An Application of Microdialysis to Drug Tissue Distribution Study: *In Vivo* Evidence for Free-Ligand Hypothesis and Tissue Binding of β -Lactam Antibiotics in Interstitial Fluids

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To prove the free-ligand hypothesis for extravascular equilibration and tissue binding mechanism of β -lactam antibiotics, the microdialysis technique has been employed for the lung, the muscle and the liver in rats. Cefminox, a cephem antibiotic, and SY5555, a new penem antibiotic, were used in the present study. During the constant infusion of each antibiotic with simultaneous infusion of antipyrine, the microdialysis studies were performed and the dialysate concentrations were determined. The dialysate concentration was extrapolated to the *in vivo* unbound concentration in tissue interstitial fluids ($C_{\text{isf},u}$) according to the extrapolation method which was derived from the clearance concept. This extrapolation method incorporates the effective dialysis coefficient of a reference compound, antipyrine, which is used to correct the difference between *in vivo* and *in vitro* permeabilities of microdialysis fiber. The values of $C_{\text{isf},u}$ values for cefminox and SY5555 in the lung, muscle and liver were close to the unbound concentrations in the venous plasma leaving these organs. Furthermore, good coincidences were obtained between the unbound concentrations of SY5555 in lung and muscle interstitial fluids estimated from the total concentrations in homogenized tissues and those extrapolated by the microdialysis studies. Consequently, the present microdialysis studies provided the *in vivo* evidence that 1) the free-ligand hypothesis for extravascular equilibration of β -lactam antibiotics is true, and that 2) β -lactam antibiotics are restricted in the interstitial space in a noneliminating organ and bind only with albumin existing in this space.

Keywords — Antipyrine; β -lactam antibiotics; extracellular model; dialysis clearance; interstitial fluid; liver; lung; microdialysis; muscle; physiological pharmacokinetics

Introduction

In pharmacokinetics, unbound drug concentration in the tissue interstitial fluid has been empirically believed to be the same as that in the

plasma estimated from an *in vitro* binding study (free-ligand hypothesis). However, recent experimental observations on the capillary transport of drugs have suggested that the enhanced dissociation on the drug-protein complex exists

Fig. 1. An Extracellular Fluid Model for the Description of the Tissue Distribution Mechanism of β -Lactam Antibiotics into a Noneliminating Organ

Arrows show the direction of blood flow. $C_{a,u}$ and $C_{a,b}$, unbound and bound concentrations in arterial plasma, respectively; $C_{v,u}$ and $C_{v,b}$, unbound and bound concentrations in venous plasma leaving the tissue, respectively; $C_{\text{isf},u}$ and $C_{\text{isf},b}$, unbound and bound concentrations in the interstitial fluid, respectively.

in the microvasculature (protein mediated transport).^{1,2)} Therefore, one of the interesting subjects for understanding the tissue distribution mechanisms of drugs is to examine whether the free-ligand hypothesis or protein mediated hypothesis is true.^{3,4)}

Recently, to describe the tissue distribution mechanisms of β -lactam antibiotics, we have proposed the extracellular fluid model utilizing the free-ligand hypothesis (Fig. 1).⁵⁻⁸⁾ This model includes several assumptions that 1) the antibiotics cannot permeate through the plasma membrane and are restricted in the interstitial fluid space of a noneliminating organ, and that 2) the antibiotics in the interstitial fluid bind only with albumin existing in this compartment. It has been shown that the total tissue concentrations predicted by this model consist well with the observed values in homogenized tissues.⁵⁻⁸⁾ However, for a lack of a suitable experimental technique, it is not easy to prove the validity of this model by separating each assumption in the *in vivo* condition.

Tissue microdialysis, a technique to collect the interstitial fluid from living animals, has been recently developed to determine several endogenous and exogenous compounds in tissue interstitial fluid compartments.⁹⁻¹²⁾ In the previous study, we have proposed a novel method to extrapolate the *in vivo* true unbound concentration in the tissue interstitial fluid from the concentration in dialysate sample.¹³⁾ Furthermore, the usefulness and the validity of this extrapolation method has been demonstrated by the brain microdialysis.¹⁴⁾ Therefore, this technology would be a promising approach to examine the microvascular exchange and the tissue distribution mechanism of drugs.

