ELSEVIER

International Journal of Pharmaceutics 128 (1996) 251–260

international journal of pharmaceutics

Evaluation of skin irritation of percutaneous absorption promoters by means of fractal dimension of rat skin structure

Jiro Akimoto[a], Kozo Takayama[a,*], Koichi Isowa[b], Tsuneji Nagai[a]

[a]*Department of Pharmaceutics, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan*
[b]*The Center of Japan Biological Chemistry Co. Ltd., Fukue 52, Kaizu-cho, Kaizu-gun, Gifu-ken 503-06, Japan*

Received 23 February 1995; revised 10 June 1995; accepted 11 August 1995

Abstract

A fractal dimension of skin structure was determined in order to evaluate the pathological damages evoked by percutaneous absorption promoters such as *d*-limonene and Azone in the hairless rat. As a model dermatological system, indomethacin hydrogels containing each of these enhancers were applied to the hairless rat abdominal skin. The application site of the skin was excised, fixed with glutaraldehyde and osmic acid, and then lyophilized. Boundary lines between the outermost lipid layer and the underlying protein parts in the skin cross section were taken by the CCD camera as image data, and these images were fed into a computer to estimate the fractal dimension. The boundary line of the skin cross section was observed to be a typical fractal morphology and the structure changes of skin evoked by the promoters could be quantified as a non-integral fractal dimension. A linear relationship between the fractal dimension and pathological irritation score was clearly observed, suggesting the usefulness and reliability of the fractal dimension as a novel method for evaluating the skin damage.

Keywords: Percutaneous absorption; Skin irritation; Fractal dimension; Color reflectance; *d*-Limonene; Azone

1. Introduction

A transdermal drug delivery system, which provides a desirable route of drug administration, can be developed by promoting the permeability of drugs through the skin. Many compounds such as Azone and its analogues (Okamoto et al., 1988; Okamoto et al., 1990; Michniak et al., 1993), polyunsaturated fatty acids (Cooper, 1984), sulphoxides and cyclic monoterpenes (Okabe et al., 1989; Okabe et al., 1992; Obata et al., 1990) have been found to be effective as percutaneous absorption promoters (Santus and Baker, 1993). However, serious skin damage is often caused by the application of these absorption promoters. Thus, the skin irritation evoked by these promoters should be evaluated in detail in addition to their enhancing activity. Primary irritation scores judged by the naked eyes or microscopic findings of the skin cross section have widely been used as a direct index in order to evaluate the skin irrita-

* Corresponding author.

SSDI 0378-5173(95)04316-3

tion (Okabe et al., 1990; Takayama and Nagai, 1991; Quan et al., 1991). One of the shortcomings of these methods is the subjectivity of judging the skin damages as an irritation score. Namely, it is difficult to obtain objective and quantitative indices by using such methods.

Visual assessments for the skin irritation are roughly classified into two categories; erythema and eschar formation, and edema formation. The skin damage such as the hydration and/or edema formation induced by the application of absorption promoters may be accompanied with the morphological change of skin surface in a greater or lesser degree. In this study, we applied the fractal geometry introduced by Mandelbrot, 1983 to quantify the morphology of boundary lines between outermost lipid layers and underlying protein parts in the skin vertical cross section. In the fractal image analysis, a general concept of integral dimensions is extended so as to include non-integral dimensions which account more accurately for the irregularities of boundary images often observed in nature. The concept of fractal dimension has widely been used to quantify the irregular morphology in the pharmaceutical sciences (Koch, 1993; El-Arini, 1993; Bonny and Leuenberger, 1993). A possibility of fractal analysis which can objectively and quantitatively assess skin damages such as the hydration and/or edema formation was investigated. To estimate the fractal dimension of skin cross sectional image data, we employed a box-counting algorithm (Koch, 1993). If the boundary line being analyzed is covered with squares of different sizes (r), then the number of boxes (N) required to cover the boundary line increases indefinitely according to the relation $N \propto r^{-D}$, where D is the fractal dimension. Thus, the fractal dimension could be used as an index of roughness in the cross sectional skin structure. Further, color changes on the skin surface brought about by the application of promoters were monitored with a chromameter, since the skin color reflectance has been reported as suited for quantification of erythema (Wilhelm et al., 1989; Serup and Agner, 1990; Lahti et al., 1993). As a model dermatological system, indomethacin hydrogels containing *d*-limonene or Azone were applied to the hairless rat abdominal skin. Finally, the applicability of these indices such as the fractal dimension and the skin color reflectance was examined by comparison with the microscopic observation of the skin damage caused by the application of these promoters.

