The Use of an Artificial Skin Model to Study Transdermal Absorption of Drugs in Inflamed Skin

Shinji Oshima,* Chihiro Suzuki, Rina Yajima, Yuya Egawa, Osamu Hosoya, Kazuhiko Juni, and Toshinobu Seki

Faculty of Pharmaceutical Sciences, Josai University; 1–1 Keyakidai, Sakado, Saitama 350–0295, Japan.
Received September 20, 2011; accepted November 22, 2011; published online November 25, 2011

Studies on drug disposition in inflamed skin are important for safe and effective application of topical drugs. Here, the absorption of flurbiprofen (FP) through inflamed skin was examined *in vivo* and in a skin-mimicking artificial model system. The model skin system consisted of a silicone membrane acting as a model stratum corneum, laminated dialysis membranes acting as a model of viable skin, and 2 microdialysis probes—one used for determination of FP concentration and one acting as a model vessel. This model system could be used for quantitative evaluation of complicated permeation processes. In the *in vivo* experiments, FP absorption was suppressed in rats with inflamed skin induced by an intracutaneous injection of a mixed solution of λ-carrageenan, zymosan, and casein. Bovine serum albumin solution was placed between the dialysis membranes in the model skin system to mimic protein leaching in skin; the results suggested that the delayed absorption of FP in inflamed skin was due to binding to serum proteins leaching in the tissue. Such a combination of *in vivo* experiments and a model skin system is useful for understanding complex phenomena in inflamed and damaged skin and reduces experimental animal use.

Key words skin absorption; model skin system; inflamed skin; protein binding; flurbiprofen

Although permeation through the stratum corneum is the rate-limiting step for transdermal absorption of drugs, the kinetics of drug transfer in the viable epidermis and dermis could influence the pharmacological effects of a drug systemically as well as locally. Prolonged retention of the drug in the epidermis could delay onset of the therapeutic effect and/or cause undesirable side effects in the local tissue. Binding of drugs to tissue components in the skin could relate to retention of the drugs. In the case of inflamed skin, the leaching of plasma proteins with drug-binding ability could also affect the disposition of the drugs in local tissue.[1,2] In the drug of low protein binding ratio, the effect of plasma proteins leached into the local tissue will be small, the drug of high protein binding ratio are conversely susceptible to leach of plasma proteins. Although most skin formulations are intended for sound skin without injury or inflammation, prolonged or repeated application can bring about skin inflammation; also, some topical preparations are applied specifically to treat skin with inflammation. Therefore, studies on drug disposition in inflamed skin are important for safe and effective application of topical drugs.

Excised animal skin is usually used for *in vitro* permeation studies in the early stages of the formulation development of new products. However, *in vitro* experimental systems using such membranes are not suitable for evaluating the drug concentration profiles in skin because of the lack of local blood flow and physiological response.[3—5] Various experimental designs, including *in situ* skin permeation experiments and *in vitro* isolated skin perfusion experiments, have been developed to study the disposition of drugs in skin after topical application.[6—8] Although such experimental systems provide new knowledge about factors in the uptake of drugs into the blood flow in the skin, large numbers of experimental animals are required and experience surgical operation stress. Reduction of the use of experimental animals is an important issue today. Well-designed alternatives to animal testing and experimentation are valuable not only to reduce the use of experimental animals but also to allow better control of experimental conditions. Some types of artificial membranes have been investigated as alternatives to skin.[9—11] The development of a new skin-mimicking *in vitro* experimental system as an alternative experimental technique that can artificially create local inflammation would be useful for basic studies of the absorption of drugs through the inflamed skin.

In our previous work, a skin-mimicking artificial membrane—consisting of a silicone membrane acting as a model stratum corneum and laminated dialysis membranes acting as a model of viable skin—was prepared to imitate the layered structure of skin.[2,12] The permeability of flurbiprofen (FP), selected as a model drug, through the laminated membranes was tested to confirm the validity of this experimental system.[2,12] The FP concentration in the laminated membranes determined by microdialysis was similar to that calculated according to Fick's law of diffusion. When a solution of bovine serum albumin (BSA) was placed between the dialysis membranes to mimic protein leaching in skin, the FP concentration–time profiles in a region between the membranes showed a dependence on the site of the inserted BSA, whereas the FP permeation profile through the membranes was independent of the site. In addition, when ketoprofen was introduced into the microdialysis probe to inhibit the BSA binding of FP during the permeation process, the FP permeation through the laminated membranes and the free concentration of FP in BSA solution in the laminated membranes were increased, suggesting a competitive interaction of FP and ketoprofen involving protein binding.[2] These results suggest that the skin-mimicking laminated membranes would be useful for evaluating drug permeation quantitatively and imitating some events that can happen in inflamed skin tissues without requiring the use of animals.