Accordingly, the purpose of the present study is to prove the free-ligand hypothesis for extravascular equilibration of β -lactam antibiotics by the direct measurement of the *in vivo* unbound concentration in tissue interstitial fluid with microdialysis. Moreover, in order to further validate our extracellular fluid model, we compared the unbound concentration predicted from the apparent tissue concentrations in tissue homogenates with those determined by the microdialysis studies.

Theoretical

Extrapolation of Unbound Concentration in Tissue Interstitial Fluid with Tissue Microdialysis

To analyze the data of microdialysis, the steady-state clearance concept was applied, as described previously in detail.¹³⁾

$$CL_{\text{vitro}} = F C_d / C_r = F \{1 - \exp(-PA_{\text{vitro}}/F)\} \quad (1)$$

$$CL_{\text{vivo}} = F C_d / C_{\text{isf,u}} = F \{1 - \exp(-PA_{\text{vivo}}/F)\} \quad (2)$$

where CL , F , C and PA represent the dialysis clearance, the dialysis flow rate, the concentration and the permeability rate constant of dialysis fiber, respectively. The subscriptions of vitro, vivo, d, r, isf and u represent the *in vitro*, *in vivo*, dialysate, reservoir, interstitial fluid and unbound, respectively. Additionally, the effective dialysis coefficient, R_d , was defined as follows:

$$R_d = PA_{\text{vivo}} / PA_{\text{vitro}} \quad (3)$$

It is not easy to obtain the *in vivo* permeability rate constant of the drug. Therefore, the effective dialysis coefficient of a reference compound ($R_{d,\text{ref}}$) was used to correct the difference between PA_{vivo} and PA_{vitro} of drug on the basis of the assumption that $R_{d,\text{ref}}$ is equal to R_d of drug. This assumption is based on the experimental findings reported previously¹³⁾ (it will be discussed in detail in a later section). Antipyrine which shows no binding to the plasma protein and no displacing or enhancing effects on the drug-plasma protein complex was used as a reference compound. Moreover, the concentration of antipyrine in the tissue interstitial fluid was considered to be the same as that in the plasma at steady-state; *i.e.* $C_{\text{isf,ref}} = C_{\text{p,ref}}$. Then, the *in vivo* permeability rate constant of antipyrine ($PA_{\text{vivo,ref}}$), $R_{d,\text{ref}}$, and PA_{vitro} of β -lactam antibiotics can be expressed as follows:

$$CL_{\text{vivo,ref}} = F C_{d,\text{ref}} / C_{\text{p,ref}} = F \{1 - \exp(-PA_{\text{vivo,ref}}/F)\} \quad (4)$$

$$R_{d,\text{ref}} = PA_{\text{vivo,ref}} / PA_{\text{vitro,ref}} \quad (5)$$

$$PA_{\text{vivo}} = R_{\text{d,ref}} PA_{\text{vitro}} \quad (6)$$

where the subscription of ref represents the reference compound.

Rearrangement of Eqs. (2) and (6) yields the following equation to determine the unbound concentration of β -lactam antibiotics in the tissue interstitial fluid, $C_{\text{isf,u}}$.

$$C_{\text{isf,u}} = C_{\text{d,vivo}} / \{1 - \exp(-PA_{\text{vitro}} R_{\text{d,ref}} / F)\} \quad (7)$$

Prediction of Unbound Concentration in Tissue Interstitial Fluid from Homogenized Tissue Concentration

Regarding the binding in the tissue interstitial fluid of β -lactam antibiotics, the extracellular fluid model shown in Fig. 1 assumes that the antibiotic restricted in the interstitial space can bind only with albumin existing in this space, in a similar manner as with plasma albumin.⁵⁾ Then, the total tissue concentration (homogenized tissue) of noneliminating organ at steady-state ($C_{\text{T,ss}}$) can be expressed as follows⁵⁾:

$$C_{\text{T,ss}} = IS \left(1 + \frac{n AR P K_a}{1 + K_a C_{\text{isf,u}}} \right) C_{\text{isf,u}} \quad (8)$$

