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Pharmacokinetics of loxoprofen and its active metabolite after dermal application of loxoprofen gel to rats

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This study was conducted to evaluate the pharmacokinetics of loxoprofen (LX) and its active metabolite (*trans*-OH form) after a single dermal application of LX gel (LX-G) to rats. In the skin at the treated site, generation of the *trans*-OH form was detected and both LX and the *trans*-OH form remained at high concentrations for 24 h after dermal application. Furthermore, both LX and the *trans*-OH form also remained in the skeletal muscle over 24 h after the single dermal application, while they eliminated rapidly after the single oral administration. The area under the curve up to the last measurable point ($AUC_{0\text{-}t}$) for plasma concentrations of LX or the *trans*-OH form after dermal application of LX-G was less than 11% of that after oral administration of LX. In addition, C_{max} and $AUC_{0\text{-}t}$ increased in a saturable manner while increasing the dose. Overall, these results demonstrated that the *trans*-OH form was generated at the treated site with the process of dermal absorption of LX and it remained at the target site for a long period with low systemic exposure compared to oral administration.

1. Introduction

Loxoprofen (LX) is a prodrug of the pharmacologically active *trans*-alcohol form (*trans*-OH form), which shows a strong inhibitory effect on prostaglandin synthesis (Matsuda et al. 1984). LX has first been marketed as an oral drug, with a strong anti-inflammatory effect against rheumatoid arthritis, backache, tooth pain, and so forth (Honma 1994). However, retention in the body of both LX and the *trans*-OH form is not for long, because the elimination of both LX and the *trans*-OH from the body is rapid after oral administration. To solve this problem, an administration route which shows longer-term retention in the body than oral administration was desired. Therefore, we developed a topical formulation of LX, LX gel (LX-G) (Loxonin® gel 1%, Daiichi Sankyo Co., Ltd., Tokyo, Japan). It is used widely in Japan for the treatment of muscle sores or pain due to osteoarthritis, sprains, and contusions, and so forth (Matsubara et al. 2011; Kajita et al. 2012).

A known merit of dermal treatment is that the drug concentration achieves a therapeutically effective level in the tissues subjacent to the treated site after dermal application of some drugs (Heyneman et al. 2000). In addition, maintaining a low systemic concentration by dermal application mitigates the risk of serious systemic toxicities of NSAIDs (Heyneman et al. 2000). Therefore, it is essential to examine the pharmacokinetics of the pharmacological active form after dermal application from the viewpoint of both efficacy and safety to develop a promising formulation.

While pharmacokinetics of LX after intravenous and oral administration of LX to rats were reported (Koo et al. 2005), pharmacokinetics of LX and the *trans*-OH form after dermal application of LX-G to rats have not been reported yet. The purpose of this study is to examine the difference in the pharmacokinetics of LX and the *trans*-OH form in rats between the single dermal application of LX-G and single oral administration of LX. In addition, we evaluated dose proportionality of systemic exposure of LX and the *trans*-OH form after dermal application of LX-G.

2. Investigations and results

2.1. Pharmacokinetic study

First, we performed a pharmacokinetic study of the single dermal application of LX-G to rats to examine the dose proportionality of systemic exposure of LX and the *trans*-OH form after dermal application. The plasma concentration profiles of LX and the *trans*-OH form after the single dermal application of LX-G are shown in Fig. 2. The pharmacokinetic parameters for each dosing are summarized in Table 1. Regarding pharmacokinetic parameters of LX, both C_{max} and $AUC_{0\text{-}t}$ of LX after dermal application of LX-G at 2.5, 7.5 and 12.5 mg/kg as LX increased in a saturable manner while increasing the dose. Both C_{max} and $AUC_{0\text{-}t}$ of the *trans*-OH form after dermal application of LX-G at 2.5, 7.5 and 12.5 mg/kg as LX increased in a saturable manner while increasing the dose as was the case with LX.

Secondly, we performed a pharmacokinetic study of dermal application of LX-G, intravenous administration of LX and oral administration of LX, to evaluate the pharmacokinetic characteristics of LX and the *trans*-OH form after each administration. The plasma concentration profiles of LX and the *trans*-OH form after intravenous administration of 2.5 mg/kg LX are shown in Fig. 2. The plasma concentration profiles of LX and the *trans*-OH form after dermal application of LX-G at 2.5 mg/kg as LX or oral administration of 2.5 mg/kg LX are shown in Fig. 3. The