The purpose of the present study is to investigate that the simple structure of the skin-mimicking laminated membranes is able to mimic the skin conditions complicated in inflamed and damaged skin. In this study, the absorption of FP, used as

* To whom correspondence should be addressed. e-mail: soshima@josai.ac.jp

a model drug, through inflamed skin was examined in an *in vivo* experiment and in a modified skin-mimicking laminated membrane system used as a model skin system.[13—15] The modified skin-mimicking laminated membranes have 2 microdialysis probes. One is for determination of FP concentration between the laminated membranes, and the other is used as model vessel to remove FP permeating among the laminated membranes. In the *in vivo* experiments, λ-carrageenan, zymosan, and casein were first injected intracutaneously to induce inflammation.[16] Then, FP was applied to normal and inflamed skin, and the plasma concentration of FP was determined to determine FP uptake into the blood flow. In the case of the model skin system, BSA solution was placed between the dialysis membranes to mimic protein leaching in inflamed skin.[17] The microdialysis probe placed beside the BSA solution was perfused at relatively high rate, and the migrating rate of FP *via* the probe was regarded as the systemic absorption in this skin model. Because FP is a typical ligand for serum albumin site II,[18,19] the disposition of FP will be influenced by the existence of the plasma protein in the absorption process after topical application.[13]

MATERIALS AND METHODS

Materials FP and BSA (Fraction V) were purchased from Sigma (St. Louis, MO, U.S.A.). Evans blue (EB), λ-carrageenan, zymosan, and casein were purchased from Wako Pure Chemical Industries (Osaka, Japan). Carboxymethylcellulose (CMC, 500—800 mPa·s) was a product of Daicel Chemical Industries Ltd. (Osaka, Japan). Dialysis fiber (OP-100-10) was purchased from Eicom (Kyoto, Japan). Silicone membranes (50 μm thickness) and dialysis membranes (SpectraPor 7, molecular weight cut-off [MWCO]=1000, 50 μm thickness) were purchased from AS ONE (Osaka, Japan) and Funakoshi (Tokyo, Japan), respectively. All other chemicals were reagent grade.

Induction of Skin Irritation in Hairless Rats Animal studies were performed according to the guidelines for animal use approved by the Life Science Research Center, Josai University. Male WBN/ILA-Ht hairless rats (230—260 g, Life Science Research Center, Josai University, Saitama, Japan) were anesthetized by intraperitoneal injection of urethane (1.5 g/kg), and a mixed solution of λ-carrageenan (0.5%), zymosan (1.0%), and casein (1.0%) in 0.2% CMC saline solution was intracutaneously injected (50 μL) through the back skin to induce inflammation.[16] In order to confirm the induction of inflammation, EB saline solution (1.0%) was preliminarily injected (1.0 mL) intravenously, and the mixed solution was intracutaneously injected 5 min after the EB administration. The treated skin was excised 30 min after the induction, and the skin was stamped out with a punch (d=15 mm). The skin samples were kept at −20°C in a freezer until determination of EB content. In order to extract EB from the skin samples, the skin was kept in 0.5 M KOH (7.0 mL) at 37°C for 24 h, and then a mixed solution of 2.02 M H_3PO_4 : acetone=3.5 : 32.5 (18 mL) was added to the resulting suspension. The mixture was kept at 25°C for 10 min and then centrifuged (25°C, 3000 rpm, 15 min). The supernatant was analyzed for absorbance at 620 nm using a spectrophotometer (U-3000, Hitachi High Technologies, Tokyo, Japan).

***In Vivo* Skin Absorption of FP in the Inflamed Skin** The hairless rats were anesthetized by intraperitoneal injection of urethane (1.5 g/kg), and the hair of the back skin was removed using an electric razor. A circular area (d=25 mm) was marked on the skin to indicate the drug application area, and a mixed solution of λ-carrageenan, zymosan, and casein was injected into the skin tissue below the application area through the outside of the area. The solution was injected from 4 different directions (50 μL each), and then the each injected hole was closed using Aron alpha A® (Daiichi Sankyo, Tokyo, Japan) to avoid undesirable absorption *via* the holes. A commercial FP product (ZEPOLAS®pap, Mikasa Seiyaku, Tokyo, Japan) was cut out to fit the application area and applied 30 min after the mixed solution injection. Blood samples were withdrawn from the jugular vein at predetermined times for 8 h, and the plasma concentration of FP was determined using an HPLC system.

The HPLC system consisted of a pump (LC-10AD_{VP}, Shimadzu Corp., Kyoto, Japan), an auto injector (SIL-10A, Shimadzu Corp.), a column oven (CTO-10A, Shimadzu Corp.), a column (Mightysil® C18 5 μm, 4.6×250 mm, Kanto Chemical, Tokyo, Japan), and a fluorescence detector (RF-10A_{XL}, Shimadzu Corp.).[7] A mobile phase consisting of 3 : 2 (v/v) acetonitrile: 0.1% phosphoric acid solution was used for the elution. The flow rate was 1.0 mL/min, the column temperature was 35°C, and the detector was operated at an excitation/emission wavelength of 250/310 nm. The plasma sample (50 μL) was mixed with acetonitrile (150 μL), and the mixture