Where IS , AR are the interstitial space of tissue and the interstitial-to-plasma albumin concentration ratio, respectively. K_a and n are the association constant and number of the binding site regarding the plasma protein binding, respectively. P is the plasma albumin concentration. Then, the unbound concentration of β -lactam antibiotics can be obtained from $C_{\text{T,ss}}$ in homogenized tissue as follow:

$$C_{\text{isf,u}} = \frac{-B + \sqrt{B^2 + 4 K_a \frac{C_{\text{T,ss}}}{IS}}}{2 K_a} \quad (9)$$

$$B = 1 + n AR P K_a - K_a \frac{C_{\text{T,ss}}}{IS}$$

The values used for IS of muscle and lung were 0.119 and 0.204, respectively.⁸⁾ AR values used for muscle and lung were 0.6 and 0.5, respectively.⁵⁾

Materials and Methods

Animals — Adult male Wistar rats weighing 250–300 g were purchased from Sankyo Laboratory Co. (Toyama, Japan). They had free access to food and water.

Chemicals — SY5555 (MW 283.3), a new penem antibiotic (Fig. 2), and cefminox (MW 519.6), a cephem antibiotic (Fig. 2), were kindly supplied by the Suntory Bio-pharma Tech Center (Gunma, Japan) and Meiji Seika Kaisha, Ltd. (Tokyo, Japan), respectively. Antipyrine was obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan. Other chemicals were of analytical grade and used without further purification.

Microdialysis Cannula — The trans-cranial type of microdialysis cannula was prepared as described previously¹³⁾ using Cuprophane hollow-fiber (i.d. 0.2 mm, wall thickness 11 μm , MW cutoff 12500; Lento H.F., Organon Technica Corp., Oklahoma, U.S.A.) and a stainless steel tubing (o.d. 0.2 mm; MT Giken, Tokyo, Japan). Both sides of 19 or 22 mm long segment of dialysis hollow fiber were threaded on 3–5

Fig. 2. Chemical Structures of SY5555 and Cefminox

cm of stainless tubings and attached with a surgical adhesive (Aron Alpha A, Sankyo Co., Ltd., Tokyo, Japan). The distance of fiber where the dialysis takes place was 8 mm for muscle and liver dialyses and 5 mm for lung dialysis. Both ends of the stainless steel tubings were connected with a polyethylene tubing (SP10, Natsume Seisakusho Ltd., Tokyo, Japan) to make possible the connection to a perfusion pump.

In Vitro Microdialysis — *In vitro* microdialysis study was carried out in Ringer–Hepes buffer (RHB: 141 mM NaCl, 4 mM KCl, 2.8 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.40) containing SY5555 (100 $\mu\text{g}/\text{ml}$), or cefminox (500 $\mu\text{g}/\text{ml}$) with containing antipyrine (100 $\mu\text{g}/\text{ml}$) at 37 °C. The microdialysis probe was soaked in 20 ml of reservoir medium in a glass plate (i.d. 8.6 cm). Flow rate (10.0 $\mu\text{l}/\text{ml}$) was controlled with a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, U.S.A.). The reservoir medium was not stirred. The dialysate was collected over a 10-min interval sequentially three times after an appropriate time lapse for the steady-state condition. One hundred microliters of the reservoir medium were sampled at the midpoint of each collection period.

In Vivo Microdialysis — For muscle microdialysis study, rats were lightly anesthetized with ether. The left femoral vein and artery were cannulated with polyethylene tubings (SP-31) filled with heparin-saline solution (100 units/ml). The rats were kept in the supine position on a fixed board and the body temperature was maintained at 37 °C with heating lamps. The microdialysis cannula perfused by RHB at a constant flow rate of 10.0 $\mu\text{l}/\text{min}$ was implanted in the right hind leg muscle as described previously.¹³⁾ At 30 min after the fiber implantation, SY5555 was infused at a constant rate of 5.52 mg/min/kg by employing the constant infusion pump (Model 22, Harvard Apparatus). After the infusion was started, the dialysate was collected over a 5-min interval sequentially up to 90 min. Blood was withdrawn through the catheter at 2.5, 7.5, 12.5, 17.5, 27.5, 37.5, 52.5, 67.5, 77.5 and 87.5 min after the start of infusion.

For the liver microdialysis study, the abdomen of the rat was opened through a midline incision

under ether anesthesia. The microdialysis cannula perfused with RHB at a constant flow rate of 10.0 $\mu\text{l}/\text{min}$ was implanted in the central lobe of the liver. After the abdominal walls were sutured, a bolus dose (75 mg/kg) of antipyrine was administered intravenously *via* as catheter. Immediately SY5555 and antipyrine was simultaneously infused at constant rates of 5.52 mg/min/kg and 140 $\mu\text{g}/\text{min}/\text{kg}$, respectively. The dialysate was collected sequentially over a 10-min interval. Blood was collected at 2.5, 7.5, 17.5, 37.5, 62.5 and 87.5 min.

