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Evaluation of percutaneous absorption and skin irritation of ketoprofen through rat skin: in vitro and in vivo study

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Abstract

The influences of different mechanisms of penetration enhancers (such as menthol, azone, ethanol and nonivarnide) regarding the percutaneous absorption and skin irritation of ketoprofen formulations through rat skin were investigated by in vitro and in vivo study. The skin irritation degree at the end of the experiment (10 h) was deterinined by pathologic biopsy and colorimetry methods. In vitro, the menthol showed the most potent enhancing effect. Furthermore, the enhancement effect of a combination of menthol and nonivamide was higher than that of their individual use alone. In vivo the formulation containing 0.05% nonivantide, 5% menthol and 20% ethanol showed a higher penetration rate and an acceptable degree of skin irritation compared to a commercial product (Formax plus® gel containing 3% ketoprofen), indicating that it could be used in the clinical situation. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Ketoprofen; Azone; Nonivamide; Irritation; Colorimetry

1. Introduction

Ketoprofen is a non-steroidal anti-inflammatory, antipyretic and analgesic agent frequently used in the treatment of arthritis and mild to moderate pain (Kantor, 1986). Oral therapy of ketoprofen is very effective, but the clinical use is often limited because of the adverse effects such as irritation and ulceration of the gastrointestine. Ketoprofen possesses lower molecular mass (254.29) and a relatively short half-life (1–3 h) in plasma, and has the potential to be delivered topically (Jamali and Brocks, 1990). Furthermore, topical administration via the dermal route can bypass disadvantages of the oral route. Therefore, the transdermal drug delivery has been considered to be an ideal route for ketoprofen administration. However, the most difficult aspect of transdermal delivery system is to overcome the barrier of stratum corneum against foreign substances. The use of penetration enhancer is valuable and important for achieving therapeutic plasma levels for many drugs (Akhter and Barry, 1984; Wotton

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et al., 1985; Wu et al., 1996). It is generally accepted that penetration enhancer causes extensive damage to skin that is along with the large increase in transdermal penetration rate. As a result, the appropriate penetration rate and an acceptable level of irritation must be considered at the same time in design of an optimum transdermal formulation.

In vitro studies as to the enhancement effect of different mechanism of penetration enhancers (such as azone, cinnamic acid, cinnamyl alcohol, menthol and nonivamide) were first undertaken through rat skin. Then, the influence of simultaneous use of several enhancers in ketoprofen formulation on the percutaneous absorption and skin irritation was investigated by in vivo study. The skin irritation at the end of the experiment (l0 h) was determined by pathologic biopsy and colorimetry methods. The pharmacokinetic parameters (such as penetration rate, lag time and AUC) and skin damage degree after topical administration were used to assess the possibility of experimental formulations in clinical use by reference to a commercial product (Formax plus® gel containing 3% ketoprofen).

2. Materials and methods

2.1. Materials

The following reagents were used: ketoprofen (Sigma Chemical Company, USA), carbamic acid ethyl ester, hydroxypropyl cellulose (HPC), menthol, cinnamic acid, cinnamyl alcohol, cinnamic acid methyl ester and nonivamide, (TCI, Japan), Formax plus® gel containing 3% ketoprofen (Shiteh, Taiwan). All other chemicals and solvents were of analytical reagent grade.

2.2. Preparation of ketoprofen gels

Hydroxypropyl cellulose was dissolvedin a mixture of water and propylene glycol. Ketoprofen was dissolved in ethanol containing transdermal enhancers, separately. Then, then both components were mixed well and the resulting hydrogels were stored in air-tight containers at room temperature prior to use.

2.3. In vitro skin penetration experiments

The extent and rate of skin permeation of ketoprofen from gel formulations were determined using a modified glass diffusion cell fitted with excised rat skin (Hsu et al., 1994). The skin was mounted on the receptor compartment with the stratum comeum side facing upwards into the donor compartment and the dermal side facing downwards into the receptor compartment. The donor cell was filled with 2 g of 3% ketoprofen gel with or without various types or concentrations of enhancers and occluded by paraffin. The receptor compartment was filledwith 20 ml of pH 7.4 phosphate buffer containing 10% PEG 400 and its temperature was maintained at 37 ± 0.5 °C by thermostatic water pump during the experiment. The effective diffusion area was 2.54 cm^2. Approximately 0.5 ml of the receptor medium was withdrawn at determined intervals and replaced immediately with an equal volume of receptor solution to maintain a constant volume. This dilution of the receiver content was taken into account when evaluating the penetration data. The sample withdrawn from the receptor compartment was then analyzed by HPLC (Takayama and Nagai, 1991). Each data point represents the average of three determinations.