2. Materials and method

2.1. Materials

d-Limonene was purchased from Tokyo Chemical Industries Co. Ltd., Tokyo, Japan; Azone (1-dodecylazacycloheptan-2-one) was supplied from Sumisho-Nelson, Tokyo, Japan. The carboxyvinyl polymer, marketed as 'Hiviswako 105' was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Indomethacin (IMC) was purchased from Sigma Co. (St. Louis, MO, USA). Other chemicals used were of reagent grade.

2.2. Preparation of hydrogel ointments

The alcoholic hydrogel ointments were prepared as follows: 2% of carboxyvinyl polymer was first dissolved in 51.5 ~ 54.5% of distilled water and 2.5% of triethanolamine was then added to this gel solution. Separately, 1% of IMC was dissolved in 40% ethanol with 0 ~ 3% of promoters. Both components were then mixed thoroughly.

2.3. Application of hydrogel ointments

Male WBN ILA-Ht hairless rats weighing 180–200 g were used. After anesthetization with ethyl carbamate saline solution, glass cells (12 mm inner diameter, 15 mm height) were attached to the abdominal part of hairless rats. The alcoholic hydrogels (0.7 ml) under test were applied and glass cells were covered with parafilm to prevent evaporation of volatile components in the hydrogels. At 6 h after application, hydrogels were removed and then abdominal skin was excised. The excised skin was washed with 0.1 M phosphate buffer solution (pH 7.3) several times and then used for further treatments.