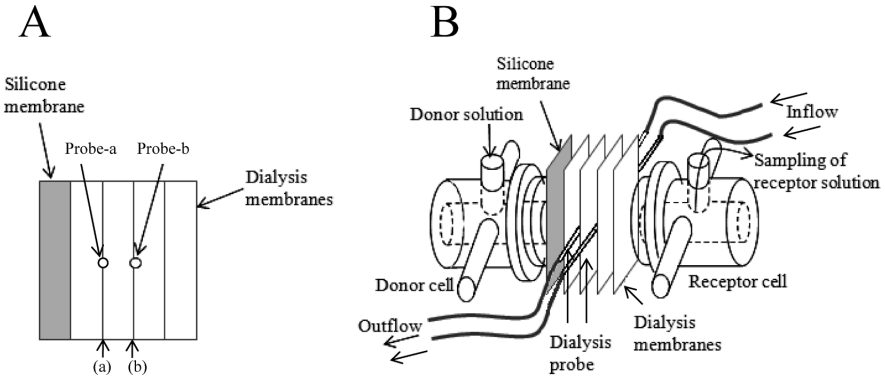

Fig. 1. Model Skin System Schematic

(A) A silicone membrane (the model stratum corneum) and 4 dialysis membranes (the model of the viable skin layer) were laminated, and microdialysis probes were placed in (a) and (b). (B) The laminated system was mounted in the diffusion cell, and the probes were perfused using a syringe pump.

was centrifuged (11000 rpm, 10 min). The resulting supernatant (50 μL) was injected to the HPLC system.

Laminated Model Skin System The skin-mimicking laminated artificial membranes experimental system (model skin system) was prepared by laminating a silicone membrane to act as a model stratum corneum and using 4 dialysis membranes to act as a model of the viable skin layer (Fig. 1). Two microdialysis probes were placed at (a) and (b) in Fig. 1A. The probe placed at (a) (probe-a) was used to determine the FP concentration near (a) using a zero-net flux method; the flow rate was fixed at 1.0 μL/min. The other probe (probe-b) was used as a model vessel, and the flow rate was set higher. The laminated membranes were mounted in a 2-chamber glass diffusion cell (effective diffusion area=0.79 cm^2, Fig. 1B).[2,12]

In this system, drug uptake into probe-b was considered systemic absorption, whereas transport into the receptor cell corresponded to migration to subcutaneous tissues. The steady state total flux (F_{tot}) through the system was calculated as the sum of the flux for uptake into the probe-b (F_{sys}) and the flux for migration into the receptor cell (F_{sub}), as shown in following equation:

$$F_{tot} = F_{sys} + F_{sub} \quad (1)$$

Determination of Uptake Clearance for Probe-b The silicone membrane and probe-a were removed from the model skin system, and the remaining 4 dialysis membranes and probe-b were mounted on the diffusion cell. The cell set was kept at 37°C, and FP solution (50 μg/mL, 2.8 mL) in an isotonic phosphate buffer (PBS; 30 mM KH_2PO_4–Na_2HPO_4 with NaCl at pH 7.4) was applied to each chamber. FP-free PBS was perfused through probe-b at 1—20 μL/min, and the concentration of FP in the outflow was determined. The uptake clearance (CL_{sys}) was calculated using Eq. 2. In order to determine the direction dependency of the value, FP-free PBS was applied to the each cell, and the FP solution was perfused through probe-b. In that case, CL_{sys} was calculated using Eq. 3.[20]

Direction=outside to inside;

$$\ln\left(1-\frac{C_O}{C_C}\right)\times(-Q) = CL_{sys} \quad (2)$$

Direction=inside to outside;

$$\ln\left(\frac{C_O}{C_I}\right)\times(-Q) = CL_{sys} \quad (3)$$

where Q is the perfusion rate (μL/min), and C_C, C_I, and C_O are the FP concentration (μg/mL) in the cell, inflow, and outflow, respectively.

Permeation Experiment with the Model Skin System The whole cell set was kept at 37°C, and FP solution (500 μg/mL, 2.8 mL) in PBS and FP-free PBS (used as a receptor medium) were applied to each chamber. Samples of medium (200—2000 μL) in the receptor chamber were removed to determine the concentration of FP at predetermined times and replaced with fresh PBS to keep the volume constant. The sampling volume was chosen to keep the FP concentration in the receptor chamber low enough to maintain sink conditions. Simultaneously, probe-a and probe-b were perfused. The perfusion rate through probe-b was 10 μL/min, and the outflow was collected in tubes. The tubes were changed every 30 min. By contrast, probe-a was used for determination of the FP concentration in the area (a) using a zero-net-flux method; the perfusion rate through probe-a was 1 μL/min. In the zero-net-flux method, the difference between C_O and C_I ($\Delta C = C_O - C_I$) is plotted against C_I, and the C_I at $\Delta C = 0$ is considered to be the free fraction concentration of the drug in the objective area.[21,22] In the model skin permeation experiment, the FP-free PBS (0—60 min) and the high-FP PBS solution (10 or 15 μg/mL, 90—150 min) were perfused, and the expected concentration of FP in area (a) ($C_{(a)}$) was calculated based on the zero-net-flux method. The samples at 30—60 min and 120—150 min were used for the calculation. The FP solution in PBS with the expected concentration was prepared, and the solution was perfused (180—240 min) to confirm the methodology.

In the FP determination for the *in vitro* experiments, the samples were mixed with the same volume of *p*-hydroxybenzoic acid isopropyl ester (internal standard) solution in methanol (2.5 μg/mL) as the supernatant (20 μL) and subjected to HPLC analysis.[7] The HPLC system was similar to that for the *in vivo* experiments, but a UV detector (SPD-10A, Shimadzu Corp.) was used instead of the fluorescence detector.