2.4. In vivo pharmacokinetics evaluation

Male Wistar rats weighting 180–200 g were anesthetized throughout the whole investigation with a 25% of carbamic acid ethyl ester solution (about 3 ml/Kg, intraperitoneally) and secured on their backs. In order to investigate the percutaneous absorption of ketoprofen gel, the dose of 3 mg/Kg was administered by bolus injection via the tail vein of rats. Subsequently, the experimental ketoprofen gel (45 mg /2.3 cm^2) was applied on the shaven abdomen by the occlusive dressing technique (ODT) (Naito and Tsai, 1981; Hsu et al., 1991). After both administrations, blood samples (500 μl) were taken via the jugular vein at appropriate intervals. Each blood sample was centrifuged for 2 m at $14\,000 \times g$ and the plasma sample (100 μl) was mixed with methanol (300 μl) containing *p*-hydroxybenzoate-*n*-butyl ester (3 μ1/

ml) as internal standard. The mixture was centrifuged (14 000 × *g*, 2 m) again to precipitate the denatured proteins. Then the supernatant solution was analyzed using an HPLC method same as in vitro. The coefficients of variation (cv%, $n = 6$) of the HPLC method were 7.5 and 1.1% for plasma concentration of 1 and 100 μg/ml in plasma respectively. The limitation of detection was 0.2 μg/ml.

2.5. Skin irritation evaluation by pathologic biopsy

Irritation evoked by experimental formulations on rat skin was microscopically judged after the end of experiments of in vivo percutaneous absorption. The site of application of each formula on the skin was excised and fixed in 10% neutral carbonated-buffered formalin for at least 24 h before routine processing. Each section was rinsed with running water, dehydrated using a graded series of ethanol solution and embedded in paraffin wax, and then frozen at − 20 °C prior to sectioning. The tissues were cut into small sections (6 μm) and stained with hematoxylin and eosin for histological evaluation. Ml sections were examined by light microscopy.

The microscopic findings were graded in to five levels of irritation, from no change (level 0) to a marked one (level 4) including the liquefaction of epidermis, edema of subepidermis, collagen fiber swelling and inflammatory cell infiltration in both the dermis and hypodermis, as well as degeneration of skin appendages (Takayama and Nagai, 1991). The total irritation score (TIS) was obtained by summation of each irritation score and used as an index of skin damage caused by the application of ketoprofen gel.

2.6. Skin irritation evaluation by colorimeter

In dermatology the description of the color of a lesion is of great importance (Neumann et al., 1991). However, visual evaluation of erythemal intensity is subjective and imprecise, especially regarding comparisons between the intensities of developed erythema (Nose and Tsurumi, 1993). The color change of skin surface may also be quantified using the colorimetric by CW system (Commission International del'Eclairage). The advantage of this system is that it is adapted to the non-linear color perception of human eye. The results may be meaningful in the clinical situation. This method is non-invasive and involves only one investigator, instead of several trained judges for visual scoring (Chan and Li Wan Po, 1992).

A colorimeter (Chroma Meter-CR 221, Minolta, Japan) was used as a measure of erythema color. The instrument records three-dimensional color reflectance including 'L', 'a' and 'b', as recommended by CIE. The luminance 'L' gives the relative brightness ranging from total black (0) to total white (100). The 'a' is the balance between red (100) and green (− 100) and 'b' represents the balance between yellow (100) and blue (− 100) (Pierard & Pierard-Franchimont, 1993). The change chroma (ΔC) and difference in color (ΔE) between the gel treated site and the untreated site as the control were described as follows (Westerhof et al., 1986; Fang et al., 1997).:

$$\Delta C = (\Delta a^2 + \Delta b^2)^{1/2}$$

$$\Delta E = (\Delta L^2 + \Delta A^2 + \Delta b^2)^{1/2}$$

2.7. Data analysis

In vitro, the cumulative amount of the drug penetration through rat skin was plotted as a flinction of time and a linear regression analysis was used to determine the flux and lag time of the drug. The effectiveness of penetration enhancers can be determined by penetration index (PI) which is expressed as PI = flux of drug with enhancers/flux of drug without enhancers (Wu et al., 1996).