For lung microdialysis, the rats were intramuscularly anesthetized with ketamine (235 mg/kg) and xylazine (2.3 mg/kg). The animals were artificially ventilated through a tracheotomy by use of a rodent respirator (Model 683, Harvard Apparatus, South Natick, U.S.A.). A tidal volume was 2.5 cm^3 and a respiration rate was 80 breaths/min. Subsequently, the thorax was opened. The microdialysis probe perfused with RHB at a constant flow rate of 10.0 $\mu\text{l}/\text{min}$ was implanted in lobus medius of pulmo dexter, and then thorax was sutured. For the study of cefminox, bolus doses of cefminox (82.5 mg/kg) and antipyrine (75 mg/kg) were simultaneously administered intravenously. Immediately, antipyrine and cefminox were infused at constant rates of 2.54 mg/min/kg and 140 $\mu\text{g}/\text{min}/\text{kg}$, respectively. On the other hand, for the study of SY5555, the drug administration, dialysate and blood collection were carried out by the same procedure as described for liver microdialysis study. The sampling times of dialysate and blood were the same as those of the liver dialysis study.

Serum Unbound Fraction — For the determination of the unbound fraction of SY5555, various doses (2.07–13.8 mg/min/kg) of SY5555 were infused into rats to achieve the desired steady-state plasma concentration (75–500 $\mu\text{g}/\text{ml}$). Blood samples (0.6 ml) were collected at 22.5 and 45 min after the start of infusion. Immediately, the infusion rate was increased 2-fold to achieve the new steady-state plasma concentration. Then, the same volume of blood was collected at 67.5 and 90 min.

For the determination of the unbound fraction of cefminox, about 1.5 ml of blood sample was withdrawn at the end of the microdialysis

study described above.

Blood samples were centrifuged at 3000 rpm for 10 min at 4 °C to obtain serum. An aliquot of 0.30 ml serum sample was introduced to an ultrafiltration device (MPS-1, Amicon Corporation, Danvers, MA) and allowed to equilibrate at 37 °C for 20 min. Serum was centrifuged at 37 °C for 5 min (1000 *g*) to determine the unbound concentration.

Tissue Concentration of SY5555 — Under the light ether anesthesia, SY5555 was infused into rats at a dose of 5.52 mg/min/kg. The arterial blood was withdrawn at 60 min after the start of infusion, and then the rat was killed by bleeding. Lung, muscle and liver were quickly excised, rinsed with ice-cooled saline, blotted, and divided into two pieces. One part was immediately frozen with dryice-acetone at –20 °C, the other was stored at 4 °C. After 2 h storage of the tissues at the respective temperatures, one gram of each tissue was homogenized with a 4-fold volume of 50% MeOH in a grinder (Ultra-Turrax, IKA-WERK, Stafen) at 4 °C. An aliquot volume (1.0 ml) of homogenate was centrifuged at 10 000 rpm for 10 min at 4 °C. Supernatants of each homogenate were analyzed by the high performance liquid chromatography (HPLC) method (see Analytical procedure).

Analytical Procedure — The concentration of SY5555 in plasma or serum was determined by the same procedure described previously.¹⁵⁾ Cefminox concentration in the plasma sample was determined by the following method. Fifty microliters of the plasma sample and 10 μ l of isotonic phosphate buffer were mixed vigorously with 60 μ l of methanol, allowed to stand for 30 min at –20 °C, and centrifuged at 10 000 rpm for 5 min at 4 °C. An aliquot of the supernatants, 50 μ l, was diluted with 200 μ l of isotonic phosphate buffer. The mixture was filtered (0.45 μ m, Nihon Millipore Kogyo, Yonezawa, Japan), and 30 μ l of the filtrate was injected onto HPLC column (TSK gel ODS-80T_M, Toso Corporation, Tokyo, Japan). Antipyrine concentration in the plasma sample was determined by the same method described previously.¹³⁾