Quantitative Examination with the Model Skin System In the skin-mimicking model system, the systemic uptake ratio (R_{sys}) was defined as the amount of FP uptake to probe-b divided by the total FP transport, and the observed values ($R_{sys\text{-}obs}$) and expected values ($R_{sys\text{-}cal}$) were expressed as following equations:

$$R_{sys\text{-}obs} = \frac{F_{sys}}{F_{tot}} = \frac{F_{sys}}{F_{sys}+F_{sub}} \quad (4)$$

$$R_{sys\text{-}cal} = \frac{CL_{sys}}{CL_{sys}+CL_{sub}} \quad (5)$$

where CL_{sub} is clearance value of FP through the 2 dialysis membranes between area (b) and the receptor. This value can be calculated using Eq. 6[2]

$$CL_{sub} = \frac{CL_{1DM}}{2} \quad (6)$$

where CL_{1DM} is the clearance value of FP through the single dialysis membrane. The value was 3.49 μL/min in a preliminary experiment.

Absolute values for the FP concentration and transport were also calculated in the model skin system. First, the FP concentration in area (b) ($C_{(b)}$) was calculated using Eq. 7.[2]

$$C_{(b)} = \frac{F_{sub}}{CL_{sub}} \quad (7)$$

Because the FP concentration in the laminated membranes was similar to that calculated according to Fick's law of diffusion.[2] The calculated total flux ($F_{tot\text{-}cal}$) was calculated using $C_{(a)}$ and $C_{(b)}$ based on Eq. 8.

$$F_{tot\text{-}cal} = CL_{1DM}(C_{(a)} - C_{(b)}) \quad (8)$$

The calculated flux values for systemic and subcutaneous transport ($F_{sys\text{-}cal}$ and $F_{sub\text{-}cal}$) were then obtained using following equations:

$$F_{sys\text{-}cal} = CL_{sys} \times C_{(b)} \quad (9)$$

$$F_{sub\text{-}cal} = F_{tot\text{-}cal} - F_{sys\text{-}cal} \quad (10)$$

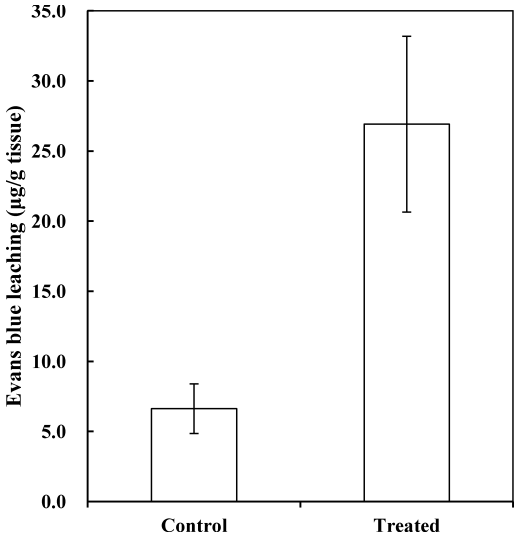

Fig. 2. Induction of Skin Inflammation in Hairless Rats

An EB saline solution (1.0%) was first injected (1.0 mL) intravenously, and a mixed solution of λ-carrageenan (0.5%), zymosan (1.0%), and casein (1.0%) was intracutaneously injected 5 min after the EB administration. The treated skin was excised 30 min after the induction. Each data set is the mean±S.D. (n=11).

Mimicking Protein Leaching in the Model Skin System Initially, BSA solution (5% in PBS, 20 μL) or PBS (20 μL) was placed in area (b). The whole cell sets were kept at 37°C, and FP solution (500 μg/mL, 2.8 mL) in PBS or PBS alone was applied to each chamber. The FP concentration in the receptor medium and the outflow from probe-b were determined at predetermined times.

RESULTS

Induction of Inflammation in Rat Skin The mixed solution of λ-carrageenan, zymosan, and casein was injected into the skin to induce inflammation. Figure 2 shows the EB leaching in the local tissue. The higher value in the treated skin shows the increased permeability of the vascular wall and the leaching of albumin into the extracellular space of the tissue.

Absorption of FP in Inflamed Skin The FP cataplasm was applied to the normal or treated skin. Figure 3 shows the plasma FP concentration of FP after skin application. In the normal (control) skin, the FP concentration rose with a short lag time and then increased, initially fast and then gradually, to reach the steady state. In the application to the inflamed skin, by contrast, the lag time for FP absorption was longer, and FP concentration was lower. If the inflammation induces an increased blood flow rate, increased permeability of vessel wall, and damage in barrier function of the stratum corneum, the systemic absorption of drugs after skin application should be enhanced. But this is not the case in the present results. Therefore, the suppressed absorption in the inflamed skin was considered to indicate the importance of other changes in the inflamed skin. In particular, we focused here on the protein binding of FP and examined the effect of protein leaching using the model skin system.[2,12]