In vivo, in order to provide a summary measure to evaluated the percutaneous absorption of experimental formulations through rat skin, the rate of penetration (*Rp*) of ketoprofen was estimated from a two-compartment model based on the assumption that the rate of penetration of ketoprofen from the gel is constant after a lag time according to the following equation (Takayama & Nagai, 1991)

$$C = \frac{R_p}{V_1 k_{10}}\left\{1 + \frac{\beta - k_{10}}{\alpha - \beta} e^{-\alpha(t - t_l)} + \frac{k_{10} - \alpha}{\alpha - \beta} e^{-\beta(t - t_l)}\right\}.$$

where C is the plasma concentration, Rp is the rate of penetration, t is time, t_L is the lag time, V_1 is the distribution volume of the central compartment, k_{10} is the elimination rate constant from the central compartment, and α & β are the hybrid first-order rate constant. The mean values of V_1, k_{10}, α and β, obtained from iv administration, were used in this study to determine Rp and t_L values (Takayama & Nagai, 1991). The $AUC_{0-10\,h}$ was calculated by trapezoidal method.

3. Results and discussion

3.1. In vitro skin penetration experiments

The cumulative amounts of drug penetration through the skin were plotted against the time as shown in Fig. 1, by which a linear relationship was obtained for each formulation ($R^2 > 0.8967$), showing that the penetration of ketoprofen from the gel was well described by the zero-order kinetics. The skin permeation parameters of ketoprofen and the penetration index (PI) of each enhancer are listed in Table 1. With the addition of 5% enhancers, it was found that menthol showed the most potent enhancing effect, followed by azone, cinnamyl alcohol and cinnamic acid, respectively. The PI of menthol was increased about 56.5 fold. Earlier reports (Williams and Barry, 1991; Kabayashi et al., 1994) indicated that the enhancing effect of menthol was mainly due to an increase in diffusion and it was speculated that the menthol might increase the partition of lipophilic drug. Moreover, menthol has the advantage of a shorter lag time; therefore, it has been broadly used in many topical formulations containing nonsterodial anti-inflammatory drugs to increase the pharmaceutical effects (Morimoto et al., 1993). A similar result, a shorter lag time was observed in this study. The flux of ketoprofen gel increased with an increase in menthol concentration and the maximum steady-state flux of ketoprofen was observed with 5% menthol formulation indicating that the value may be the optimal concentration for ketoprofen to attain the effective penetration capability. In addition, the potent effect of enhancement and skin damage of

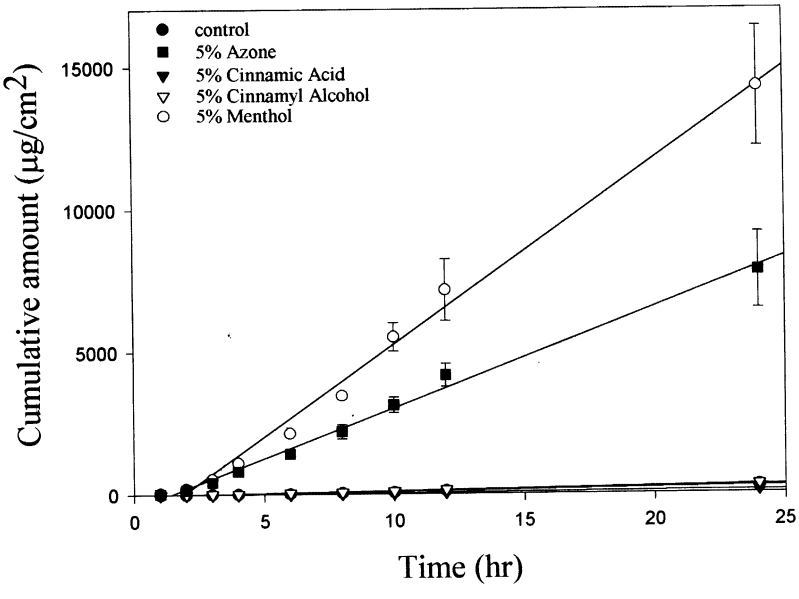

Fig. 1. In vitro penetration-time profile of 3% ketoprofen gel with or without various enhancers through rat skin. ($n = 3$).