HPLC system was constructed with a pump (TRI ROTAR-V, Jasco, Tokyo, Japan), ultraviolet (UV) spectrophotometer (UVIDEC C-

100-V, Jasco) and a data processor (C-R4A, Shimadzu Corporation, Kyoto, Japan). The analytical conditions were: flow rate, 1.0 ml/min; analytical wavelength; 313 nm for SY5555, 270 nm for cefminox and 254 nm for antipyrine. The mobile phases used for SY5555, cefminox and antipyrine were methanol–0.01 M ammonium acetate (25:75, v/v), methanol–acetonitrile–2% acetic acid (3:3:94, v/v) and acetonitrile–pH 7.2, 78 mM phosphate buffer (25:75, v/v), respectively. Peak area was used for quantification. The concentration was determined from the calibration curve prepared by the same procedure as that for the respective samples.

Results

Serum Protein Binding of Cefminox and SY5555

The serum unbound fractions of cefminox and SY5555 were determined in the *in vitro* binding study. The unbound fraction of cefminox was almost independent of the concentration, and 0.849 ± 0.005 (mean $\pm$ S.E.M., $n = 3$) at the average total concentration of 301 μ g/ml (265.7–342.9 μ g/ml). On the other hand, as shown in Fig. 3, the serum unbound fraction of SY5555 was remarkably dependent on the concentration in the range of 51 to 357 μ g/ml. Using the Scatchard equation and albumin

Fig. 3. Serum Unbound Fraction of SY5555 in the Rat Serum

Each unbound fraction was determined for the serum collected from the SY5555 infused rats. The solid line was generated using the binding parameters of $n = 1.53$ and $K_a = 4720 \text{ M}^{-1}$.

TABLE I. Effective Dialysis Coefficient ($R_{d,ref}$) of Antipyrine, a Reference Compound, in Muscle, Liver and Lung

Tissue	$R_{d,ref}^{a)}$	$n^{b)}$
Lung	0.506 ± 0.065	7
Muscle	$0.404 \pm 0.018^c)$	3
Liver	0.508 ± 0.018	3

a) Values are calculated from Eq. (5) and represent the mean $\pm$ S.E.M. *b)* Number of experiments. *c)* Cited from a previous report.¹³⁾

concentration (P) of $470 \mu\text{M}$, the number of binding sites (n) and the association constant for the binding (K_a) were estimated to be 1.53 ± 0.07 and $4720 \pm 477 \text{ M}^{-1}$, respectively.

Effective Dialysis Coefficient of Antipyrine

To obtain PA_{vivo} of β -lactam antibiotics (Eq. (6)), the effective dialysis coefficient of antipyrine ($R_{d,ref}$) was determined by the *in vivo* microdialysis of antipyrine simultaneously ad-

ministered with β -lactam antibiotics. Using the steady-state concentrations in the dialysate ($C_{d,ref}$) and in plasma ($C_{p,ref}$), the values of $PA_{vivo,ref}$ were calculated from Eq. (4). The values of $PA_{vivo,ref}$ for lung and liver were 0.259 ± 0.017 (mean $\pm$ S.E.M., $n = 7$) and 0.368 ± 0.006 ($n = 3$), respectively. The values of $PA_{vitro,ref}$ were also determined at 37°C by the respective same fibers used for lung and liver, and were 0.510 ± 0.013 ($n = 7$) and 0.737 ± 0.018 ($n = 3$), respectively. Thus, the values of $R_{d,ref}$ were calculated from Eq. (6) and were summarized in Table I along with $R_{d,ref}$ for muscle determined previously.¹³⁾ Although there was a significant difference between $R_{d,ref}$ values of muscle and liver ($p < 0.05$), no differences between $R_{d,ref}$ values of lung and muscle, and between those of lung and liver were observed.

Unbound Concentrations in Interstitial Fluid of Lung, Muscle and Liver

Fig. 4. Time Courses of Cefminox and SY5555 Concentrations in the Plasma and the Dialysate

Each symbols and vertical bar represent the mean and S.E.M., respectively. When verticals are not shown, the S.E.M. is contained within the limits of the symbol. Open and closed symbols represent the plasma and dialysate concentrations of cefminox or SY5555, respectively. Panel A represents the result of lung dialysis study for cefminox. Panels B, C and D represent the results of lung, muscle and liver dialysis studies for SY5555, respectively.