Effect of Flow Rate on Uptake Clearance of the Microdialysis Probe In order to determine the flow rate through microdialysis probe-b—the model vessel in the skin model system—the effect of the flow rate on the uptake clearance of the microdialysis probe (CL_{sys}) was examined in both directions using Eqs. 2 and 3. Figure 4 shows the flow rate dependency of the CL_{sys} values. At a low flow rate, the CL_{sys} values were 0.45—0.50 μL/min without any direction dependency. As the flow rate increased, the CL_{sys} values calculated by Eq. 2 decreased, whereas those calculated by Eq. 3 increased, suggesting a different flow rate dependency of CL_{sys} at high flow rates. The percent recovery of out-flowing solution (volume%) through the probe was 99.5±0.5, 99.8±0.9, 96.0±1.9, 94.9±0.2, and 94.0±1.0 at 1, 2, 5, 10, and 20 μL/min inflow rates, respectively, suggesting that a high flow rate could induce leakage of the solution and that the water transport *via* the probe wall could affect the CL_{sys} values. Because blood flow can vary in different vessel types, parts of skin, and physiological conditions, the flow rate in the model system cannot correspond directly with *in vivo* conditions. In this study, we chose a flow rate of 10 μL/min through probe-b to avoid the effect of water transport on the CL_{sys} value of FP. There was no significant difference between the CL_{sys} values for the different directions at 10 μL/min, whereas there was a significant difference (t-test, $p<0.05$) at 20 μL/min. This value was 10 times the value through probe-a that was used in the zero-net-flux method to determine the free-fraction of FP concentration in area (a).

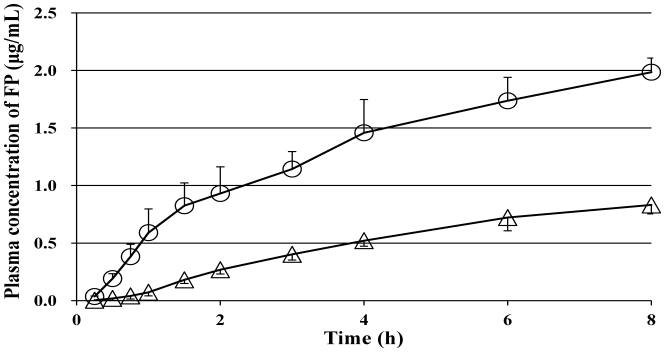

Fig. 3. FP Plasma Concentration after Application of the FP Product to Hairless Rats with or without Skin Inflammation

ZEPOLAS®pap was applied to a circular area (d=25 mm) of a hairless rat skin 30 min after the intracutaneous injection of a mixed solution of λ-carrageenan, zymosan, and casein. Symbols: ○, no injection (control); △, injection of the mixed solution (inflamed skin). Each data set is the mean±S.D. (n=3).

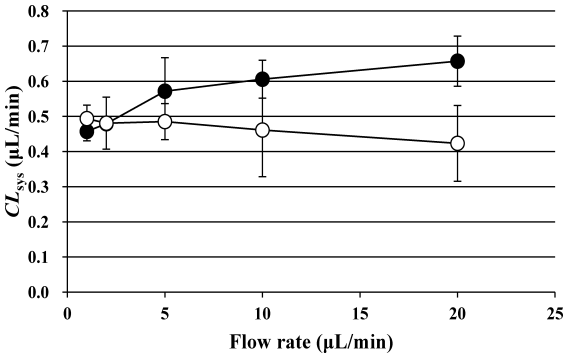

Fig. 4. Effect of Perfusion Rate in Microdialysis Probe on the Permeation Clearance of FP

Symbols: ○, values calculated using Eq. 2, direction=outside to inside; ●, values calculated using Eq. 3, direction=inside to outside. Each data set is the mean±S.D. (n=3).

Table 1. Expected and Calculated FP Concentrations in the Steady-State Transport through the Model Skin System

FP concentration (μg/mL)				
Area (a)			Area (b)	Concentration gradient[e]
Expected[a]	Outflow from probe-a[b]	ΔC[c]	Calculated[d]	
5.27±0.39	5.25±0.28	−0.01±0.18	3.49±0.46	−1.78±0.60

a) The zero-net-flux method was used for the prediction (see text). *b*) Solutions with the expected FP concentrations were perfused at 180—240 min, and the outflow was collected (210—240 min) to confirm the prediction made by the zero-net-flux method. *c*) A value near 0 indicates the reliability of the method. *d*) The value was calculated using Eq. 7. *e*) The value is the difference between the FP concentrations in areas (a) and (b).

Table 2. Evaluation of Systemic Uptake in the Model Skin System

CL_{sys} (μL/min)	CL_{sub}[a] (μL/min)	$R_{sys\text{-}cal}$[b] (%)	F_{sys} (ng/min)	F_{sub} (ng/min)	$R_{sys\text{-}obs}$[c] (%)
0.461±0.133	1.75	20.8	0.85±0.16	6.11±0.80	12.1±1.3

a) The value was calculated using Eq. 6. *b*) The value was calculated using Eq. 5. *c*) The value was calculated using Eq. 4.

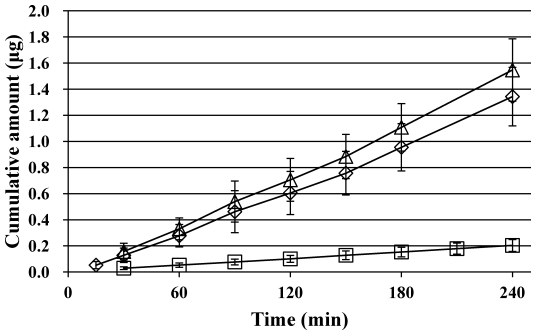

Fig. 5. FP Transport in the Model Skin System

FP solution (500 μg/mL) was applied to the silicone membrane (model stratum corneum). Symbols: □, transport *via* probe-b (hypothetical systemic absorption); ◇, transport to the receptor cell (hypothetical subcutaneous transport); △, total transport. Each data set is the mean±S.D. (n=3).