Table 1
Flux, lag time and cumulated amount of ketoprofen through rat skin from different formulations in vitro permeation studies

Formulations[a]	%	Flux (μg/cm^2 h)	Lag time (h)[c]	Cumulative amount at 24 h (μg/cm^2)	PI[b]
Control		11.66 ± 1 3.95	3.25 ± 0.08	259.71 ± 90.6	1
Azone	5	352.91 ± 54.97	1.39 ± 0.27	7814.3 ± 1333.0	30.3
Cinnamic acid	5	5.17 ± 2.29	3.56 ± 0.19	115.8 ± 52.7	0.4
Cinnamyl alcohol	5	24.84 ± 3.55	2.95 ± 0.40	553.4 ± 303.1	2.1
Menthol	1	13.54 ± 3.32	3.64 ± 0.37	306.5 ± 70.4	1.2
	3	463.25 ± 276.17	3.04 ± 0.94	10 188.8 ± 5700.9	39.7
	5	658.70 ± 92.88	1.78 ± 0.21	14 280.0 ± 2108.3	56.5
	7	682.84 ± 56.14	1.12 ± 0.74	15 220.8 ± 1206.3	58.6
	10	714.42 ± 67.77	0.73 ± 0.04	15 933.3 ± 1514.4	61.3
Nonivamide	0.025	27.66 ± 28.31	2.97 ± 0.81	622.0 ± 635.7	2.4
	0.050	50.31 ± 8.39	2.58 ± 0.34	1134.3 ± 186.7	4.3
	0.075	58.75 ± 10.42	2.63 ± 0.21	1316.5 ± 228.1	5.0
	0.100	39.87 ± 19.30	1.72 ± 1.66	870.3 ± 170.3	3.4
Menthol & Nonivamide	5.000 0.025	953.19 ± 265.66	2.28 ± 0.11	20 445.8 ± 6174.2	81.7

[a] Each value represents the mean ± S.D. ($n = 3$).
[b] PI: penetration index = $\text{Flux}_{\text{with enhancement}}/\text{Flux}_{\text{control}}$.
[c] Lag time: the intercept on the time axis of the steady state flux calculated by linear regression.

menthol derivatives on ketoprofen had reported (Obata et al., 2000). It would be presumed that menthol was a useful and available enhancer for ketoprofen.

Azone has been shown to be effective in enhancing the permeability of many compounds through the stratum corneum (Akhter and Barry, 1984; Wotton et al., 1985; Hosoya et al., 1987). The mechanism of action of azone is suggested to influence the lipid fluidizing and to alter the keratin structure on stratum corneum lipids (Lambert et al., 1989). In this study, the flux of ketoprofen for the control base was increased 30.3 fold with incorporation of 5% azone. The PI of cinnamic acid and cinnamyl alcohol were 0.4 and 2.1, respectively, showing non-enhancement effect.

Capsaicin has an antunflammatory, antinociceptive effect for treatment joint of inflammation because it can deplete sensory neurous containing substance *P*, calcitonin gene-related peptide and neurokinins, and then increase the nociceptive thresholds (Games 1982; Lahann and Farmer, 1983; Hayes et al., 1984). Moreover, Degim et al., 1999 pointed out that the Capsaicin has the enhancement effect for naproxen because of a similar chemical structure to azone, and also it has potential antunflammation activity in its own right (lowering subatance *P*). It may be possible to create synergistic effect, and formulations may be created that are more effective than capsaicin or naproxan alone. On the other hand, because of the burning pain sensation and erythema, its clinical use and commercially available concentration (0.025 and 0.075%) are limited (Fuchs et al., 1999). Nonivaminde is a synthetic analogue of capsaicin. The pharmacological and pungent profiles of nonivamide were found to be similar to those of capsaicin, and it has been used as a substitute for capsacin in neuro-physiological studies (Szolcsanyi and Jancso-Gabor, 1975; Hayes et al., 1984; Fang et al., 1995). In this study, we compared the enhancement effect of various concentrations (0.025 − 0.1%) of nonivamide. The results showed nonivamide had an enhancement effect at low concentration (0.025%), but the enhancement effect did not conspicuously increase with an increase in nonivamide concentration. The lag time of ketoprofen gel tended to decrease while the concentration of nonivamide increased. In addition, when menthol (5%) and nonivamide (0.025%) were used simultaneously (PI = 81.7), the enhancement effect was higher than that of menthol (PI = 56.5) or noni-

vamide (PI = 2.4) alone, as shown in Table 1. According to previous study (Morimoto et al., 1993), the combination of enhancers with different mechanisms or sites of action obtains a synergistic effect. Similarly, the menthol and nonivamide provided different mechanisms to enhance the penetration of ketoprofen gel through rat skin, and had a synergistic effect.

