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A rabbit ear flap perfusion experiment to evaluate the percutaneous absorption of drugs

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Abstract

A rabbit ear flap single-pass perfusion system was examined as an experimental method for studying the relationship between the physiological conditions of tissues and drug disposition after topical applications. Tyrode solutions containing bovine serum albumin (BSA) and sucrose or flurbiprofen (FP), used as a model drug, were perfused through the vessel in the ear flap to evaluate the physiological conditions prior to the application of FP to the skin surface. The extracellular volume and distribution properties of FP in the perfused ear were similar to those in an in vivo experimental system. In addition, the perfused ear flap exhibited a pharmacological response to bradykinin (BK). The amount of FP in the outflow Tyrode solution containing BSA after application to the skin surface of the perfused ear decreased with the addition of BK, while that in the tissues under the application site increased. FP binds to BSA, which leaked from the intravascular space, and could be retained in the tissues under the application site. The protein binding also affected the redistribution of FP to other tissues in the ear flap after application to the skin. The rabbit ear perfusion system is a useful method for studying the percutaneous absorption of drugs especially variations in the disposition of drugs in oedematous tissues.

Keywords: Rabbit ear; Perfusion; Flurbiprofen; Bradykinin; Topical application; Protein binding

1. Introduction

There are two types of topical formulations for drugs applied to skin according to their target site of action. One is for systemic action of drugs which are absorbed from the cutaneous microvessels; the other exhibits local effects in the skin and subcutaneous tissues. The drug disposition in the skin and the uptake from the vessels are important for both types of formulations. Since in vitro skin permeation experiments through excised skin, a popular experimental method, do not give enough information about these, new experimental methods involving microdialysis have been used for the quantitative determination of drugs in skin (Touitou et al., 1998; Kreilgaard, 2001; Clough et al., 2002). The amount and rate of uptake of the drugs from the vessels could be an important factor in determining the concentration of drugs in local tissues (Cross et al., 1999; Auclatr et al., 1991; Benowitz et al., 1992). Quantitative evaluation of drug absorption from the vessels is needed to fully understand drug disposition in the skin (Cross et al., 1999;

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Sugibayashi et al., 1999; Yanagimoto et al., 1998, 1999; Morimoto et al., 2000). Vascular perfusion experiments are useful for quantitatively evaluating drug absorption in local tissues. Some perfusion experimental systems, e.g. porcine skin flaps (Williams et al., 1990; Chang et al., 1994; Riviere et al., 1996), porcine ear flaps (Lange et al., 1992), rat hindlimb (Roberts and Cross, 1999), bovine udder (Kietzmann et al., 1995; Kietzmann and Blume, 1997) and rabbit ear flaps (Celesti et al., 1992; Bast and Kampffmeyer, 1994), have been used to study transdermal drug absorption. However, the condition of the tissues, e.g. the extracellular volume and vascular permeability, under perfusion was not examined in detail in those experiments. In our preliminary studies of a rabbit ear perfusion system, edema related to leaching of bovine serum albumin (BSA) applied to the perfusate was observed and this could be avoided by addition of norepinephrine (NE) to the perfusate. This result suggests that well established experimental methods, in which the physiological conditions of perfused tissues are controlled, are needed for studying the absorption of drugs after topical applications.

In the present study, the rabbit ear perfusion experimental system was compared with an in vivo experimental system to examine the physiological conditions and the distribution properties of flurbiprofen (FP), used as a model drug (Mathy et al., 2001). FP is a nonsteroidal anti-inflammatory drug and its distribution in local tissues is important for its pharmacological effect. Although the rabbit ear is not popular for studying skin permeation of drugs, it has a good vascular system for perfusion and has been used for a long time in studies on arteriovenous anastomoses (Clark and Clark, 1932; Pollock et al., 1996), and rabbits are more popular as an experimental animal compared with pigs and cows. The pharmacological effect of bradykinin (BK), which has strong effects on the peripheral vascular system (Eisen, 1970), was also examined to evaluate the physiological response of the perfused ear flap. If the rabbit ear perfusion experimental system showed a response to BK, it would be useful for studying the disposition of drugs in oedematous tissues. In the initial part of the experiment, FP solution was perfused through the ear flap to evaluate the physiological conditions. In the second part, FP gel was applied to the skin to evaluate the usefulness of the experimental system.

2. Materials and methods

2.1. Materials

FP and NE were purchased from Sigma Chemical Co. (St. Louis, MO). Evans blue (EB), sucrose, inulin and hydroxypropylcellulose (HPC, HPC-H) were purchased from Wako Pure Chemical Industries (Osaka, Japan). BSA (Fr. V) was purchased from Serologicals (IL). Dextran T40 (DexT40) was purchased from Pharmacia Biotech (Uppsala, Sweden). BK was purchased from Peptide Institute (Osaka, Japan). Other chemicals were of reagent grade.

2.2. Methods

2.2.1. Evaluation of the in vivo extracellular volume of the rabbit ear flap

Male albino rabbits (JW strain, 2.1–3.2 kg, Saitama Experimental Animals Supply, Saitama, Japan) were anaesthetised with pentobarbital (s.c., 50 mg/kg) and then an isotonic 2% inulin solution was intravenously infused to obtain a mean steady-state plasma inulin concentration of 0.5 mg/ml. Blood samples were taken from an ear vein 2.5–3.5 h after the start of infusion and then the rabbit was sacrificed using CO_2 gas according to the NIH standards as described in "Principles of Laboratory Animal Care." A piece of ear flap (3.14 cm^2, site (A) in Fig. 1) was excised and homogenised with 2 ml PBS (pH 7.4) to determine the inulin content. An aliquot of the homogenate (200 μl) was mixed with 40 μl 10% trichloroacetic acid and then the mixture was centrifuged at 12,000 × g for 3 min. The supernatant (100 μl) was mixed with 1.5% cysteine hydrochloride (20 μl), 78% sulphuric acid (600 μl) and 0.12% carbazole ethanol solution (20 μl). The mixture was kept at 40 °C for 30 min and then the absorbance at 560 nm was determined. The amount of inulin per gram of tissue was divided by the inulin concentration in the plasma to give the distribution volume of inulin in the ear flap. The distribution volume of inulin was regarded as the extracellular volume of the ear flap.

