (1971).
9) Goto S., Nakata K., Miyakawa T., Zhang W., Uchida T., *Yakugaku Zasshi*, **111**, 702—708 (1991).
10) Szoka F., Papahadjopoulos D., *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194—4198 (1978); Szoka F., Olson F., Health T., Vail W., Mayhew E., Papahadjopoulos D., *Biochim. Biophys. Acta*, **601**, 559—571 (1980).
11) Huang C., Mason J. T., *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 308—310 (1978)
12) Okada J., "Yakuhin Kougaku," Asakura Shoten, Tokyo, 1972, p. 33.
13) Craig L. C., *Advan. Anal. Chem. Instr.*, **4**, 35—74 (1966).
14) Ohsawa F., Kasai M., Matsuura T., Toyoshima Y., Kishimoto U., "Maku No Kinou," Kyouritsu Shuppan, Tokyo, 1977, p. 9.
15) Yamaoka K., Tanigawara Y., Nakagawa T., Uno T., *J. Pharmacobio-Dyn.*, **4**, 879—885 (1981).
16) Akaike H., Computer in Biomedicine, Suppl. 5th Hawaii Int. Conf. on System Sciences, 1972 pp. 99—101; Akaike H., *IEEE Trans. Automat. Contr.*, **19**, 716—723 (1974).