3.2. In vivo pharmacokinetics evaluation

From above in vitro results, the menthol showed the highest enhancement effect. Since nonivamide possessed both enhancing effect for ketoprofen and pharmacological activity, it may be possible to create synergistic effects when combined with ketoprofen. The amount of solvent in the formulation was another important influencing factor for penetration. In order to easily design an optimum formulation, the amount of menthol (0–10%), nonivamide (0–0.1%) and ethanol (20–40%) were simultaneously added in various experimental formulations as shown in Table 3. The penetration effect and skin irritation of these formulations were evaluated through rat in vivo and compared to commercially available product of ketoprofen gel.

In pharmacokinetic study, the solution of ketoprofen in pH 7.4 phosphate buffer was administrated by bolus injection in rats (3 mg/Kg). The plasma concentrations of ketoprofen after iv administration declined in a biexponential manner as shown in Fig. 2. The plasma level of ketoprofen was adequately described using a two-compartment open model ($R^2 > 0.980$) by utilizing the least-square fit program (PCNONLIN, SCI, Software, USA). The calculated parameters are listed in Table 2. The pharmacokinetics of percutaneous absorption of drugs have been widely discussed and several effective models have been developed for understanding the absorption behavior of drug through the skin (Naito and Tsai, 1981; Guy et al., 1982; Takayama and Nagai, 1991; Ogiso et al., 1989; Huang et al., 1993). From above in vitro results, the penetration profile of ketoprofen exhibited a zero-order permeation at a constant penetration rate, so a two-compartment model, which was consistent with the zero-order penetra-

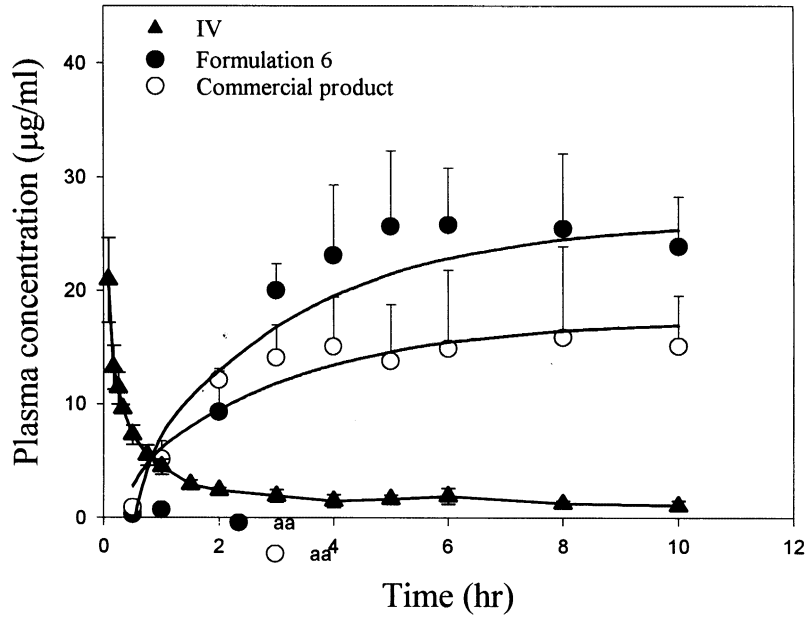

Fig. 2. Plasma concentration—time profile after iv (3 mg/Kg) and topical (45 mg /2.3 cm^2) administration. The solid line shows simulated curve for ketoprofen gels. ($n = 3$).