2.2.2. Procedure for rabbit ear flap single-pass perfusion experiments

Male albino rabbits were sacrificed as described in the in vivo experiment. Whole ear flaps were excised

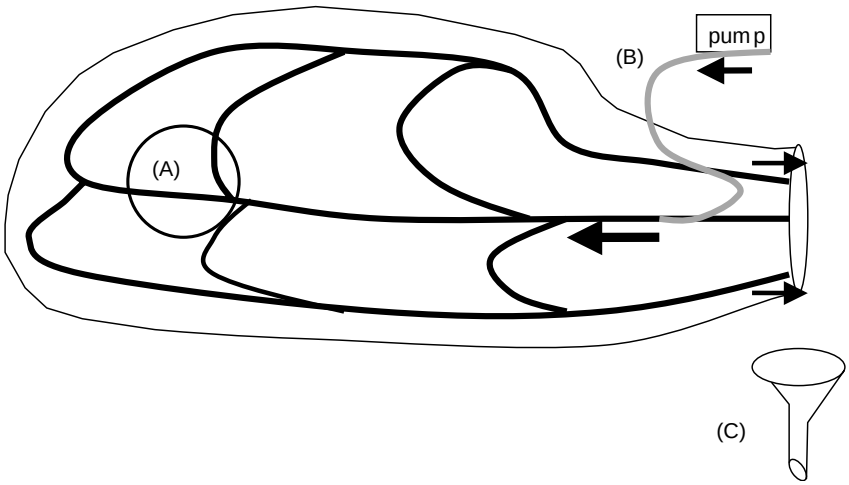

Fig. 1. Schematic diagram of rabbit ear flap for the experiments. (A) is the site for sampling in the perfusion experiments and for FP application in the skin absorption experiments. Tyrode solution was perfused at 1 ml/min via a polyethylene (B) cannula inserted into the central artery. All perfused solutions were collected using a funnel (C).

and a polyethylene (PE50) cannula was then inserted into the central artery (Fig. 1). Blood was washed out using saline solution containing heparin. The ear flap was then perfused with oxygenated (95% O_2/5% CO_2) Tyrode solution at 1 ml/min. The ear flap and Tyrode solution were kept at 37 °C in an incubator. The composition of the Tyrode solution was as follows: sodium chloride (137 mM), potassium chloride (1.5 mM), calcium chloride (1.8 mM), magnesium chloride (1.0 mM), sodium bicarbonate (12 mM), monobasic sodium phosphate (0.4 mM), D-glucose (5.0 mM), BSA (4.7%) and NE (50 ng/ml). When other additives were added, the content of sodium chloride was reduced to keep the osmotic pressure constant.

2.2.3. Evaluation of in vitro extracellular volume of the rabbit ear flap

Tyrode solution containing sucrose (0.1%) was used to determine the distribution volume of sucrose in the rabbit ear flap. The Tyrode solution was perfused for 30 or 180 min. An aliquot of the outflow was collected to check the sucrose concentration. A piece of ear flap (site A) was excised and the sucrose content was determined in the same way as for inulin. Since the sucrose concentrations in the outflow and the distribution volume of sucrose were similar at 30 and 180 min, this showed that distribution equilibrium was achieved by 30 min. The distribution volume of sucrose was regarded as the extracellular volume of the ear flap. In order to examine the effect of BK on the extracellular volume in the excised ear flap, Tyrode solution containing 0.1 μM BK was used as the perfusate (Eisen, 1970).

2.2.4. Evaluation of the in vivo distribution properties of FP in the rabbit ear flap

Rabbits were anaesthetised and then isotonic FP solution (167 μg/ml) was intravenously infused to obtain a mean plasma steady-state FP concentration of 10 μg/ml (Mathy et al., 2001). Blood samples were taken from an ear vein 2.5–3.5 h after the start of infusion and the rabbit was then sacrificed in the same way as in the inulin experiment. A piece of ear flap (site A) was excised and then homogenised with 2 ml PBS to determine the FP content. The homogenate was mixed with 50 μl of a methanolic solution of *p*-hydroxybenzoic acid isopropyl ester (50 μg/ml), as an internal standard, and 2 ml acetonitrile and then the mixture was centrifuged at 12,000 × *g* for 3 min. The supernatant (20 μl) was subjected to HPLC. The HPLC system consisted of a pump (LC-10AT, Shimadzu, Kyoto, Japan), an injector (7125, Rheodyne, CA), a stainless-steel column (4 mm × 250 mm) packed with LiChrospher 100 RP-18(e) (5 μm, Merck, Darmstadt, Germany), an oven (CTO-10AC,

Shimadzu), a UV detector (SPD-6A, Shimadzu) and an integrator (C-R5A, Shimadzu). The mobile phase consisted of 50:50 (v/v) acetonitrile:0.1% phosphoric acid solution, and the flow rate was 1.0 ml/min. The detector was operated at 245 nm. The concentration of FP in the tissue was calculated as the amount of FP in the piece of the ear flap (3.14 cm^2) divided by its weight. The partition coefficient, K_p, was defined as the concentration of FP in the tissue/concentration of FP in plasma in the in vivo experiments.

2.2.5. Evaluation of the in vitro distribution properties of FP in the rabbit ear flap

Tyrode solution containing FP (10 μg/ml) was used to evaluate the distribution of FP in the perfused rabbit ear flap. The Tyrode solution was perfused for 180 min. An aliquot of outflow was collected to check the FP concentration. A piece of ear flap (site A) was excised and the content of FP was determined in the same way as in the in vivo experiments. The partition coefficient, K_p, was defined as the concentration of FP in the tissue/concentration of FP in the outflow in the in vitro experiments.

2.2.6. Measurement of the in vitro distribution volume of EB in the rabbit ear flap

Tyrode solution containing EB (100 μg/ml) was used to evaluate the effect of BK on the vascular permeability in the perfused rabbit ear flap. EB was used as a marker of BSA. The Tyrode solution was perfused for 30 min. A piece of ear flap (site A) was excised and the content of EB was determined by measurement of the absorption at 620 nm. The equilibrium of the distribution of EB and BSA was verified by comparison of the EB concentration in the inflow and outflow. The amount of EB in the ear flap was divided by the EB concentration in the outflow to give the distribution volume of EB in the ear flap. When Tyrode solution without BK was used, the distribution volume of EB could be regarded as the intravascular volume of the ear flap.