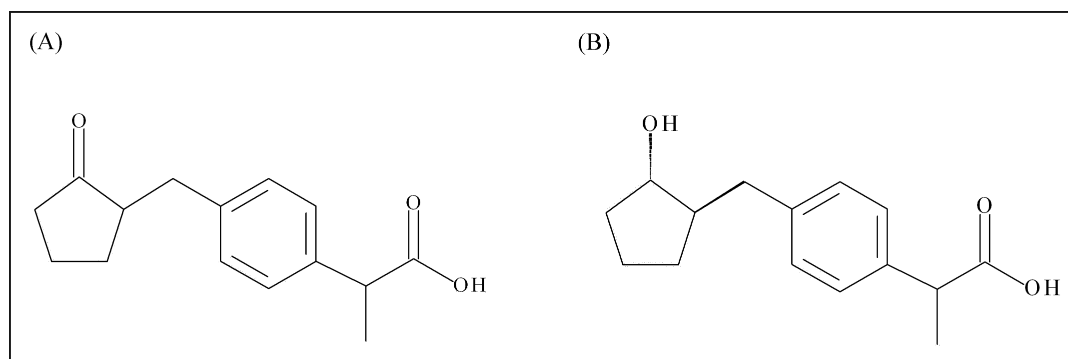

Fig. 1: Chemical structures of LX (A) and the *trans*-OH from (B).

Table 1: Pharmacokinetic parameters of LX (A) and the *trans*-OH form (B) in plasma after single dermal application of LX-G at 2.5, 7.5 and 12.5 mg/kg as LX, or single intravenous or oral administration of 2.5 mg/kg LX to rats

(A)

		Intravenous	Oral	Dermal		
		2.5 mg/kg	2.5 mg/kg	2.5 mg/kg	7.5 mg/kg	12.5 mg/kg
		Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
$AUC_{0\text{-}t}$*	h•ng/mL	2460 ± 90	1850 ± 290	202 ± 22	331 ± 38	535 ± 139
$AUC_{0\text{-}\infty}$	h•ng/mL	2560 ± 80	1890 ± 250	N.C.**	N.C.**	N.C.**
C_{max}	ng/mL	2940 ± 510	1290 ± 330	23.1 ± 4.7	59.5 ± 44.4	80 ± 29.7
$t_{1/2}$	h	1.36 ± 0.07	3.18 ± 2.04	11.7 ± 2.1	14.6 ± 6.6	11.8 ± 7.6
T_{max}	h	–	0.25 ± 0	0.813 ± 0.375	1 ± 0	0.75 ± 0.289
CL_{tot}	mL/min/kg	15 ± 0.5	–	–	–	–
$V_{d,\ ss}$	mL/kg	1420 ± 160	–	–	–	–
F	%	100	73.8 ± 9.9	–	–	–
$AUC_{0\text{-}t}$ ratio	%	100	75.2 ± 11.8	8.21 ± 0.89	–	–
$AUC_{0\text{-}t}$/Dose		–	–	80.8	44.1	42.8
Cmax/Dose		–	–	98.8	55.7	54.1

(B)

		Intravenous	Oral	Dermal		
		2.5 mg/kg Mean ± S.D.	2.5 mg/kg Mean ± S.D.	2.5 mg/kg Mean ± S.D.	7.5 mg/kg Mean ± S.D.	12.5 mg/kg Mean ± S.D.
$AUC_{0\text{-}t}$*	h•ng/mL	1710 ± 200	1620 ± 200	157 ± 16	208 ± 42	379 ± 117
$AUC_{0\text{-}\infty}$	h•ng/mL	1990 ± 120	1630 ± 200	N.C.**	N.C.**	N.C.**
C_{max}	ng/mL	1110 ± 100	666 ± 125	12 ± 0.4	35.1 ± 25.2	44.4 ± 16.2
$t_{1/2}$	h	2.04 ± 0.31	4.03 ± 1.23	27.5 ± 12.8	14.4 ± 5.1	16.1 ± 9.7
T_{max}	h	0.208 ± 0.085	0.313 ± 0.125	1.25 ± 0.5	1.25 ± 0.5	1.25 ± 0.5
$AUC_{0\text{-}t}$ ratio	%	100	94.7 ± 11.7	9.18 ± 0.94	–	–
$AUC_{0\text{-}t}$/Dose		–	–	62.8	27.7	30.3
Cmax/Dose		–	–	4.8	4.68	3.55

*: Area under the curve of plasma concentrations up to the last measurable point
*: Not calculated
Each value is the mean ± standard deviation (S.D.) of four animals.

pharmacokinetic parameters for dermal, intravenous and oral dosing are summarized in Table 1. The plasma concentration of both LX and the *trans*-OH form at 24 h after intravenous administration was less than the lower limit of quantification (LLOQ). The total clearance (CL_{tot}) and the distribution volume ($Vd_{,\ ss}$) of LX were 15 mL/min/kg and 1420 mL/kg, respectively. The absolute bioavailability of LX after oral administration was 73.8%. The $AUC_{0\text{-}\infty}$ of both LX and the *trans*-OH form after dermal application was not shown because the elimination phase of their plasma concentrations was not evaluated for a long enough time period after the application. The $AUC_{0\text{-}t}$ ratio of LX after dermal application was 8.21%, while that after oral administration was 75.2%. The $AUC_{0\text{-}t}$ ratio of plasma *trans*-OH form concentrations between dermal application and intravenous administration was 9.18%, while that between oral administration and intravenous administration was 94.7%. After oral administration of LX, both LX and the *trans*-OH form were rapidly eliminated from the plasma compared to dermal application of LX-G.