Table 2
Pharmacokinetic parameters of ketoprofen after intravenous administration (3 mg/Kg)

Parameters	Value[b]
α (h^{-1})[a]	6.32 ± 12.21
β(h^{-1})[a]	0.35 ± 0.25
$t_{1/2}$ (h)[a]	3.12 ± 2.51
k_{12} (h^{-1})[a]	3.64 ± 1.40
k_{21} (h^{-1})[a]	1.76 ± 1.29
k_{10} (h^{-1})[a]	1.27 ± 0.50
V_t (ml)[a]	20.92 ± 6.00
V_{ss}(ml)[a]	76.18 ± 28.00
AUC_{0-10} (μg h/ml)[a]	24.70 ± 3.50
$AUC_{0-\infty}$ (μg h/ml)[a]	26.83 ± 11.32

[a] α and β are the hybrid first-order rate constant; $t_{1/2}$ elimination half-life at β phase; k_{12} is rate constant from the central to tissue compartment; k_{21} is rate constant from the tissue to central; compartment; k_{10} is elimination rate constant from the central compartment; V_t and V_{ss} are the distribution volume of central and tissue compartment; AUC_{0-10} was calculated by the trapezoidal method; $AUC_{0-\infty}$ $A/\alpha + B\beta$.
[b] Each value represents the mean 15.D. ($n = 4$).

tion rate, was employed to explain the plasma-time curve for ketoprofen after topical administration in this study (Takayama and Nagai, 1991). As shown in Fig. 2, the plasma levels of ketoprofen appeared to be described adequately using this two-compartment open model (AIC = 10–40). The pharmacokinetic parameters of ketoprofen through topical administration including penetration rate (P_R) and lag time (t_L) are listed in Table 3. There were significant differences in these formulations: P_R from 14 to 693 μg/h and t_L from 0.03 to 0.57 h, respectively. Formulation 6 had the highest penetration rate, which was about 50 times higher than that of Formulation 4. This revealed that the concentration of excipients greatly affected the penetration absorption of ketoprofen gel through rat skin. As shown in Table 3, the penetration rate increased with an increase in nonivamide content from 0 to 0.1% and the maximum penetration rate observed was 0.05%. In addition, increasing the menthol content from 0 to 10% led to an increase in penetration rate. These results were consistent with the above in vitro study. In contrast, the penetration rate declined when concentration of ethanol was increased from 20 to 40%. This result might be due to the fact that a large amount of alcohol would increase the affinity of lipophilic

Table 3
Experimental design formulations and permeation parameters of 3% ketoprofen gels through rat in vivo

Trial[e]	NVA[a] (%)	Menthol (%)	Alcohol (%)	Penetration rate[a,*] (μl/h)	Lag time[b,*] (h)	AUC_{0-10}* [c](μg h/ml)	TIS[f]
1	0	5	30	20.96 ± 4.43	0.41 ± 0.01	4.97 ± 1.9	7.00 ± 2.00
2	0.05	5	30	$169.40 \pm 81/25$	0.57 ± 0.02	37.6 ± 21.9	10.00 ± 5.29
3	0.10	5	30	125.03 ± 82.11	0.55 ± 0.02	31.0 ± 21.2	7.67 ± 1.15
4	0.05	0	30	14.33 ± 1.86	0.03 ± 0.06	4.3 ± 0.5	7.33 ± 1.15
2	0.05	5	30	169.40 ± 1.86	0.57 ± 0.02	37.6 ± 21.9	10.00 ± 5.29
5	0.05	10	30	386.91 ± 53.59	0.57 ± 0.00	99.9 ± 13.1	11.67 ± 4.04
6	0.05	5	20	693.17 ± 168.30	0.52 ± 0.01	181.7 ± 44.9	13.00 ± 3.00
2	0.05	5	30	169.40 ± 81.25	0.57 ± 0.02	37.6 ± 21.9	10.00 ± 5.29
7	0.05	5	40	23.90 ± 6.78	0.26 ± 0.12	6.5 ± 1.7	6.00 ± 6.29
8	Commercial product			462.23 ± 162.53	0.17 ± 0.28	128.5 ± 41.6	12.67 ± 0.58

* Significant differences among these formulations (Anova, $P < 0.01$).
[a] 4,1,7,3,2, 5,8, 6: there are non-significant differences among these formulations underlined and significant differences with other formulations.
[b] 4,8,7, 7,1, 1,6,3,2,5: there are non-significant differences among these formulations underlined and significant differences with other formulations.
[c] 4,1,7,3,2, 5,8, 6: there are non-significant differences among these formulations underlined and significant differences with other formulations
[e] NVA, Nonivamide.
[f] TIS, total irritation score.

drug (ketoprofen) in the hydrophilic gel bases, and then decrease the release of drug from the bases.