2.2.7. Application of FP gel to the surface of the perfused ear flap

An FP gel was used for application to the surface of the perfused ear flap. The FP gel consisted of 1% FP and 3% HPC-H in PBS (Yanagimoto et al., 1998). A glass cell (available area = 3.14 cm^2) was fixed on the outer side of the ear flap (site (A) in Fig. 1). The FP gel (1.0 g) was applied to the cell 1 h after the start of perfusion and the outflow was collected every 15 min for 2 h. The FP gel and cell were removed carefully from the surface and then the surface was washed with ethanol to avoid contamination with unabsorbed FP. The stratum corneum of the surface was removed by tape stripping (20×) and then the tissue of ear flap under the site of the applied drug (site A) was excised. The content of FP in the piece of ear flap was determined in the same way as in the other experiments.

2.2.8. Evaluation of the redistribution of FP from perfusate to tissue after application of FP to the surface of the ear flap

The FP content in the rest of the ear flap after excision of the application site was determined to evaluate the redistribution of FP from perfusate to tissue in the FP gel application experiments. The ear flap was homogenised with 15 ml PBS. The homogenate was mixed with 70 ml acetonitrile and then the mixture was centrifuged at 12,000 × g for 3 min. The supernatant (20 μl) was subjected to HPLC using a fluorescence detector (RF-535, Shimadzu). The detector was operated at Ex 262 nm and Em 312 nm. The redistribution ratio (RR) was defined by the following equation:

$$\mathrm{RR} = \frac{\text{FP in rest of ear flap}}{\text{FP in perfusate} + \text{FP in rest of ear flap}}. \quad (1)$$

In order to examine the effect of protein binding on the redistribution of FP, DexT40 was added to a Tyrode solution instead of BSA. The osmotic pressure and viscosity of the Tyrode solution containing 3.95% DexT40 (288 mOsm/kg, 1.38 mPa s) were similar to that containing 4.7% BSA (286 mOsm/kg, 1.41 mPa s).

2.2.9. Determination of the diffusion coefficient of FP in the medium

In order to examine the effect of protein binding on the mobility of FP, the diffusion coefficient (D) of FP in the medium was determined by a chromatographic broadening method (CBM; Seki et al., 2003a). A stainless-steel tube (10 m, 0.8 mm i.d., Supelco Inc., PA) was fitted to the HPLC system instead of the column. Phosphate buffer (10 mM, pH 7.4) or buffer containing 0.2% BSA was used as the mobile phase. The flow rate was 0.1 ml/min, the oven was kept at 37 °C

and the detector was operated at Ex 262 nm and Em 312 nm. FP was dissolved in mobile phase (20 μg/ml) and the solution (10 μl) was injected into the system. The values of the residence time (t_R) and the eluted peak width at half height ($W_{1/2}$) were obtained from the integrator. The D values were calculated from the following equation, where r is the radius of the capillary tube (0.426 mm) determined from the calibration runs (Seki et al., 2003b):

$$D = \frac{0.231 r^2 t_R}{W_{1/2}^2}. \quad (2)$$

2.2.10. Statistical analysis

All results are expressed as means + S.E. Student's *t*-test or the Tukey–Kramer test was used to examine differences between each group.

3. Results

3.1. Comparison of the extracellular volume in the in vivo and in vitro perfusion experiments

In order to evaluate the physiological conditions of the perfused ear flap, the extracellular volume was compared first with the in vivo situation. In the case of the in vivo experiments, a constant inulin concentration (0.494 ± 0.150 mg/ml) in plasma at 2.5–3.5 h was obtained by inulin infusion (120 mg/h/kg). The extracellular volume of site (A) was 0.365 ± 0.039 ml/g (Fig. 2). On the other hand, the extracellular volume of the perfused ear flap was 0.407 ± 0.029 ml/g (Fig. 2). These results suggest that the extracellular space of the perfused ear flap remained normal for a 3-h period.

3.2. Comparison of the distribution of FP in the ear flap in the in vivo and in vitro perfusion experiments

The distribution of FP in the perfused ear flap was also compared with the situation in vivo. In the in vivo experiments, FP solution was infused (1.0 mg/h/kg) to obtain a steady-state plasma concentration of about 10 μg/ml. The FP concentrations in plasma at 2.5–3.5 h were constant and the mean value was 10.2 ± 1.3 μg/ml. The amount of FP in site (A) and the calculated K_p value were 0.297 ± 0.032 μg per 3.14 cm^2 and 0.0965 ± 0.0129 ml/g, respectively. In the case of the in vitro single-pass perfusion experiment, Tyrode solution containing 10 μg/ml of FP was perfused. The FP concentration in the outflow at 3 h was 10.1±0.7 μg/ml and this suggests that tissue equilibrium for the distribution of FP was achieved. The amount of FP in site (A) and the calculated K_p value of the perfused ear flap were 0.297 ± 0.054 μg per 3.14 cm^2 and 0.0920 ± 0.0300 ml/g, respectively. The distribution of FP in the perfused ear flap is similar to that in vivo.

3.3. Effect of BK on the perfused ear flap

BK was added to the perfusate at a concentration of 0.1 μM to examine the effect on the ear flap. This concentration is high enough for BK to affect the vascular permeability (Eisen, 1970). EB was used as a marker of vascular permeability. The vascular permeability of EB was low under normal conditions and the distribution volume of EB was equal to the intravascular volume of the ear flap. When the vascular permeability increased, EB distributed in the extracellar space. The distribution volume of EB in site (A) was 0.0608±0.0241 ml/g under control conditions (Fig. 3). When the intravascular solution in the perfused ear was removed by air, about 85% of the EB was removed. This suggests that most of the EB was distributed in the intravascular space under control conditions. On the other hand, the distribution volume of EB in the perfused ear flap was 0.514 ± 0.091 ml/g, when BK was added to the perfusate (Fig. 3). This suggests that the perfused ear flap exhibits a response to BK.

The effects of BK on the extracellular volume and the distribution of FP in the perfused ear flap were also examined (Figs. 2 and 4). When perfusate containing BK was used, the extracellular volume of site (A) was 0.485 ± 0.021 ml/g (Fig. 2). When the extracellular volume was expressed as volume/sampling area (3.14 cm^2), the volume was 321 ± 13 μl per 3.14 cm^2 and the value was significantly higher than that without BK (157 ± 5 μl per 3.14 cm^2). The edema is due to the enhanced vascular permeability produced by the pharmacological effect of BK. The amount of FP in site (A) and the K_p value also increased following the addition of BK to the perfusate (Fig. 4). Since BSA applied to the perfusate could leak from the intravascular to the extracellular space in edema, FP bound to BSA could also move to the extracellular space.