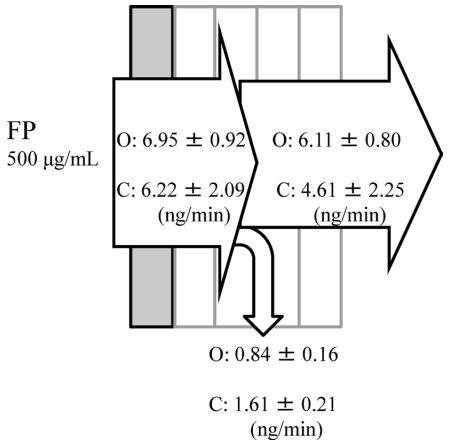

Fig. 6. Steady State Mass Transport of FP in the Model Skin System

"O" indicates observed values, and "C" indicates values calculated using Eqs. 8—10.

Quantitative Evaluation of FP Transport in the Model Skin System The FP solution (500 μg/mL) was applied to the donor cell (on the silicone-membrane side representing the stratum corneum), and both microdialysis probe-a and probe-b were perfused. Figure 5 shows the mass transfer of FP in the model system. The mass transfer *via* probe-b—the hypothetical systemic absorption—was smaller than that to the receptor cell—the hypothetical subcutaneous transport. The total mass transfer is the sum of both types of transport. The mass transfer *via* probe-a was 0.13±0.10 μg/min and was ignored in the calculation of the total amount. Table 1 shows the FP concentration in areas (a) and (b) as calculated using the zero-net-flux method and Eq. 7.[2,12] In the zero-net-flux method, solutions with the expected FP concentrations were perfused for 180—240 min, and the outflow was collected (210—240 min) to confirm the methodology. The mean FP concentration in the outflow was close to 0, suggesting that the method is reliable. Table 2 shows the observed and calculated ratios of uptake to probe-b (the hypothetical systemic absorption). The ratio calculated from the CL values of the microdialysis probe and the double dialysis membranes was higher than the observed ratio. Figure 6 shows the observed and calculated mass transfer of FP in the model skin system. The calculated values were based on the concentration gradient between areas (a) and (b). Although the calculated uptake to probe-b was over-estimated, the good agreement for the observed and calculated total mass transfer suggests that the FP concentrations estimated using the zero-net-flux method and Eq. 7 were correct and that the model skin system is a useful experimental tool for basic studies on skin absorption of drugs.

Effect of BSA on the Mass Transfer of FP in the Model Skin System In order to examine the effect of protein leaching into the tissue on the transfer of FP in the absorption process, BSA solution was placed in area (b) of the laminated membranes in the model skin system (Fig. 1). Figure 7 shows the FP permeation to the receptor cell (A) and the uptake into probe-b (B). FP permeation into the receptor cell in the BSA system was suppressed in the initial phase, but there was no significant difference from the control in the steady-state permeation rate of FP. The uptake into probe-b was also suppressed by the BSA. Such delayed absorption of FP must be due to the binding of FP to BSA. The placement of BSA solution was limited to area (b) in the model skin system, whereas the protein leaching occurred throughout the skin of the application area. That might explain why the suppressed absorption was observed only in the initial phase in the model skin system. The uptake rate of FP into probe-b was decreased in the later phase both with and without the placement of BSA solution in area (b). Condition of the area placed the probe and the solution might change with time. Several possibilities are considered, for example, the interaction of the FP and BSA in area (b) reached saturation, and the concentration of FP in

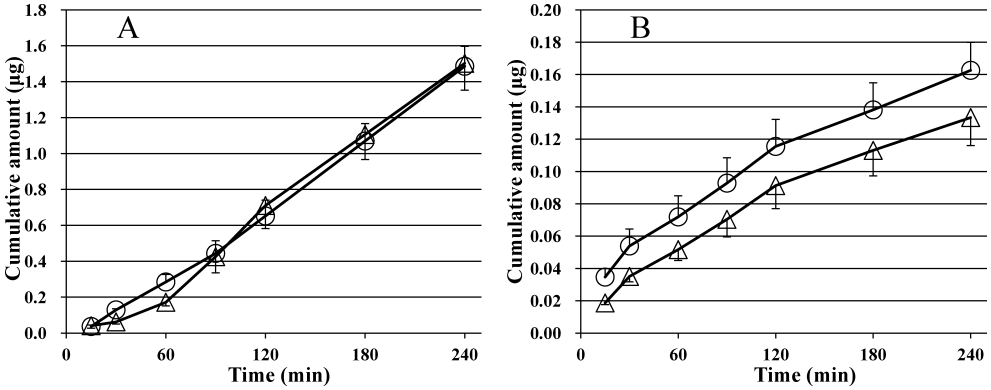

Fig. 7. Effect of BSA Solution Placed in Area (b) on the Transfer of FP in the Model Skin System

(A) transfer to the receptor cell; (B) uptake into probe-b. Symbols: ○, PBS (20 μL) placed in area (b); △, 5% BSA in PBS (20 μL) placed in area (b). Each data set is the mean±S.D. (n=3).

PBS reached to steady-state.