2.2. *Tissue distribution study*

We performed a tissue distribution study of dermal application of LX-G to rats to evaluate the concentrations of LX and the *trans*-OH form in the treated skin or in the skeletal muscle at the treated site (a target site of efficacy) after the single dermal application. The skin concentration-time profiles of LX and the *trans*-OH

Table 2: Pharmacokinetic parameters of LX and the *trans*-OH form in the skin (A) or the skeletal muscle (B) at the treated site after single dermal application of 3% LX-G at the dose of 7.5 mg/kg as LX or single oral administration of 7.5 mg/kg LX to rats

(A)

		AUC_{0-t}* ng•h/g tissue	Dermal/oral AUC_{0-t} ratio	C_{max} ng/g tissue	Dermal/oral C_{max} ratio	T_{max} h
LX	Dermal	675300	359	–**	54.4	–**
	Oral	1879		708		0.5
Trans-OH form	Dermal	10849	6.59	620	1.27	0.5
	Oral	1646		490		1

(B)

		AUC_{0-t}* ng•h/g tissue	Dermal/oral AUC_{0-t} ratio	C_{max} ng/g tissue	Dermal/oral C_{max} ratio	T_{max} h
LX	Dermal	936	1.43	177	0.600	1
	Oral	656		295		0.5
Trans-OH form	Dermal	466	0.468	94.7	0.306	1
	Oral	995		309		1

*: Area under the curve of skin or skeletal muscle concentrations up to the last measurable point
**: Skin C_{max} and T_{max} of LX after dermal application are not shown because the decrease of skin concentrations was not observed during 24 h.
Each parameter was calculated using the mean concentration data.

form after dermal application of LX-G at 7.5 mg/kg as LX or oral administration of 7.5 mg/kg LX are shown in Fig. 4. The pharmacokinetic parameters for skin concentrations of LX and the *trans*-OH form are summarized in Table 2A. Regarding LX concentrations in the skin, the AUC_{0-t} value in the treated skin site after dermal application (675300 ng•h/g tissue) was much

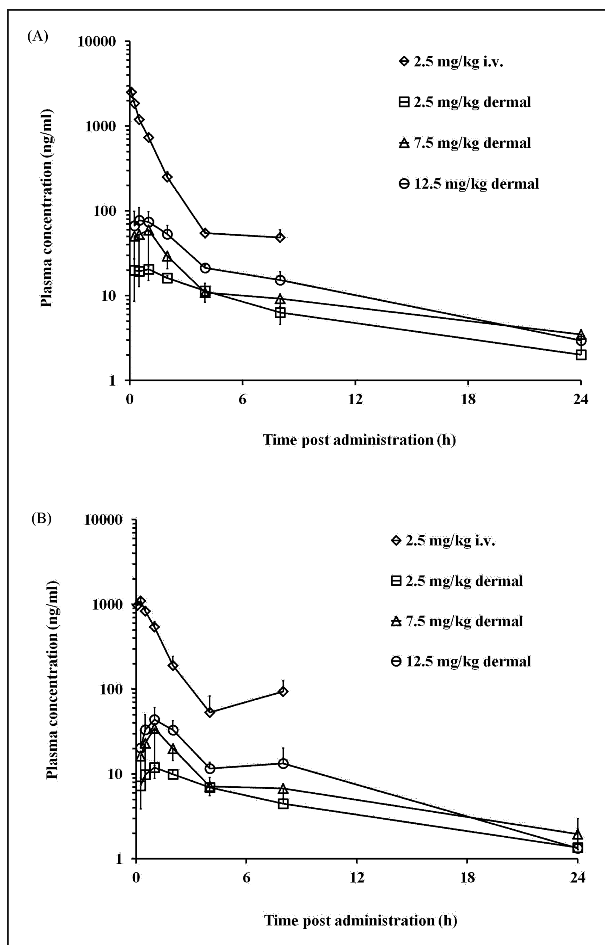

Fig. 2: Plasma concentration of LX (A) and the *trans*-OH from (B) after single dermal application of LX-G at 2.5, 7.5 and 12.5 mg/kg as LX, or single intravenous administration of 2.5 mg/kg LX to rats. Symbols and vertical bars represent the mean ± S.D. from four rats.