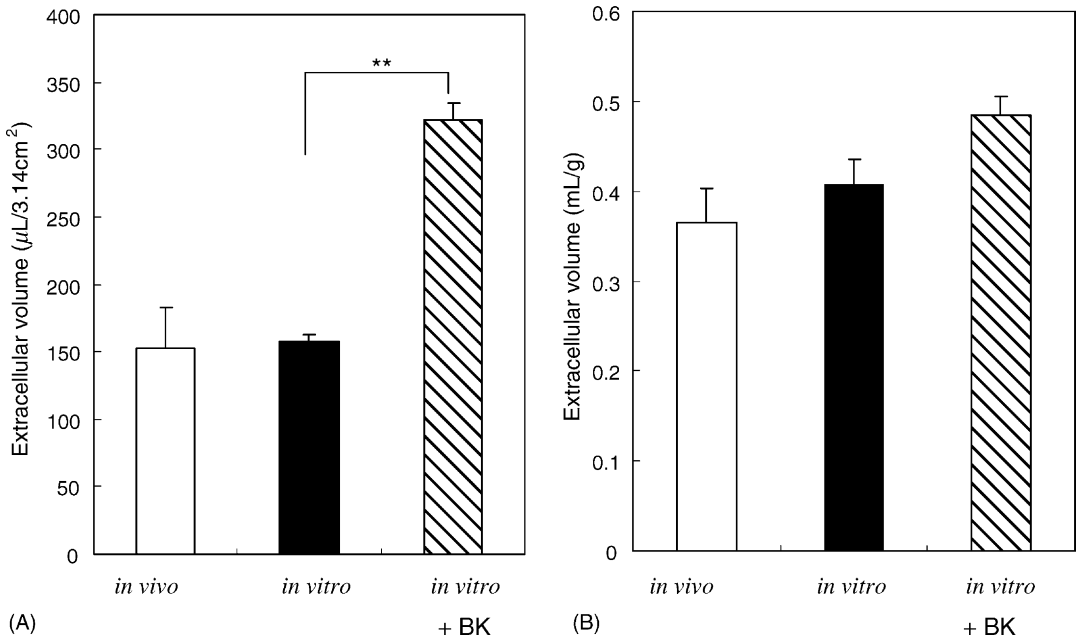

Fig. 2. Comparison of the extracellular volume in in vivo and in vitro perfusion experiments. (A) Extracellular volume of site (A) in Fig. 1; (B) extracellular volume per gram tissue. An isotonic 2% inulin solution was intravenously infused to obtain a mean steady-state inulin plasma concentration of 0.5 mg/ml. Tyrode solution containing sucrose (0.1%) and BSA (4.7%), with or without BK (0.1 μM), was perfused at 1 ml/min in the in vitro experiments. Each set of data is the mean ± S.E. ($n = 3$–4). $^{**}P < 0.01$ in Tukey–Kramer test.

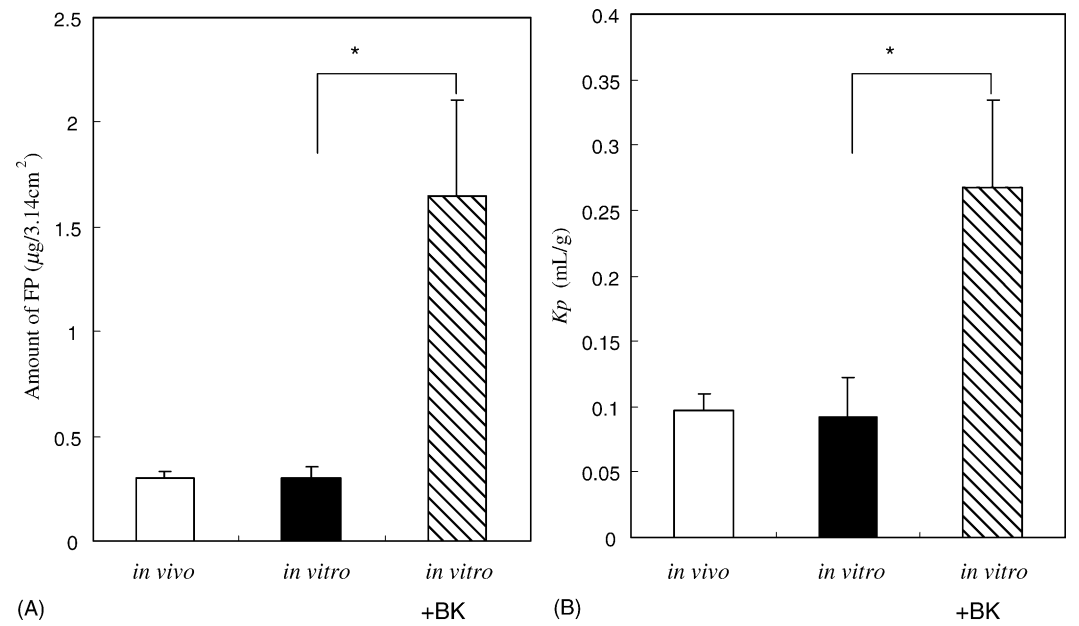

Fig. 3. Effect of BK on the distribution volume of EB in the in vitro rabbit ear perfusion experiments. Tyrode solution containing EB (100 μg/ml) and BSA (4.7%), with or without BK (0.1 μM), was perfused at 1 ml/min. Each data set is the mean ± S.E. ($n = 3$–10). $^{***}P < 0.0001$ in Student's t-test.