Figure 4 shows the plasma and dialysate concentrations *versus* time profiles obtained by the microdialysis studies for SY5555 and cefminox. In the lung microdialysis study for cefminox, the steady-state concentrations were attained both for plasma and dialysis samples at 15 min after the infusion ((A) in Fig. 4). The steady-state dialysate concentration of cefminox was $1.90 \pm 0.15 \mu\text{g/ml}$ (mean $\pm$ S.E.M., $n = 21$). On the other hand, in the lung, muscle and liver microdialysis studies for SY5555, the steady state condition was attained at 25 min after the infusion ((B), (C), (D) in Fig. 4). The steady-state dialysate concentrations of SY5555 for lung, muscle and liver were $1.45 \pm 0.20 \mu\text{g/ml}$ (mean $\pm$ S.E.M., $n = 24$), $1.58 \pm 0.11 \mu\text{g/ml}$ ($n = 36$) and $1.78 \pm 0.19 \mu\text{g/ml}$ ($n = 18$), respectively.

In the study for cefminox, the unbound concentration in the lung interstitial fluid ($C_{\text{isf,u}}$) was estimated from the steady-state dialysate

concentration (C_d), and PA_{vitro} and $R_{d,\text{ref}}$ values. On the other hand, the unbound concentration in the venous plasma leaving tissue ($C_{v,u}$) was predicted from the steady-state total plasma concentration and the unbound fraction determined in the *in vitro* binding study. As shown in Table II, a good coincidence was obtained between $C_{\text{isf,u}}$ and $C_{v,u}$.

Similarly, in the studies for SY5555, the values of $C_{\text{isf,u}}$ in lung, muscle and liver were estimated from Eq. (7). Results are summarized in Table II. The values of $C_{v,u}$ were predicted from the steady-state total plasma concentration, serum unbound fraction determined in the *in vitro* study (Fig. 1) and the tissue extraction ratio determined in the previous report.¹⁵⁾ The values of extraction ratios used for the prediction were 0.104 for muscle and 0.120 for liver. Results were also summarized in Table II. The values of $C_{\text{isf,u}}$ were close to those of $C_{v,u}$. There were no

TABLE II. Comparison of Unbound Concentrations in Tissue Interstitial Fluid ($C_{\text{isf,u}}$) and Tissue Venous Plasma ($C_{v,u}$)^{a)}

Drug	Tissue	Unbound concentration ($\mu\text{g/ml}$)	
		$C_{\text{isf,u}}$ ^{b)}	$C_{v,u}$ ^{c)}
Cefminox	Lung	252.5 ± 14.7 (21)	251.8 ± 5.8 (12)
SY5555	Lung	68.7 ± 2.9 (24)	89.4 ± 3.7 (9)
	Muscle	72.7 ± 4.9 (36)	89.9 ± 16.4 (15)
	Liver	64.2 ± 5.3 (18)	74.9 ± 7.8 (9)

a) Values are the mean $\pm$ S.E.M. and the number in parenthesis represents number of points. b) Values are estimated by Eq. (7) from the dialysate concentration obtained by the tissue microdialysis studies. c) Values are predicted from the total plasma concentration in artery, the unbound fraction and the tissue extraction ratio reported previously.¹⁵⁾

TABLE III. Tissue Concentrations of SY5555, and Comparison between Unbound Concentrations Estimated from Tissue Homogenate and that Determined by Microdialysis^{a)}

Tissue	$\frac{C_{T,\text{ss}}}{C_a}$ ^{b)}		$\frac{C_{\text{isf,u}}}{C_a}$ ^{c)}	
	Storage temperature ^{d)}		Homogenate ^{e)}	Microdialysis ^{f)}
	4 °C	−20 °C		
Lung	0.00723 ± 0.00211	0.0940 ± 0.0288	0.244 ± 0.084	0.270 ± 0.012
Muscle	0.0746 ± 0.0079	0.0645 ± 0.004	0.275 ± 0.048	0.320 ± 0.013
Liver	0.995 ± 0.065	0.917 ± 0.119	—	0.287 ± 0.012

a) Values are the mean $\pm$ S.E.M. of three to four experiments. b) Values are represented by the ratio of the concentration in tissue and that in arterial plasma. c) Values are represented by the ratio of the concentration in interstitial fluid and that in the arterial plasma. d) Tissues were stored at 4 °C and −20 °C for 2 h before the assay. e) The unbound concentrations were estimated according to Eq. (9) from the total concentration in homogenized tissue. f) The unbound concentrations were estimated according to Eq. (7) with microdialysis.

significant differences between the values of $C_{\text{isf,u}}$ and $C_{\text{v,u}}$ in muscle and liver, but significant difference between $C_{\text{isf,u}}$ and $C_{\text{v,u}}$ in lung was observed.