DISCUSSION

Although most skin formulations are intended for sound skin without injury or inflammation, formulations for skin diseases and topical inflammation are often applied to damaged skin.[1] Therefore, studies on drug disposition in inflamed skin are important for the safe and effective application of drugs to such skin. In inflamed skin, damaged barrier functionality of the stratum corneum, varied blood flow rates, increased permeability of vessel walls and tissue fluids, and leaching plasma proteins with drug binding ability could all affect drug disposition in the local tissue. In this study, skin absorption of FP through inflamed skin was examined in both *in vivo* experiments and a model skin system. In the *in vivo* experiments, λ-carrageenan, zymosan, and casein were preliminarily injected intracutaneously to induce inflammation, and the plasma concentration of FP was calculated in order to determine FP uptake into the blood flow after FP application to normal and inflamed skin. In the model skin system, a BSA solution was placed between the laminated dialysis membranes to mimic protein leaching in inflamed skin.

The FP plasma concentration after the application of ZEPOLAS®pap, a commercial FP cataplasm product, was suppressed in the rats with inflamed skin. Because increased blood flow rate, increased permeability of vessel wall, and damaged barrier functionality of the stratum corneum in the inflamed skin could all enhance the systemic absorption of FP after skin application, the suppressed absorption considered to be due to decreased local blood flow and/or increased binding of FP in the local area. We then examined the effect of protein binding on the transfer of FP in the permeation process in the model skin system.[2,12] A suppressed permeation of FP into the receptor cell and a delayed uptake into probe-b were observed in the model skin system with the inflammation-mimicking BSA solution between the laminated membranes. This model skin system can be considered useful for the study of factors affecting disposition of drugs in skin with various conditions.

This study was limited in several ways. In this work, the MWCO of the microdialysis probes was 50 kDa, and BSA could not be permeate the membrane. Thus, only the unbound fraction of FP could be taken out *via* the probes. However, in inflamed skin, the permeability of serum proteins through vessel walls increases, and drugs bound to serum proteins can migrate into the blood flow. Such migration must not be ignored to fully understand drug disposition in inflamed skin.[13] To mimic such migration, microdialysis probes with higher MWCOs (such as 1000 kDa) could be used for probe-b in the model skin system.[23] Such increased permeability of the vessel and probe wall could cause fluid migration *via* the wall. As shown in Figure 4, such fluid migration can also affect drug disposition in the local area. A combination system of a push pump (syringe pump) and a pull pump (perista pump) is available for microdialysis systems and uses high-permeability probes to control the fluid migration. In this study, using only the push pump, the flow rate through probe-b was limited to avoid fluid migration. If the combined pump system were used in the model skin system, the effects of the flow rate on the R_{sys} values could be examined over a wide range; the results would be helpful in understanding the effects of blood flow rate on the systemic absorption of drugs after topical application.[5] In addition, drugs bound with serum proteins can diffuse into the extracellular space of the inflamed tissue, whereas the protein-binding FP in the model skin system was kept in area (b) because of the low MWCO of the laminated membranes. Such a property of the model skin system might be good for simplification of drug disposition in local tissues with inflammation. If there is a need to mimic diffusion of protein-binding drugs in local tissues, hydrogels, such as agar gel, could be used as the model dermis.

In order to understand drug disposition in inflamed tissues after topical application, well-designed *in vivo* experiments are needed because of the complex physiological response involved in inflammation. Such complex *in vivo* phenomena, however, are difficult to understand fully. Some *in situ* and *in vitro* experimental methods for the study of drug disposition in inflamed tissue after skin application have been reported and provide valuable information about influencing factors in the phenomena.[6—8] These experimental methods, including *in vivo* methods, require the sacrifice of experimental animals. Yet there is pressure from society to reduce the use of experimental animals, and alternative methods to animal experiments are needed. Because physiological responses in medical conditions are complicated, alternative methods must be focused on specific phenomena. In this study, the model

skin system was focused on the protein binding of FP in the permeation process, and the permeation into the receptor cell and uptake into the microdialysis probe were examined in the inflammation-mimicking system. The model skin system is expandable and has the capability to recreate events involved in the complicated physiological responses of inflamed skin. Such modified model systems would be useful in understanding drug disposition in local tissue after skin application and would also reduce the use of experimental animals.

CONCLUSION

In this study, the skin absorption of FP, a highly protein-binding drug, through inflamed skin was examined in both *in vivo* experiments and a skin-mimicking model system. In the model skin system, microdialysis probes were used to determine FP concentration between the laminated membranes and to act a model vessel to mimic systemic uptake *via* local blood flow. In the *in vivo* experiments, suppressed FP absorption was observed in the rats with inflamed skin. The experiments using the model skin system suggested that the delayed absorption of FP in the inflamed skin was due to FP binding to serum proteins leaching in the tissue. Because skin conditions are variable and factors related to drug disposition are complicated in inflamed and damaged skin, the combination of *in vivo* experiments and well-designed model systems, as done in this report, is useful to understand the complex phenomena and to reduce experimental animal use.

Acknowledgments This work was supported by Japan Society for the Promotion of Science, KAKENHI (23590197).