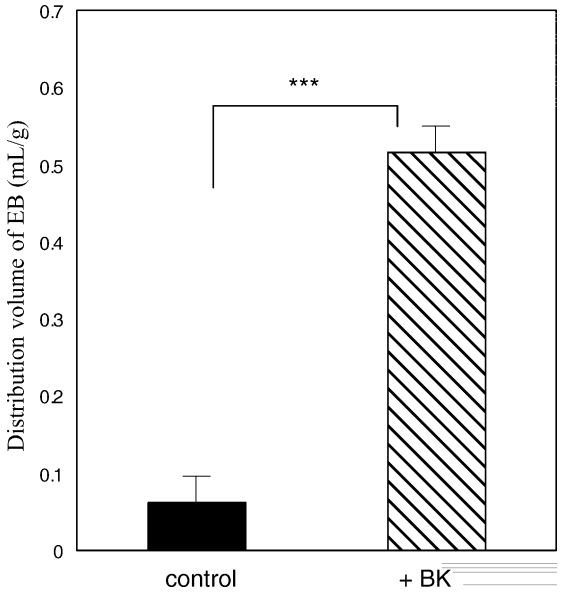

Fig. 4. Comparison of the amount distributed (A) and the K_p values (B) of FP in in vivo and in vitro perfusion experiments. An isotonic FP solution (167 μg/ml) was intravenously infused to obtain a mean steady-state FP plasma concentration of 10 μg/ml. Tyrode solution containing FP (10 μg/ml) and BSA (4.7%), with or without BK (0.1 μM), was perfused at 1 ml/min in the in vitro experiments. Each data set is the mean±S.E. ($n = 3$–4). $^{*}P < 0.05$ in Tukey–Kramer test.

3.4. Distribution of FP after application of FP gel to the skin surface of the perfused ear flap

In order to evaluate the perfused rabbit ear flap as an experimental method to examine the absorption of drugs after application to skin, FP gel (1%) was used (Yanagimoto et al., 1998). One gram FP (1%) gel was applied to the skin surface of the perfused ear flap 1 h after the start of the perfusion to evaluate the distribution of FP during the absorption process. The perfusate (outflow) was collected every 15 min for 2 h after application, and then the FP content of site (A) and the other ear site were determined. In order to evaluate the effect of BK on the distribution, the Tyrode solution containing BK (0.1 μM) was also used as a perfusate. Fig. 5 shows the cumulative amounts of FP in the outflow after application. When the Tyrode solution containing BK was used as the perfusate, the amount of FP after 2 h was significantly lower than that in the control experiments. Fig. 6 shows the distribution of FP in the perfusate, site (A) and the other site 2 h after application (3 h after the start of the perfusion). The total amount is equal to the amount of FP that has permeated through the stratum corneum of site (A) 2 h after application to the skin surface. There is no significant difference in the totals, with and without BK, in the Tyrode solutions. While the amount of FP in the outflow decreased, the amount in site (A) increased,

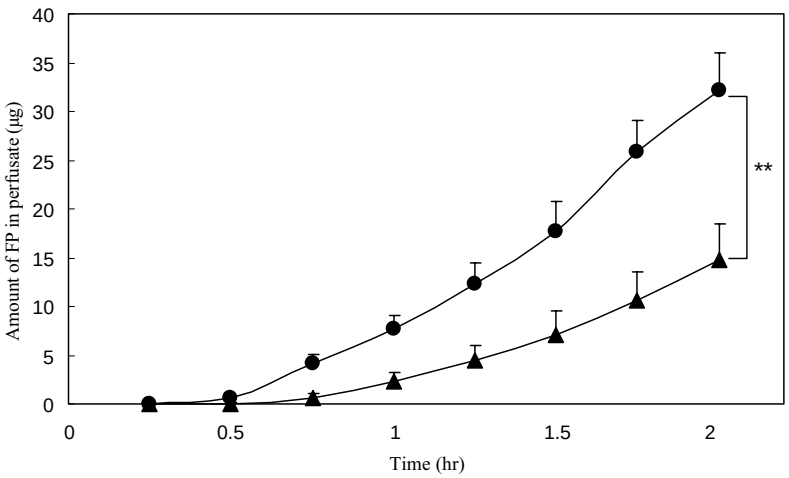

Fig. 5. Effect of BK on the cumulative amount of FP in the perfusate after application of FP gel (1%) to the skin surface of the rabbit ear flap. Tyrode solution containing BSA (4.7%), with or without BK (0.1 μM), was perfused at 1 ml/min. Closed circles, without BK; closed triangles, with BK. Each data set is the mean ± S.E. ($n = 6$). **, $P < 0.01$ in Student's t-test.

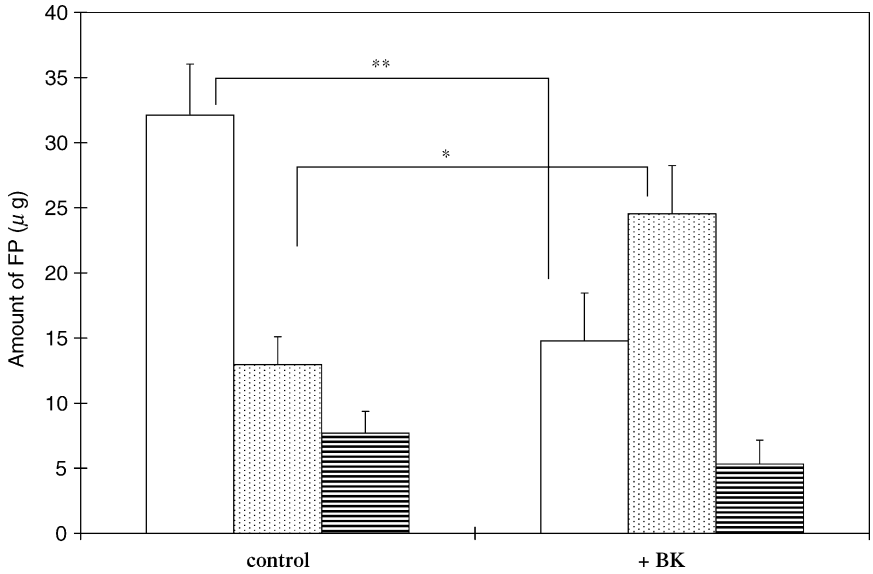

Fig. 6. Effect of BK on the distribution of FP 2 h after application of FP gel (1%) to the skin surface of rabbit ear flap. Open bars, in perfusate; dotted bars, in tissue under the application site; striped bars, in the rest of the ear flap tissue. Tyrode solution containing BSA (4.7%), with or without BK (0.1 μM), was perfused at 1 ml/min. Each data set is the mean ± S.E. ($n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$ in Student's *t*-test.

when BK was added to the Tyrode solutions. BK could increase the vascular permeability and the leakage of BSA into the extracellular space of site (A). Since FP could bind to the leaked BSA during the absorption process, the FP in site (A) could be increased and the absorption of FP into the perfusate could be decreased by the addition of BK to the perfusate. Although the vascular permeability could increase in all the sites of the perfused ear flap, the FP in the other site did not increase following the addition of BK.