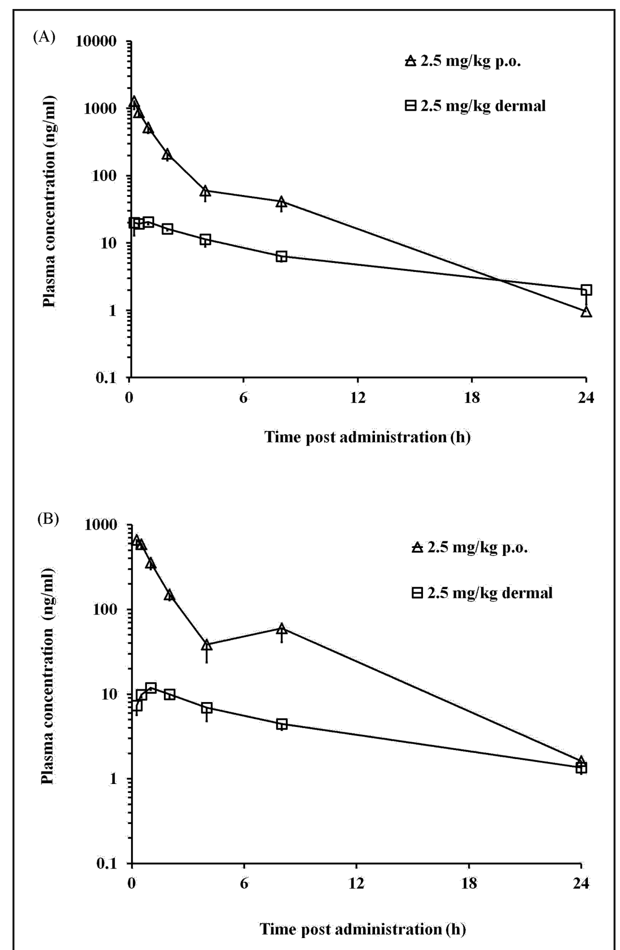

Fig. 3: Plasma concentration of LX (A) and the *trans*-OH from (B) after single dermal application of LX-G at 2.5 mg/kg as LX, or single oral administration of 2.5 mg/kg LX to rats. Symbols and vertical bars represent the mean ± S.D. from four rats.

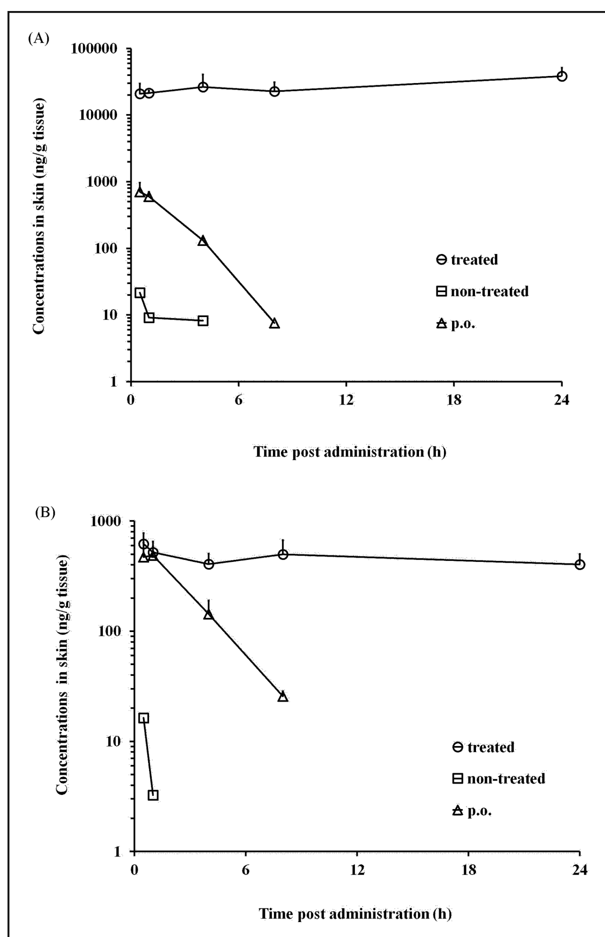

Fig. 4: Skin concentration of LX (A) and the *trans*-OH from (B) after single dermal application of LX-G at 7.5 mg/kg as LX, or single oral administration of 7.5 mg/kg LX to rats. Symbols and vertical bars represent the mean ± S.D. from four rats.