REFERENCES

1) Ortiz PG, Hansen SH, Shah VP, Menné T, Benfeldt E. The effect of irritant dermatitis on cutaneous bioavailability of a metronidazole formulation, investigated by microdialysis and dermatopharmacokinetic method. *Contact Dermat.*, **59**, 23—30 (2008).
2) Oshima S, Sashida T, Egawa Y, Hosoya O, Juni K, Seki T. Study of a drug interaction involving the permeation process using an artificial skin model as an alternative to animal skin. *AATEX*, **15**, 97—105 (2010).
3) Roberts MS, Cross SE. Percutaneous absorption of topically applied NSAIDS and other compounds: role of solute properties, skin physiology and delivery systems. *Inflammopharmacology*, **7**, 339—350 (1999).
4) Cevc G., Vierl U. Spatial distribution of cutaneous microvasculature and local drug clearance after drug application on the skin. *J. Control. Release*, **118**, 18—26 (2007).
5) Clough GF, Boutsiouki P, Church MK, Michel CC. Effects of blood flow on the in vivo recovery of a small diffusible molecule by microdialysis in human skin. *J. Pharmacol. Exp. Ther.*, **302**, 681—686 (2002).
6) Sugibayashi K, Yanagimoto G, Hayashi T, Seki T, Juni K, Morimoto Y. Analysis of skin disposition of flurbiprofen after topical application in hairless rats. *J. Control. Release*, **62**, 193—200 (1999).
7) Yanagimoto G, Hayashi T, Seki T, Juni K, Sugibayashi K, Morimoto Y. A new *in-situ* experimental method to evaluate the cutaneous disposition of flurbiprofen after topical application in hairless rats. *Pharm. Pharmacol. Commun.*, **4**, 261—265 (1998).
8) Seki T, Hosoya O, Yamazaki T, Sato T, Saso Y, Juni K, Morimoto K. A rabbit ear flap perfusion experiment to evaluate the percutaneous absorption of drugs. *Int. J. Pharm.*, **276**, 29—40 (2004).
9) de Jager MW, Gooris GS, Ponec M, Bouwstra JA. Lipid mixtures prepared with well-defined synthetic ceramides closely mimic the unique stratum corneum lipid phase behavior. *J. Lipid Res.*, **46**, 2649—2656 (2005).
10) Ottaviani G, Martel S, Carrupt PA. Parallel artificial membrane permeability assay: a new membrane for the fast prediction of passive human skin permeability. *J. Med. Chem.*, **49**, 3948—3954 (2006).
11) Wasdo SC, Juntunen J, Devarajan H, Sloan KB. A correlation of flux through a silicone membrane with flux through hairless mouse skin and human skin *in vitro*. *Int. J. Pharm.*, **373**, 62—67 (2009).
12) Seki T, Sashida T, Oshima S, Egawa Y, Nakagawa H, Hosoya O, Juni K. Novel experimental system as an alternative to animal experiments to study the skin permeation of drugs. *AATEX*, **14**, 887—897 (2009).
13) Morimoto Y, Hayashi T, Kawabata S, Seki T, Sugibayashi K. Effect of L-menthol–ethanol–water system on the systemic absorption of flurbiprofen after repeated topical applications in rabbits. *Biol. Pharm. Bull.*, **23**, 1254—1257 (2000).
14) Sugawara S, Ohno H, Ueda T, Ishigami M, Fujiwara E, Hashimoto S, Sato H, Yamazaki K. Studies on drug concentration in the articular fluid and other tissues of patients undergoing application of flurbiprofen for external use (FP-A). *Ther. Res.*, **6**, 289—294 (1987).
15) Ueno K, Kitagawa H. Characteristics in percutaneous absorption and preparations of flurbiprofen-containing patches, and analysis of factors affecting oragnisms. *Ther. Res.*, **3**, 1078—1085 (1985).
16) Tanaka M, Futaki N, Takahashi S, Yokoyama M, Yamakawa Y, Higuchi S. Anti-inflammatory activities of TS-410, a topical anti-inflammatory steroid (2nd report). *Clinical Pharmacology and Therapy*, **5**, 1599—1605 (1995).
17) Evrard PA, Cumps J, Verbeeck RK. Concentration-dependent plasma protein binding of flurbiprofen in the rat: an *in vivo* microdialysis study. *Pharm. Res.*, **13**, 18—22 (1996).
18) Zhang Q, Huang Y, Zhao R, Liu G, Chen Y. Determining binding sites of drugs on human serum albumin using FIA-QCM. *Biosens. Bioelectron.*, **24**, 48—54 (2008).
19) Borgå O, Borgå B. Serum protein binding of nonsteroidal anti-inflammatory drugs: a comparative study. *J. Pharmacokinet. Biopharm.*, **25**, 63—77 (1997).
20) Seki T, Wang A, Yuan D, Saso Y, Hosoya O, Chono S, Morimoto K. Excised porcine skin experimental systems to validate quantitative microdialysis methods for determination of drugs in skin after topical application. *J. Control. Release*, **100**, 181—189 (2004).
21) Kehr J. A survey on quantitative microdialysis: theoretical models and practical implications. *J. Neurosci. Methods*, **48**, 251—261 (1993).
22) Whitehead KJ, Manning JP, Smith CGS, Bowery NG. Determination of the extracellular concentration of glycine in the rat spinal cord dorsal horn by quantitative microdialysis. *Brain Res.*, **910**, 192—194 (2001).
23) Hotta H, Uchida S, Kagitani F. Stimulation of the nucleus basalis of Meynert produces an increase in the extracellular release of nerve growth factor in the rat cerebral cortex. *J. Physiol. Sci.*, **57**, 383—387 (2007).