3.5. Redistribution of FP from perfusate to tissue after application of FP to the surface of the ear flap

In order to examine the factors involved in the redistribution of FP to the other site, the values of RR were calculated as described in Section 2.2 and then compared. RR is a parameter related to the leakage of drugs from the perfusate to the extravascular space under the assumption that the direct diffusion of drugs from site (A) to the other site is negligible. Although the value of RR with the Tyrode solution containing BK was higher than that under control conditions, there was no significant difference between them (Fig. 7). A Tyrode solution containing DexT40 and BK was used instead of that containing BSA and BK to evaluate the effect of protein binding on the redistribution process. The value of RR with the Tyrode solution containing DexT40 was higher than that with the Tyrode solution containing BSA alone or BSA and BK (Fig. 7). The vascular permeability of FP binding to BSA could be low. In order to evaluate the effect of binding to BSA on the diffusivity of FP, the diffusion coefficient of FP in 0.2% BSA solution was determined by CBM. The diffusion coefficients of FP in the buffer (pH 7.4) and the buffer containing BSA were $8.05 \times 10^{-6} \pm 0.03 \times 10^{-6}$ cm^2/s and $1.08 \times 10^{-6} \pm 0.02 \times 10^{-6}$ cm^2/s, respectively. The diffusion coefficient of FP in the BSA solution is similar to that of BSA (1.04×10^{-6} cm^2/s; Salmon et al., 1984). This result suggests that FP could be transferred slowly along with BSA in the Tyrode solution containing BSA.

4. Discussion

The local tissue concentrations of drugs after topical application could be affected by many factors, e.g. the physicochemical properties of the drugs, local tissue binding and cutaneous blood flow (Clough et al.,

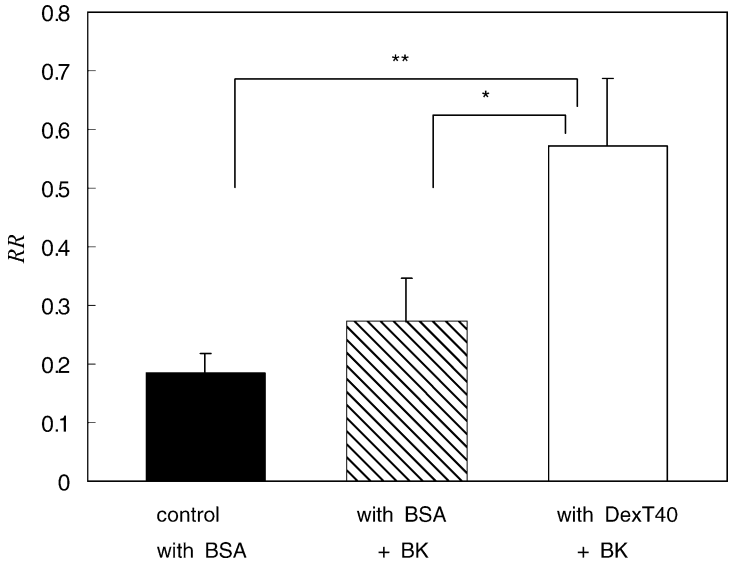

Fig. 7. Effect of BK on the redistribution of FP from perfusate to tissue 2 h after application of FP gel (1%) to the skin surface of rabbit ear flap. The ratio of redistribution (RR) is defined in the text. Tyrode solution containing BSA (4.7%), with or without BK (0.1 μM) or DexT40 (3.95%) with BK (0.1 μM), was perfused at 1 ml/min. Each data set is the mean ± S.E. ($n = 4$–6). $^{*}P < 0.05$; $^{**}P < 0.01$ in Tukey–Kramer test.

2002; Singh and Roberts, 1994). The rate and amount of absorption of drugs from the cutaneous microvessels could be important factors in determining the concentration profiles of the drugs in skin. Therefore, vascular perfusion experiments could provide useful information for a better understanding of the disposition of drugs in local tissues after topical application.

In order to study the percutaneous absorption of drugs, rabbit skin has been used for both drug permeation experiments through excised skin and also for perfusion experiments (Celesti et al., 1992, 1993; Bast and Kampffmeyer, 1994; Mura et al., 1994; Nicoli et al., 2003). Although drug metabolism and tissue integrity were evaluated in those perfusion studies, the tissue conditions of the perfused ear, such as the extracellular volume and vascular permeability, were not fully discussed. In the present study, the rabbit ear flap single-pass perfusion system was examined as an experimental method for studying the relationship between the physiological conditions of the tissue and drug disposition after topical applications.

In the initial part of the experiment, the extracellular volume of a perfused rabbit ear and the distribution of FP, used as a model drug, in the perfused ear were examined and compared with those in an in vivo experimental system. Tyrode solutions containing sucrose or FP were perfused through the ear vessels to evaluate the distribution properties (Weiss et al., 1997; Oliver et al., 1997). The distribution volume of sucrose in the perfused ear, which is considered to be the extracellular volume, was similar to the volume observed in the in vivo studies (Fig. 2). Celesti et al. (1992) observed progressive edema in perfused ear flaps. In our perfusion system, edema was not serious over the perfusion period of 3 h. The K_p of FP in the perfusion system was about 0.1 ml/g and this value was equivalent to that under in vivo conditions (Fig. 4). The low K_p values could be related to the higher protein binding of FP (Borga and Borga, 1997). The vascular permeability of FP binding to BSA could be low allowing movement into the extravascular space under controlled conditions (Cross et al., 1994).

The pharmacological response of the perfused ear to vasoactive agents could be one criterion for experimental methods evaluating the disposition of drugs in tissues (Rogers and Riviere, 1994). In the present studies, BK was chosen as the vasoactive agent. Since BK markedly increases vascular permeability, the extracellular volume and distribution of FP in the perfused ear will be changed by the addition of BK to the

perfusate. The vascular permeability was evaluated by determination of the distribution volume of EB in the perfused ear. The distribution volume of EB in the perfused ear without BK, which is considered to be the intravascular volume, was very low, while that with BK was higher than the extracellular volume without BK (Fig. 3). This result means that BSA leaks from the intravascular space into the intercellular space following the addition of BK to the perfusate. The extracellular volume of the perfused ear, which was determined using sucrose, also increased following the addition of BK (Fig. 2). The distribution volume of EB was equal to the extracellular volume in the perfused ear with BK, suggesting uniform distribution of BSA in the extracellular space. In addition, the K_p value also increased when Tyrode solution containing BK was perfused (Fig. 4). FP could easily move to the extracellular space due to the increased vascular permeability. These results suggest that edema can be created in the perfused ear flap. As the relationship between changes in tissue conditions and the disposition of drugs is of great interest (Vaden et al., 1996), the rabbit ear perfusion system is a useful method for studying it.