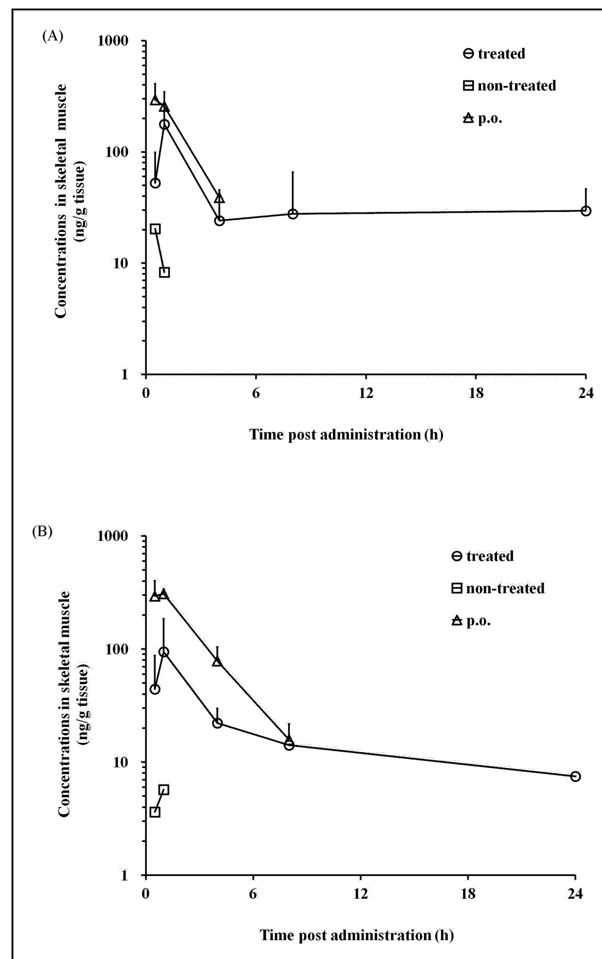

Fig. 5: Skeletal muscle concentration of LX (A) and the *trans*-OH from (B) after single dermal application of LX-G at 7.5 mg/kg as LX, or single oral administration of 7.5 mg/kg LX to rats. Symbols and vertical bars represent the mean ± S.D. from four rats.

larger than the $AUC_{0\text{-}t}$ value in the skin after oral administration (1879 ng•h/g tissue). We did not calculate the C_{max} and T_{max} of LX in the treated skin site after dermal application because the decrease in skin concentrations was not observed during 24 h. The C_{max} value of LX in the skin was 708 ng/g tissue after oral administration. Regarding the *trans*-OH form concentrations in the skin, the $AUC_{0\text{-}t}$ value in the treated skin site after dermal application (10849 ng•h/g tissue) was larger than the $AUC_{0\text{-}t}$ value in the skin after oral administration (1646 ng•h/g tissue). The C_{max} value of the *trans*-OH form in the treated skin site after dermal application (620 ng/g tissue) was almost similar to that in the skin after oral administration (490 ng/g tissue). Both LX and the *trans*-OH form remained at high concentrations in the treated skin over a period of 24 h postdose, while they eliminated rapidly from the skin after oral administration and their concentrations were below the LLOQ (10 ng/g tissue) after 8 h postdose. The *trans*-OH form was detected in the treated skin within 0.5 h after dermal application.

The skeletal muscle concentration-time profiles of LX and the *trans*-OH form after dermal application of LX-G at 7.5 mg/kg as LX or oral administration of 7.5 mg/kg LX are shown in Fig. 5. The pharmacokinetic parameters for skeletal muscle concentrations of LX and the *trans*-OH form are summarized in Table 2B. Regarding LX concentrations in the skeletal muscle, the $AUC_{0\text{-}t}$ value at the treated site after dermal application (936 ng•h/g tissue) were almost similar to the $AUC_{0\text{-}t}$ value after oral administration (656 ng•h/g tissue). The C_{max} value of LX in the skeletal muscle at the treated site after dermal application (177 ng/g tissue) was lower than the C_{max} value in the skeletal muscle after oral administration (295 ng/g tissue). Regarding the *trans*-OH form concentrations in the skeletal muscle, the $AUC_{0\text{-}t}$ value at the treated site after dermal application (466 ng•h/g tissue) was lower than the $AUC_{0\text{-}t}$ value after oral administration (995 ng•h/g tissue). The C_{max} value of the *trans*-OH form in the skeletal muscle at the treated site after dermal application (94.7 ng/g tissue) was also lower than the C_{max} value in the skeletal muscle after oral administration (309 ng/g tissue). The concentrations of LX and the *trans*-OH form in the skeletal muscle at the treated site after dermal application were maintained at more than 10 ng/g tissue for more than 8 h, while the concentrations of LX and the *trans*-OH form in the skeletal muscle after oral administration decreased rapidly where the concentrations were below 10 ng/g tissue at 4 h or thereafter. The *trans*-OH form was detected within 0.5 h in the skeletal muscle after dermal application as was the case with skin.

At the non-treated site, small amounts of LX and the *trans*-OH form were detected in both skin and skeletal muscle after dermal application.

3. Discussion

We performed pharmacokinetic and tissue distribution studies after a single dermal application of LX-G or single oral administration of LX using rats to evaluate the administration route difference in the pharmacokinetic characteristics of LX and the *trans*-OH form.