Comparison between Unbound Concentrations in Interstitial Fluid Estimated from Homogenized Tissue and That from the Dialysate

The tissue homogenized concentrations of SY5555 in the lung, muscle and liver at the steady state were determined and are listed in the left hand side of Table III. For the muscle and liver, no significant differences were observed between the concentration stored at 4 °C and that at -20 °C. However, the concentration in lung stored at 4 °C for 2 h was significantly lower than that at -20 °C. Using the values of IS and AR , the binding parameters (n and K_a), and the concentrations in the lung, muscle and liver stored at -20 °C, the unbound concentrations of SY5555 in the lung and muscle interstitial fluids were estimated from Eq. (9). The values were normalized by the respective arterial plasma concentration ($C_{\text{isf,u}}/C_a$), and are summarized in Table III. Additionally, the unbound concentrations in the interstitial fluid determined directly by the microdialysis are also listed in Table III as the values normalized by the respective arterial concentration. Statistically no significant difference was observed between both values of unbound concentration.

Discussion

For the effective chemotherapy, to know the *in vivo* unbound concentration of antibiotics in the interstitial fluid of the infectious organ is as important as to know the *in vitro* antimicrobial activity. To determine the *in vivo* unbound antibiotic concentration in the interstitial fluid, several techniques such as dialyzing sacs, clots, wicks and blister have been attempted.¹⁶⁾ However, these techniques are known to alter the normal capillary physiology owing to the relatively large size of the device and are applicable only to the limited tissues such as skin.¹⁷⁾ However, the microdialysis technique can be applied to virtually any tissues and organs.¹⁰⁾ Moreover, since the microdialysis cannula can be implanted in the vicinity of the microvessel,

this technique would provide a more exact basis for understanding the microvascular exchange of drugs. These merits would help to obtain the direct *in vivo* evidence for the enhanced dissociation of drugs^{1,2)} and hormones^{18,19)} in the capillary microvessel or for the free ligand hypothesis of drugs. Then, the microdialysis technique was employed to determine the *in vivo* unbound concentration of β -lactam antibiotics in tissues and to prove directly the free ligand hypothesis for the distribution of these antibiotics. Three organs were selected, that is, the lung as a target organ for the pulmonary infection, the muscle as a depot organ for the antibiotic distribution and the liver as an eliminating organ. Cefminox and SY5555 were used in the present study.

To obtain the *in vivo* true unbound concentration in the interstitial fluid from the dialysate concentration obtained by microdialysis, several extrapolation methods have been recently proposed.^{13,14,20-24)} Among these methods, the characteristic of our proposed extrapolation method (Eq. (7)) is that the effective dialysis coefficient of a reference compound ($R_{\text{d,ref}}$) corrects the difference of the permeability rate constant of drug between the *in vivo* and the *in vitro* microdialysis. This idea is based on the previous experimental findings that the R_d values obtained in muscle tissue at steady-state are not significantly different among the compounds with different molecular weights such as [³H]water, [¹⁴C]urea, [¹⁴C]sucrose and antipyrine.¹³⁾ Moreover, R_d values of several compounds determined in the erythrocyte suspensions were independent of the molecular weight in the range of 18–1039.¹³⁾ These results suggest that R_d is independent of the plasma membrane permeability since sucrose does not permeate through the cell membrane while the other compounds do. Therefore, although antipyrine penetrates the cell membrane while β -lactam antibiotics do not, it would be reasonable to assume that the R_d values of both compounds are the same. The validity of this extrapolation method has been recently demonstrated by brain microdialysis using model compounds such as [³H]water, caffeine and aminopyrine.¹⁴⁾ On the other hand, we have shown previously that the R_d value is predominantly dependent on the interstitial fluid

space.¹³⁾ Since the lung, muscle and liver interstitial fluid spaces of rats have been reported to be 0.204,⁸⁾ 0.119⁸⁾ and 0.163,⁵⁾ respectively, the comparatively lower R_d value of muscle than those of lung and liver would be due to relatively small interstitial spaces of muscle tissue.