FP gel was applied to the skin surface of site (A) of the perfused ear flap to evaluate the effects of BK on the disposition of FP after topical application. The amount of FP in the outflow decreased and the amount in site (A) increased when BK was added to the Tyrode solutions. FP could bind to the BSA leaking into the intercellular space. The FP in site (A) could increase and the absorption of FP into the perfusate could be decreased following binding to BSA. This phenomenon should be taken into account in the disposition of drugs following their application to inflamed tissues.

Transport of drugs to deeper tissues via blood flow contributes to the distribution of drugs in the underlying tissues after topical application (Cross et al., 1999; Monteiro-Riviere et al., 1993). Therefore, redistribution of absorbed drugs to other tissues can be a valuable way of evaluating the perfusion system. In this study, the values of RR were used for the evaluation on the assumption that direct diffusion of drugs from site (A) to the other site was negligible. Since the vascular permeability was increased by the addition of BK to the perfusate, this suggests that a higher RR could be obtained during the perfusion with BK. However, there was no significant difference in RR, with and without BK (Fig. 7). In the initial phase of the perfusion, when Tyrode solution containing BK was used as the perfusate, the medium could leak prompting flow into the intercellular space. BSA could move into the space due to this flow. Since the FP gel was applied to the skin surface 1 h after the start of the perfusion, the medium flow into the intercellular space during the period of the transdermal absorption experiments could be lower than that in the initial phase of the perfusion. After equilibrium of the BSA distribution, exchange of BSA between the intravascular and extravascular space could only be achieved by diffusion. The disposition of FP, which binds strongly to BSA, could be affected by BSA (Mathy et al., 2001; Evrard et al., 1996). The results of the determinations of the diffusivity of FP suggest that FP diffuses slowly in the presence of BSA. In order to examine the effect of the binding of FP to BSA on the distribution of FP, DexT40 was added to the Tyrode solution instead of BSA (Roberts and Cross, 1999; Wu et al., 1997). DexT40 produces an oncotic pressure but does not bind to FP. The RR value for the Tyrode solution containing DexT40 and BK was higher than that for the Tyrode solution containing BSA alone or BSA and BK (Fig. 7). FP could move easy into the extravascular space through the vessel wall, the permeability of which could be increased by BK. The disposition of drugs bound to proteins should be considered not only in applications of drugs to inflamed tissues but also in drug delivery systems used to target drugs to inflamed tissues. Further studies could be needed to understand the pharmacokinetics of drugs and plasma proteins in local tissues.

In conclusion, the rabbit ear flap single-pass perfusion system was examined as an experimental method for studying the relationship between the physiological conditions of tissue and drug disposition after topical applications. When the rabbit ear perfusion experimental system was compared with the in vivo experimental system they were found to be equivalent in terms of the tissue condition properties and the distribution of FP. In addition, the perfused ear flap exhibited a pharmacological response to BK. The disposition of FP after application to the skin surface of the perfused ear varied depending on the composition of the Tyrode solutions. The rabbit ear perfusion system is useful for studying the percutaneous absorption of drugs, especially for variations in the disposition of drugs in inflamed tissues.

References

Auclatr, F., Besnard, M., Dupont, C., Wepierre, J., 1991. Importance of blood flow to the local distribution of drugs after percutaneous absorption in the bioediculated dorsal flap of the hairless rats. Skin Pharmacol. 4, 1–8.

Bast, G.E., Kampffmeyer, H.G., 1994. Absorption and metabolism of hydrocortisone 21-butyrate, 21-hemisuccinate and hydrocortisone by skin of the rabbit ear during single-pass perfusion. Xenobiotica 24, 1029–1042.

Benowitz, N.L., Jacob, P., Olsson, P., Johansson, C.-J., 1992. Intravenous nicotine retards transdermal absorption of nicotine: evidence of blood flow-limited percutaneous absorption. Clin. Pharmacol. Ther. 52, 223–230.

Borga, O., Borga, B., 1997. Serum protein binding of nonsteroidal antiinflammatory drugs: a comparative study. J. Pharmacokin. Biopharm. 25, 63–77.

Celesti, L., Murratzu, C., Valoti, M., Sgaragli, G., Corti, P., 1992. The single-pass perfused rabbit ear as a model for studying percutaneous absorption of clonazepam. I. General characteristics. Meth. Find. Exp. Clin. Pharmacol. 14, 701–709.

Celesti, L., Murratzu, C., Valoti, M., Sgaragli, G., Corti, P., 1993. The single-pass perfused rabbit ear as a model for studying percutaneous absorption of clonazepam. II. Influence of hydrogel-borne propylene glycol and skin pretreatment with lauryl alcohol. Meth. Find. Exp. Clin. Pharmacol. 15, 49–56.

Chang, S.K., Williams, P.L., Dauterman, W.C., Riviere, J.E., 1994. Percutaneous absorption, dermatopharmacokinetics and related bio-transformation studies of carbaryl, lindane, malathion, and parathion in isolated perfused porcine skin. Toxicology 91, 269–280.

Clark, E.R., Clark, M.G., 1932. Observations on living performed blood vessels as seen in a transparent chamber inserted into the rabbit's ear. Am. J. Anat. 49, 441–447.

Clough, G.F., Boutsiouki, P., Church, M.K., Michel, C.C., 2002. 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.

Cross, S., Wu, Z., Roberts, M.S., 1994. Effect of perfusion flow rate on the tissue uptake of solutes after dermal application using the rat isolated perfused hindlimb preparation. J. Pharm. Pharmacol. 46, 844–850.

Cross, S.E., Megwa, S.A., Benson, H.A.E., Roberts, M.S., 1999. Self promotion of deep tissue penetration and distribution of methylsalicylate after topical application. Pharm. Res. 16, 427–433.

Eisen, V., 1970. Plasma kinins in synovial exudates. Br. J. Exp. Pathol. 51, 322–327.

Evrard, P.A., Cumps, J., Verbeeck, R.K., 1996. Concentration-dependent plasma protein binding of flurbiprofen in the rat: an in vivo microdialysis study. Pharm. Res. 13, 18–22.