We found that LX permeated rapidly through the skin because LX appeared in the plasma within 0.5 h after dermal application (Fig. 2, 3), suggesting that LX has good physical properties for dermal application. In addition to the good permeability of LX, it was considered that the *trans*-OH form was formed in the skin because the skin concentrations of the *trans*-OH form at the treated site were much higher than those at the non-treated site (Fig. 4). Furthermore, it was deduced that both LX and the *trans*-OH form permeated from skin to skeletal muscle because both LX and *trans*-OH form concentrations in the skeletal muscle at the treated site were much higher than those at the non-treated site (Fig. 5). Both LX and the *trans*-OH form concentrations in the treated skin site after dermal application remained for a long period at high concentrations compared to oral administration (Fig. 4). The $AUC_{0\text{-}t}$ of LX in the treated skin site after dermal application was more than 350-fold larger than that in the skin after oral administration (Table 2A). It was thought that the *trans*-OH form would be generated from LX continuously in the skin after dermal application. Although the C_{max} of the *trans*-OH form in the treated skin site after dermal application was similar to that in the skin after oral administration, $AUC_{0\text{-}t}$ of the *trans*-OH form in the treated skin site after dermal application was more than 6-fold larger than that in the skin after oral administration (Table 2A). The possibility that the *trans*-OH form would migrate from the treated skin to the skeletal muscle continuously was considered. In fact, both LX and the *trans*-OH form concentrations in the skeletal muscle after dermal application remained for a long period compared to oral administration (Fig. 5). One of the reasons was assumed that both LX and the *trans*-OH form permeated from the skin to the skeletal muscle continuously in the treated site.

The *trans*-OH form concentrations in the skeletal muscle at the treated site after dermal application were lower than those in the skeletal muscle after oral administration (Fig. 5), however, the inflammation was suppressed in rats when the concentrations of the *trans*-OH form in the skeletal muscle were around 10 ng/g tissue after dermal application of LX-G (data not shown). In the tissue distribution study, the concentrations of the *trans*-OH form in the skeletal muscle at 24 h after dermal application were about 10 ng/g tissue, while those after oral administration were less than 10 ng/g tissue. This meant that the *in vivo* efficacy after dermal application would be observed for a long period of time compared to oral administration at the same dose. From these results, it was deduced that dermal application of LX-G would excel oral administration of LX in topical treatment from the viewpoint of duration of efficacy. However, pains at the body sites to which LX-G cannot be applied (e.g. teeth, head, bronchial tubes, etc.) are cured by oral administration of LX. Therefore, an appropriate administration route of LX (oral administration or dermal application) should be selected that suits each pain treatment.

Non-steroid anti-inflammatory drugs (NSAIDs) tend to have several side effects after oral administration, such as anaphylaxis, bleeding, kidney damage, and so forth. It is reported that these side effects correlate with plasma exposure of the parent drug or its metabolites after oral administration (Sánchez-Borges. 2010; van der Klauw et al. 1996; Harirforoosh et al. 2009; Lacroix et al. 2004). Therefore, systemic exposure of the NSAIDs after dermal application is also thought to be an indication of systemic toxicity as is the case with oral administration. Regarding the systemic exposure, the plasma concentrations of LX and the *trans*-OH form after dermal application were much lower than those after oral administration (Fig. 3, Table 1). The value of $AUC_{0\text{-}t}$ of LX and the *trans*-OH form after dermal application were 10.9% and 9.7% of that after oral administration, respectively. Therefore, it was deduced that dermal application of LX-G might decrease the risk of systemic side effects, although the frequency of the systemic toxicity by LX is very low even in oral administration (Honma et al. 1994).

There are some reports of pharmacokinetics of LX and the *trans*-OH form in rats (Koo et al. 2005). Regarding pharmacokinetic parameters of LX after intravenous administration, each value of CL_{tot} and $Vd_{,ss}$ reported by Koo et al. was 5.35 ± 0.212 mL/min/kg and 736 ± 184 mL/kg, respectively (Koo et al. 2005). One of the reasons why both CL_{tot} (15 ± 0.5 mL/min/kg) and $Vd_{,ss}$ (1420 ± 160 mL/kg) which we obtained were higher than the reported values, was considered to be the strain difference because Koo et al. (2005) used Sprague-Dawley rats and we used Wistar-Imamichi rats. Regarding $AUC_{0\text{-}\infty}$ of the *trans*-OH form after intravenous administration, the $AUC_{0\text{-}\infty}$ value at a dose of 2.5 mg/kg was calculated to be 177 ± 87.5 μg•min/mL from the value reported by Koo et al. (2005). The $AUC_{0\text{-}\infty}$ value which we obtained (119 ± 7.2 μg•min/mL) was almost similar to the reported values considering the variability of the reported value. As for the pharmacokinetic parameters after oral administration of LX to rats, the absolute bioavailability value which we obtained (73.8 ± 9.9%) was lower than the reported value by Koo et al. (99%). The strain difference was deduced as one of the reasons as was the case with CL_{tot}.

When we compared the pharmacokinetic parameters in healthy volunteers (Daiichi Sankyo Co., Ltd., 2012) to those in the rats, plasma C_{max} of both LX and the *trans*-OH form in healthy volunteers was within two-fold of that in rats after dermal application of LX-G at almost the same dose. T_{max} of both LX and the *trans*-OH form in healthy volunteers was larger than that in rats. The reasons were thought to be the difference in thickness of the *stratum corneum* or in the amount of skin surface lipids between humans and rats (Sato et al. 1991).