As reported previously,⁵⁾ a well-mixed state exists in the capillary bed for the tissue distribution of β -lactam antibiotics. Therefore, total antibiotic concentrations in venous plasma leaving the tissues should be the same as those in the capillary bed. If the free-ligand hypothesis is acceptable for the microvascular equilibration of β -lactam antibiotics, the unbound concentration in the tissue interstitial fluid determined with microdialysis ($C_{isf,u}$) would be the same as that in the venous plasma ($C_{v,u}$). As shown in Table II, a good coincidence for cefminox and relatively good coincidences for SY5555 were observed between the values of $C_{isf,u}$ and $C_{v,u}$. This suggests that the free-ligand hypothesis was directly proven by the microdialysis study for these tissues and these antibiotics.

The dehydropeptidase-I (DHP-I), an enzyme to metabolize penem antibiotics, has been found in several organs of the rat.^{25,26)} Recently, we have reported that the metabolic activity of DHP-I for SY5555 in homogenized tissues of rats decreases in the order of lung > liver > muscle. Results in the left hand side of Table III show that DHP-I in the lung tissue has the extremely high activity to SY5555 despite the storage at relatively low temperature (4 °C) without homogenization. In contrast to these *in vitro* findings, the prediction by the physiological pharmacokinetic model of SY5555 has shown that the unbound concentration in lung interstitial fluid in the *in vivo* intact organ would likely be the same as that in the plasma.¹⁵⁾ This would be attributed to the instantaneous and the continuous supply of the unbound form of SY5555 by the rapid plasma flow through the lung tissue. The present results of Table II support directly the above prediction in the *in vivo* state.

It is of great interest to further validate our extracellular fluid model shown in Fig. 1 on the basis of the results of the microdialysis study. For this purpose, we compared the unbound concentration in the interstitial fluid determined by the

microdialysis studies with those predicted from the total concentration in the homogenized tissue according to Eq. (9). As shown in the right hand side of Table III, the ratios of the unbound concentrations of SY5555 in lung and muscle and the respective arterial plasma concentrations agree well with those determined by the microdialysis studies. This suggests that the extracellular fluid model is valid for the description of the binding in the interstitial fluid and the free-ligand hypothesis of β -lactam antibiotics. This is perhaps the first *in vivo* evidence that the tissue distribution mechanisms of β -lactam antibiotics were clarified by the direct determination of the unbound concentration in tissue interstitial fluid.

As shown in Table II, the mean values of $C_{isf,u}$ of SY5555 estimated by the microdialysis technique tended to be slightly lower than that estimated by the plasma concentration ($C_{v,u}$). This discrepancy may be accounted for by the following reason. Since SY5555 is metabolized by DHP-I in several tissues,¹⁵⁾ the degradation in the unstirred water layer around the microdialysis fiber membrane would give the smaller PA_{vivo} value than that estimated from Eq. (6) using $R_{d,ref}$ of antipyrine. Significant difference between $C_{isf,u}$ and $C_{v,u}$ of SY5555 in lung which has the highest metabolic activity to this antibiotic¹⁵⁾ is considered to be due to the large difference between the true PA_{vivo} value and the PA_{vivo} value estimated from Eq. (6). On the other hand, $C_{isf,u}$ value estimated from the homogenized tissue concentration according to Eq. (9) is known to be very sensitive to the value of the interstitial space (IS), as reported previously.⁷⁾ As seen in Tables II and III, the slight underestimation of $C_{isf,u}$ from the homogenized tissue concentration of SY5555 may be due to the difference between the reported value of IS and the IS value of the animals used in this study. However, it should be noted that the values of $C_{isf,u}$ estimated by the present microdialysis method were close to those estimated from the homogenized tissue concentration and the plasma concentration.

In conclusion, the free-ligand hypothesis for extravascular equilibration of β -lactam antibiotics was proven by the present microdialysis technique. Moreover, it was demonstrated, by

comparing the unbound concentration in the tissue interstitial fluid predicted from the total tissue concentrations with those estimated by the microdialysis studies, that our previously proposed extracellular fluid model is valid for the description of tissue distribution of β -lactam antibiotics.

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