A comparison was made between the experimental formulation and commercially available preparations (Formax plus® gel) containing 3% ketoprofen. As shown in Table 3, the penetration rate and $AUC_{0-10\,h}$ of Formulation 6 was higher than that of the commercial product. The lag time of commercial product (0.17 h) was shorter than for Formulation 6 (0.52 h). Without direct information about ingredients of the commercial formulation, it is not possible to state precisely what ingredients strategies have been used. However, the above results indicate that the experimental Formulation 6 containing menthol and nonivamide as enhancers possessed good penetration and is worthy of study.

3.3. Skin irritation evaluation

To evaluate the influence of ingredients on the skin irritation, the skin was pathologically investigated after application of the ketoprofen gels. Fig. 3A, B and C are microscopic photographs of rat skin at 10 h after with or without treatment with Formulatiom 6 and the commercially available product, respectively. It was found that the main region of destruction was in the epidermis, dermis and hypodermis of skin. The total irritation scores (TSI) of skin are listed in Table 3. The results revealed that the damage degree of skin (TSI) tended to increase slightly with the increases in penetration rate (P_R). There was no significant difference ($P > 0.05$) in TIS between the Formulation 6, the penetration of which was highest, and the commercial product, indicating that the damage degree of Formulation 6 was acceptable.

According to previous study (Kilo et al., 1994; Fang et al., 1997), the skin is reddened and slightly oedematous with treatment by capsaicin and its analogues. In order to quantify and compare the skin erythema and inflammation, colorimetric method is utilized in this study. As shown in Fig. 4, the value of color difference (ΔL, Δa, Δb, ΔC and ΔE) between the gels treated site and the neighboring normal skin represents the degree of erythema. There was no significant difference between the experimental formulations and the commercial product except for the Formulation 5, in which Δb was higher. The results indicated that these experimental formulations containing noni-

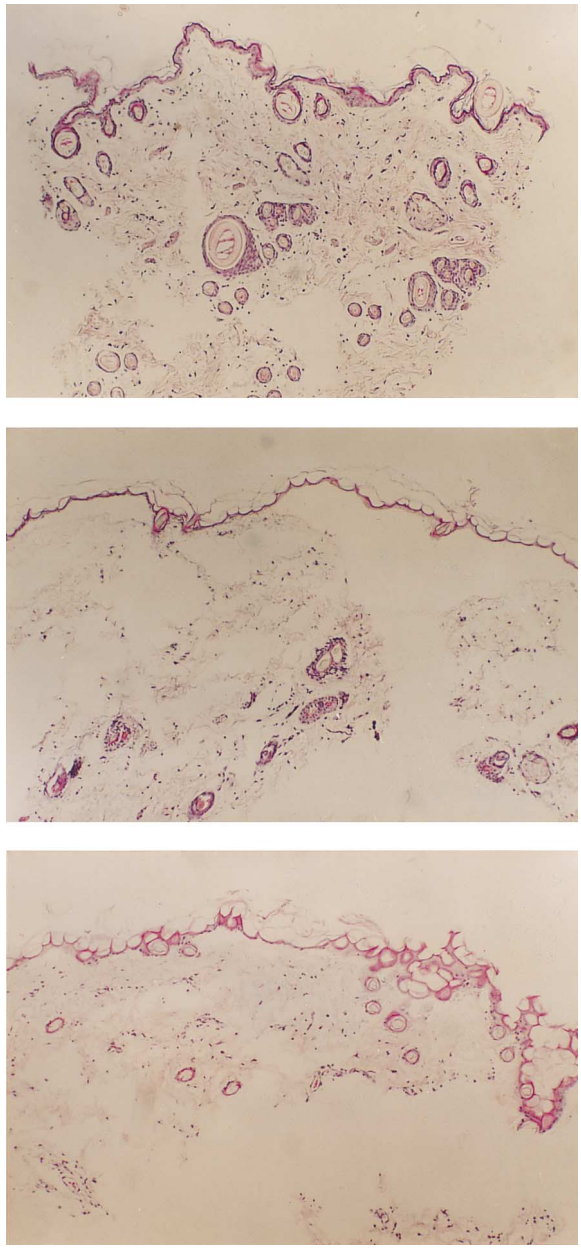

Fig. 3. Microscopic photos of rat skin at 10 h after with and without treatment with Formulation 6 and the commercial available product. A: without treatment; B: treated with Formulation 6; C: treated with the commercial available product.