The plasma C_{max} and $AUC_{0\text{-}t}$ of LX increased in a saturable manner while increasing the dose (Fig. 2, Table 1). One of the factors is considered to be the permeation process of LX through the *stratum corneum* because it is well known that the *stratum corneum* plays a role of the skin's barrier to protect underlying tissue from infection, dehydration and chemicals (Trommer et al. 2006). The plasma C_{max} and $AUC_{0\text{-}t}$ of the *trans*-OH form also increased in a saturable manner while increasing the dose. However, the generation of the *trans*-OH form was not thought to be saturated because the $AUC_{0\text{-}t}$ ratio between LX and the *trans*-OH form at each dose was almost 1:1.

We did not show the concentrations of the *cis*-OH form in the plasma, skin and skeletal muscle, although we determined them because the pharmacological activity of the *cis*-OH form was weak (Matsuda et al. 1984) and its concentration was very low compared with LX and the *trans*-OH form.

In conclusion, we found that LX-G overcomes the short duration and high systemic exposure of LX and the *trans*-OH form in oral administration of LX by examining the difference in pharmacokinetics between dermal application of LX-G and oral administration of LX. LX-G is used widely in Japan as a topical gel containing 1% LX (Matsubara et al. 2011; Kajita et al. 2012). Our preclinical studies supported the development of LX-G.

4. Experimental

4.1. Chemicals

LX, the *trans*-OH form, and their stable isotopes (d_3-LX and d_3-*trans*-OH form) were synthesized in Chemtech Labo., Inc. (Tokyo, Japan). The chemical structures of LX and the *trans*-OH form are shown in Fig. 1. Propyl p-hydroxybenzoate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LX-G containing 1, 3 or 5% LX was provided from Toko Yakuhin Kogyo Co., Ltd. (Osaka, Japan). Soluene-350 and Hionic-Fluor were purchased from Packard Instrument Company, Inc. (Meriden, CT, USA). All reagents and solvents were of reagent grade or HPLC grade and used without further purification.

4.2. Animals

Male Wistar-Imamichi rats at six weeks old were obtained from the Imamichi Institute for Animal Reproduction (Ibaraki, Japan) and placed in the SPF animal room set at 23 ± 2^oC and humidity of $55 \pm 15\%$, under a 12-h cycle of light/dark artificial lightning. A certified rodent diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were provided *ad libitum*. The animals at seven weeks old were used. All the rats were fasted for 16 h before the administration. The animals were used for a single treatment. The body weight of each rat was between 160–230 g. All animal experimental procedures were performed in accordance with the institutional animal care guidelines.

4.3. Preparation of treated site of skin

The dorsal hair of rats was clipped with an electric hair clipper and then shaved with an electric shaver avoiding damage to the skin under phenobarbital (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) anesthesia on the day before administration.

4.4. Preparation of LX-G and dosing solution

LX-G was prepared using LX and vehicle for LX-G in Toko Yakuhin Kogyo Co., Ltd. (Osaka, Japan). The vehicle for LX-G was made of ethanol, 1,3-butylene glycol, hydroxypropylmethylcellulose, carboxy vinyl polymer and triethanolamine. For the dosing solution for intravenous and oral administration, LX was dissolved in saline to create a solution at a final concentration of 1.25 or 3.75 mg LX/mL. Each solution was administered at a dose of 2.5 or 7.5 mg/kg, respectively.

4.5. Administration methods

LX-G (250 μL/kg) was applied to the shaved area surrounded with adhesive plaster for a patch test (Torii Pharmaceutical Co., Ltd., Tokyo, Japan) which consisted of a square hole at $2 \times 2\,cm^2$, for single dermal application. LX-G at 1, 3, or 5% was used for the dose levels of 2.5, 7.5, or 12.5 mg/kg, respectively. The rats were housed individually in cages after the application. The single dosing solution (2 mL/kg) was administered intravenously *via* the jugular vein or orally using a metal stomach tube at a dose of 2.5 mg/kg (pharmacokinetic study) or 7.5 mg/kg (tissue distribution study) to rats.

4.6. Pharmacokinetic study

LX-G was applied to the skin of the rats at 2.5, 7.5 and 12.5 mg/kg as LX, or LX was administered intravenously or orally at 2.5 mg/kg as LX (n = 4) as described above. A 0.2-mL aliquot of the blood sample was sequentially collected from the jugular vein under light disthyl ether anesthesia with a heparinized syringe at 5 min, 0.25, 0.5, 1, 2, 4, 8 and 24 h postdose. Blood sampling at 5 min was conducted only after intravenous administration. The blood samples were centrifuged at 14,000 rpm (himac CF15D, Hitachi Koki Co., Ltd., Tokyo, Japan) for 2 min at $4\,^oC$ to obtain the plasma samples. The plasma samples were stored frozen at $-20\,^oC$ until analysis.