Kietzmann, M., Wenzel, B., Loscher, W., Lubach, D., Muller, B.W., Blume, H., 1995. Absorption of isosorbide dinitrate after administration as spray, ointment and microemulsion patch. An in-vitro study using the isolated perfused bovine udder. J. Pharm. Pharmacol. 47, 22–25.

Kietzmann, M., Blume, H., 1997. Percutaneous absorption of betamethasone from different formulations using the isolated perfused bovine udder. In Vitro Toxicol. 10, 11–15.

Kreilgaard, M., 2001. Dermal pharmacokinetics of microemulsion formulations determined by in vivo microdialysis. Pharm. Res. 18, 367–373.

Lange, J., Eck, P., Elliott, G.R., Lort, W.L.A.M., Wolthuis, O.L., 1992. The isolated blood-perfused pig ear: an inexpensive and animal-saving model for skin penetration studies. J. Pharmacol. Toxicol. Meth. 27, 71–77.

Mathy, F.-X., Preat, V., Verbeeck, R.K., 2001. Validation of subcutaneous microdialysis sampling for pharmacokinetic studies of flurbiprofen in the rat. J. Pharm. Sci. 90, 1897–1906.

Monteiro-Riviere, N.A., Inman, A.O., Riviere, J.E., McNeill, S.C., Francoeur, M.L., 1993. Topical penetration of piroxicam is dependent on the distribution of the local cutaneous vasculature. Pharm. Res. 10, 1326–1331.

Morimoto, Y., Hayashi, T., Kawabata, S., Seki, T., Sugibayashi, K., 2000. Effect of l-menthol–ethanol–water system on the systemic absorption of flurbiprofen after repeated topical applications in rabbits. Bio. Pharm. Bull. 23, 1254–1257.

Mura, P., Nassini, C., Valoti, M., Santoni, G., Corti, P., 1994. The single-pass perfused rabbit ear as a model for studying percutaneous absorption of clonazepam. III. Influence of vehicle composition on drug permeation. Eur. J. Pharm. Biopharm. 40, 90–95.

Nicoli, S., Cappellazzi, M., Colombo, P., Santi, P., 2003. Characterization of the permselective properties of rabbit skin during transdermal iontophoresis. J. Pharm. Sci. 92, 1482–1488.

Oliver, R.E., Heatherington, A.C., Jones, A.F., Rowland, M., 1997. A physiologically based pharmacokinetic model incorporating dispersion principles to describe solute distribution in the perfused rat hindlimb preparation. J. Pharmacokin. Biopharm. 25, 389–412.

Pollock, D.C., Li, Z., Koman, L.A., Gordon, E.S., Smith, T.L., 1996. Control of arteriovenous anastomoses in rabbit ear model of digital perfusion. Am. J. Physiol. 271, H2007–H2013.

Riviere, J.E., Brooks, J.D., Williams, P.L., McGown, E., Francoeur, M.L., 1996. Cutaneous metabolism of isosorbide dinitrate after transdermal administration in isolated perfused porcine skin. Int. J. Pharm. 127, 213–217.

Roberts, M.S., Cross, S.E., 1999. A physiological pharmacokinetic model for solute disposition in tissues below a topical application site. Pharm. Res. 16, 1392–1398.

Rogers, R.A., Riviere, J.E., 1994. Pharmacologic modulation of the cutaneous vasculature in the isolated perfused porcine skin flap. J. Pharm. Sci. 83, 1682–1689.

Salmon, E.D., Saxton, W.M., Leslie, R.J., Karow, M.L., McIntosh, J.R., 1984. Diffusion coefficient of fluorescein-labeled tubulin in the cytoplasm of embryonic cells of a sea urchin: video image analysis of fluorescence redistribution after photobleaching. J. Cell Biol. 99, 2157–2164.

Seki, T., Okamoto, M., Hosoya, O., Aiba, D., Morimoto, K., Juni, K., 2003a. Effect of chondroitin sulfate on the diffusion coefficients of drugs in aqueous solutions. S.T.P. Pharma. Sci. 13, 215–218.

Seki, T., Mochida, J., Okamoto, M., Hosoya, O., Juni, K., Morimoto, K., 2003b. Measurement of diffusion coefficients of parabens and steroids in water and 1-octanol. Chem. Pharm. Bull. 51, 734–736.

Singh, P., Roberts, M.S., 1994. Skin permeability and local tissue concentrations of nonsteroidal anti-inflammatory drugs after topical application. J. Pharmacol. Exp. Ther. 268, 144–151.

Sugibayashi, K., Yanagimoto, G., Hayashi, T., Seki, T., Juni, K., Morimoto, Y., 1999. Analysis of skin disposition of flurbiprofen after topical application in hairless rats. J. Control. Release 62, 193–200.

Touitou, E., Meidan, V.M., Horwitz, E., 1998. Methods for quantitative determination of drug localized in the skin. J. Control. Release 56, 7–21.

Vaden, S.L., Page, R.L., Riviere, J.E., 1996. An in vitro–in vivo validation of the isolated perfused tumor and skin flap preparation as a model of cisplatin delivery to tumors. J. Pharmacol. Toxicol. Meth. 35, 173–177.

Weiss, M., Koester, A., Wu, Z.Y., Roberts, M.S., 1997. Distribution kinetics of diazepam, lidocaine and antipyrine in the isolated perfused rat hindlimb. Pharm. Res. 14, 1640–1643.

Williams, P.L., Carver, M.P., Riviere, J.E., 1990. A physiologically relevant pharmacokinetic model of xenobiotic percutaneous absorption utilizing the isolated perfused porcine skin flap. J. Pharm. Sci. 79, 305–311.

Wu, Z., Smithers, B.M., Roberts, M.S., 1997. Tissue and perfusate pharmacokinetics of melphalan in isolated perfused rat hindlimb. J. Pharmacol. Exp. Ther. 282, 1131–1138.

Yanagimoto, G., Hayashi, T., Seki, T., Juni, K., Sugibayashi, K., Morimoto, Y., 1998. A new in-situ experimental method to evaluate the cutaneous disposition of flurbiprofen after topical application in hairless rats. Pharm. Pharmacol. Commun. 4, 1–5.

Yanagimoto, G., Hayashi, T., Hasegawa, T., Seki, T., Juni, K., Sugibayashi, K., Morimoto, Y., 1999. Skin disposition of drugs after topical application in hairless rats. Chem. Pharm. Bull. 47, 749–754.