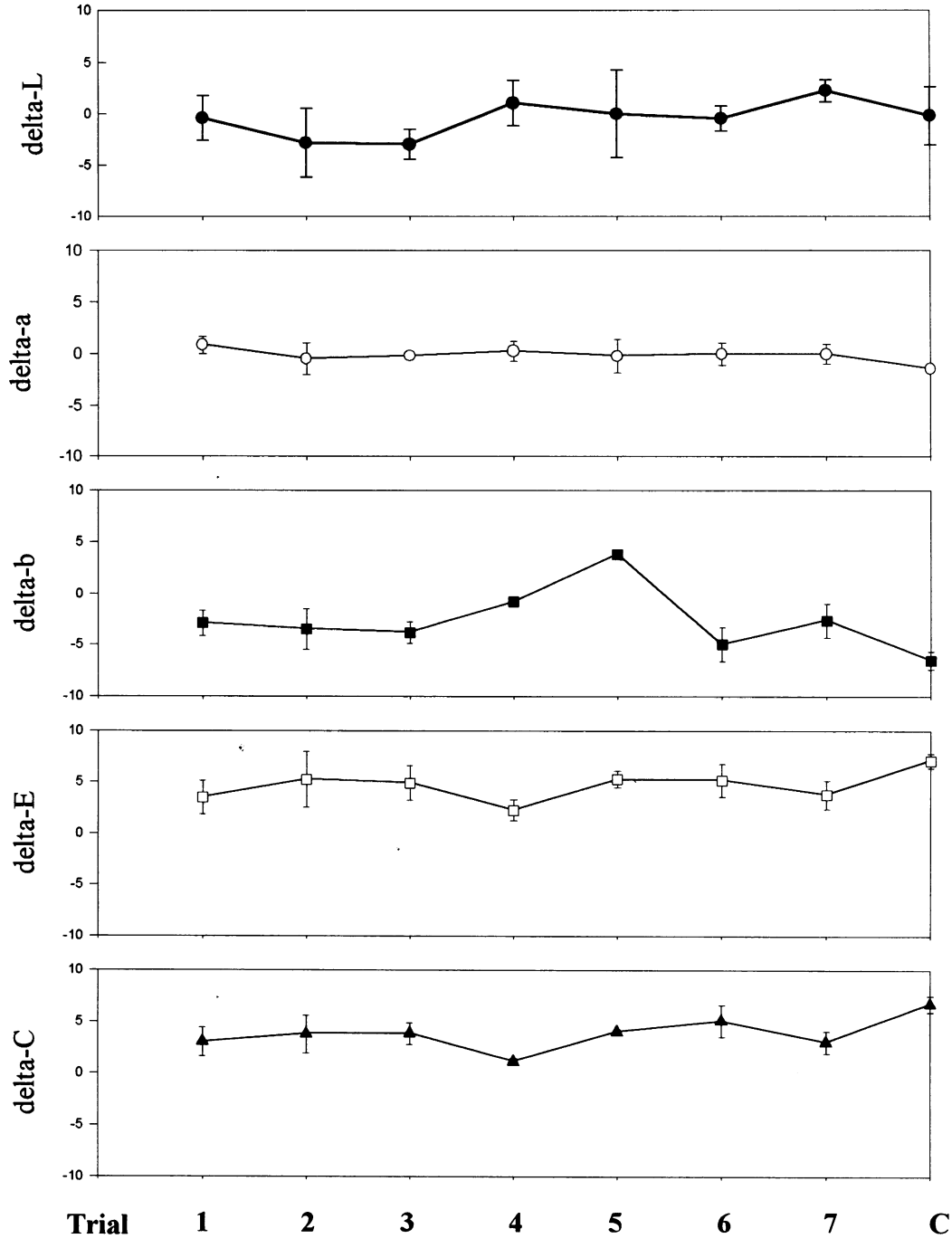

Fig. 4. Color differences measured by simpler indexes of colorimetry between treated sites and neighboring untreated sites after topical administration (45 mg /2.3 cm^2) (trial 1–7 and C). C: commercial product. ($n = 3$).

vamide did not induce the irritation reaction of skin. It might be suggested that the combination of ketoprofen that possessed the inhibitory effect of erythema led to a decrease in irritation of skin evoked by nonivamide (Otterness et al., 1979). However, change of skin color of Formulation 5 and Formulation 6, which had higher penetration rates, were similar with that of the commercial product, indicating that the experimental formulations could be used in the clinical situation.

Acknowledgements

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