4.7. Tissue distribution study

LX-G was applied to the skin, or LX was administered orally at 7.5 mg/kg as LX (n = 4) as described above. The rats were sacrificed by exsanguination from the abdominal aorta under ether anesthesia at 0.5, 1, 4, 8 and 24 h postdose. In dermal application to rats, $8\,cm^2$ of the skin area, and skeletal muscle from the backs (treated site) and legs (non-treated site) were sampled. After oral administration, skin and skeletal muscle samples were obtained from the legs at the same time points. The skin and skeletal muscle samples were stored frozen at $-20\,^oC$ until analysis. On the day of analysis, the skin and skeletal muscle samples were homogenized in four volumes (v/w) of 0.1 M phosphate buffer (pH 7.0).

4.8. Analytical method

The determination of LX and the *trans*-OH form in rat plasma and tissue homogenates were performed by an LC-MS/MS system consisting of Quattro Micro API coupled to Quattro LC (Waters Corporation, MA, USA). One hundred μl of 50 ng/mL of deuterium-labeled LX (d_3-LX), 100 μL of 50 ng/mL of the d_3-*trans*-OH form, and 50 μL of 1N HCl were added to the plasma samples (100 μL) and then the samples were mixed well using a vortex mixer. After adding an additional 4 mL of diethyl ether, the sample were shaken mechanically for 5 min. The samples were centrifuged at $1{,}700 \times g$ for 5 min at $4\,^oC$ and the supernatant was transferred to another vial. After evaporating the diethyl ether under a nitrogen gas stream at about 30^oC, the residue was dissolved in 100 μL of toluene/methanol (80:20, v/v) and 25 μL of ca. 10% solution of trimethylsilyldiazomethane in hexane by mixing with a vortex gently for methylation. After 1 h, the samples were evaporated under a nitrogen gas stream at about 30^oC. The residue was reconstituted to 100 μL with an HPLC mobile phase.

LX and the *trans*-OH form were determined applying an isocratic (10 mM CH_3COONH_4:CH_3CN = 60/40 (v/v)) through a XterraTM MS column (5 μm, 2.1 by 150 mm; (Waters Corporation, MA, USA)) at a flow rate of 0.2 mL/min and a column oven temperature of $40\,^\circ C$. LX, the *trans*-OH form and their d_3-compounds were detected in positive-ion mode using the mass transitions of m/z 278→201 for LX, m/z 280→185 for the *trans*-OH form, m/z 281→204 for d_3-LX and m/z 283→188 for the d_3-*trans*-OH form. The following compound parameters were used: desolvation temperature, $300\,^oC$; source block temperature, $120\,^oC$; nebulizer flow, 83 L/min; desolvation gas flow: 500 L/min; gas cell, 1.1×10^{-5} mBar. No interference peaks were observed in the blank plasma skin and skeletal muscle. The peak area ratios of analytes to internal standards were linear over a concentration range of 1-500 ng/mL in the plasma and 10-2500 ng/g tissue in the skin or skeletal muscle. In case the sample concentration was above the upper limit of quantification, the sample was diluted within the range of the determination. The correlation coefficient for the calibration curve of LX and its metabolites in the plasma, skin and skeletal muscle was 1.00 for all compounds. The intra-assay precision and accuracy was less than $\pm 15\%$ at all the concentrations except for the LLOQ. The precision and accuracy at LLOQ was less than $\pm 20\%$. The LLOQ for all compounds in the plasma was 1 ng/ml. The LLOQ for all compounds in the skin and skeletal muscle was 10 ng/g tissue. For the calculation of the mean values and the PK parameters, the concentrations below the LLOQ were taken to be zero.

4.9. Data analysis

Elimination terminal half-life ($t_{1/2}$), time to reach maximum concentration (T_{max}), maximum concentration (C_{max}), area under concentration-time curve up to the last measurable concentration ($AUC_{0\text{-}t}$), area under concentration-time curve ($AUC_{0-\infty}$), total clearance (CL_{tot}) and volume of distribution at a steady state (Vd_{ss}) were calculated individually using non-compartmental analysis techniques in WinNonlin Standard (ver. 2.1, Pharsight Corp. CA, USA). The plasma pharmacokinetic parameters were expressed as mean ± S.D. of four animals. The skin and skeletal muscle pharmacokinetic parameters were calculated using the mean concentration of LX and the *trans*-OH form in the skin or skeletal muscle. Absolute bioavailability (F) after oral administration was calculated according to the following equation:

$$\text{Absolute bioavailability} = \frac{AUC_{0-\infty(\text{p.o.})}}{AUC_{0-\infty(\text{i.v.})}} \times 100\%$$

Ratio of the $AUC_{0\text{-}t}$ after oral administration or dermal application to that after intravenous administration was calculated according to the following equation:

$$AUC_{0-t}\ \text{ratio} = \frac{AUC_{0-t(\text{p.o. or dermal})}}{AUC_{0-t(\text{i.v.})}} \times 100\%$$

Dose proportionality of the C_{max} and $AUC_{0\text{-}t}$ was evaluated by dividing the parameter by the dose.

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