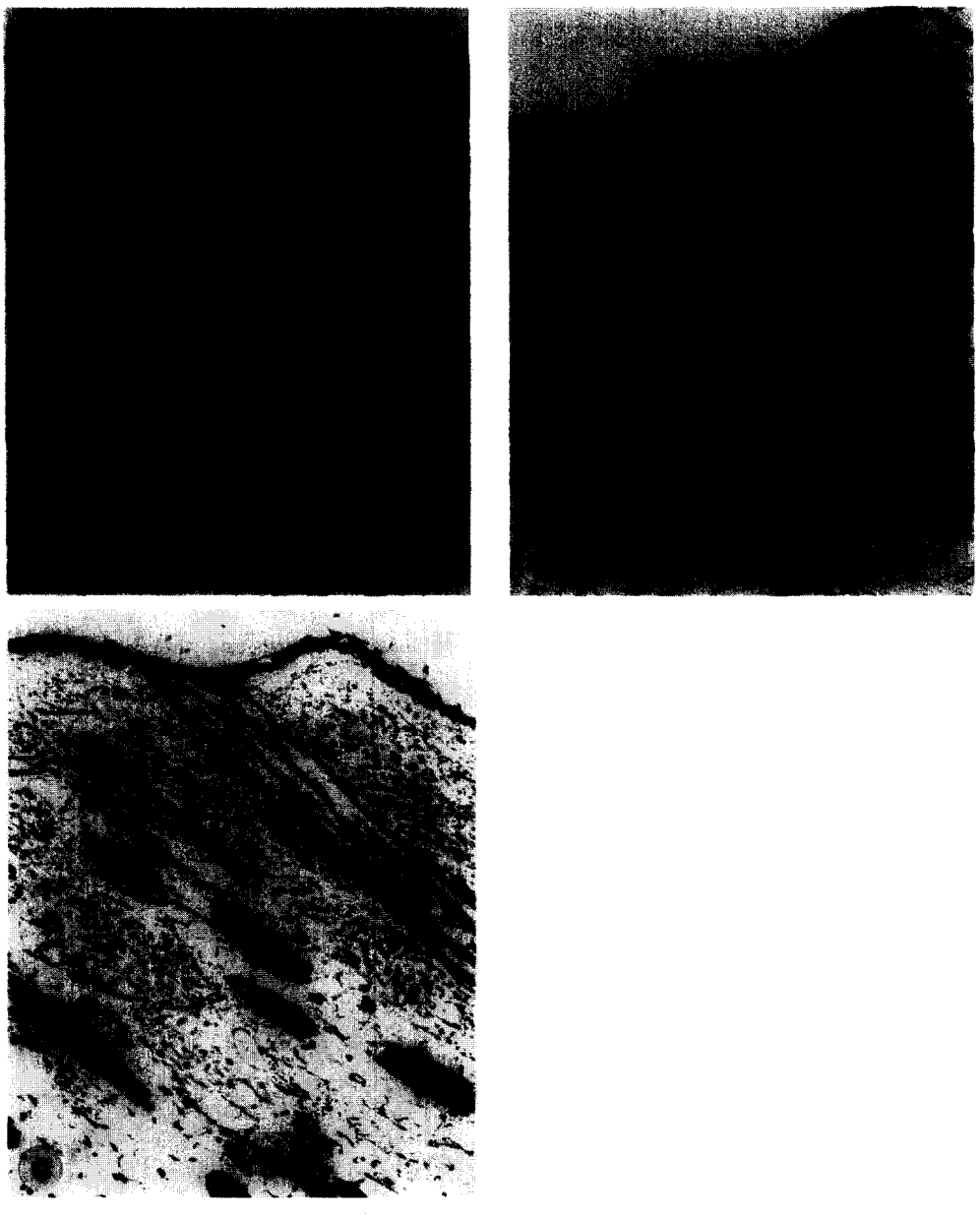

Fig. 1. Microphotographs of vertical section of hairless rat abdominal skin at 6 h after application of indomethacin hydrogels. H and E stain. × 100. (a) without promoter, (b) 2% *d*-limonene (c) 2% Azone.

2.4. Pathological study

The separated skin was fixed in 10% neutral carbonate-buffered formalin for at least 24 h before routine processing and then cut vertically against the skin surface at the central region in 4-mm widths. Each section was dehydrated using a graded series of ethanol solutions and embedded

Table 1
Pathological findings of hairless rat abdominal skin at 6 h after application of indomethacin hydrogels containing 0-3% *d*-limonene and 40% ethanol

Pathological findings	Concentration of *d*-limonene in hydrogels (%)			
	0	1	2	3
Epidermis: liquefaction	0 0 0 0 0	2 2 2 2 3	3 3 3 3 3	3 3 3 3 3
Subepidermis: edema	0 0 0 0 0	2 2 2 2 2	3 3 2 2 3	3 3 3 2 2
Dermis: collagen fiber swelling	0 0 0 0 0	2 2 2 2 2	2 3 2 2 3	3 3 3 3 3
Hypodermis: collagen fiber swelling	0 0 0 0 0	0 2 1 0 2	2 3 2 3 3	4 4 3 3 3
Hypodermis: inflammatory cell infiltration	0 0 0 0 0	0 0 0 0 0	1 2 0 2 2	2 2 2 1 2
Skin appendages: degeneration	0 0 0 0 0	0 0 0 0 0	1 2 0 2 3	3 3 2 2 2
Total irratation score: mean ± S.D.	0	7.2 ± 1.3	13.8 ± 3.3	16.2 ± 1.8

0, No change; 1, very slight; 2, slight; 3, moderate; 4, marked.

Table 2
Pathological findings of hairless rat abdominal skin at 6 h after application of indomethacin hydrogels containing 0-3% Azone and 40% ethanol

Pathological findings	Concentration of Azone in hydrogels (%)			
	0	1	2	3
Epidermis: liquefaction	0 0 0 0 0	4 3 3 1 4	3 4 4 4 4	4 4 4 4 4
Subepidermis: edema	0 0 0 0 0	4 3 3 1 3	3 2 3 3 4	4 3 2 2 2
Dermis: collagen fiber swelling	0 0 0 0 0	3 3 3 2 3	3 3 3 3 4	4 4 4 4 4
Hypodermis: collagen fiber swelling	0 0 0 0 0	3 0 0 0 2	3 3 3 3 4	4 4 4 4 4
Hypodermis: inflammatory cell infiltration	0 0 0 0 0	0 2 1 0 3	2 1 3 3 3	1 2 2 3 3
Skin appendages: degeneration	0 0 0 0 0	3 2 0 0 3	3 3 3 3 3	4 4 3 4 4
Total irritation score: mean ± S.D.	0	12.4 ± 5.7	18.6 ± 2.3	20.6 ± 0.9

0, No change; 1, very slight; 2, slight; 3, moderate; 4, marked.

in paraffin wax. Tissues were divided into small pieces (about 3 μm in thickness) and stained with hematoxylin and eosin. All sections were examined by optiphoto light microscopy.

2.5. Determination of fractal dimension

The excised skin samples were immersed in 2% glutaraldehyde solution for the protein fixation for 2 h. After being soaked in 7.5% saccharose buffer solution for 24 h, the samples were again immersed in 1% osmic acid solution for the fixation of lipid layer (mainly stratum corneum) for 2 h. The samples were dehydrated using a graded series of ethanol solution (60 ~ 100%) and then ethanol was replaced with *t*-butanol. After lyophilization (ES-2030, Hitachi Ltd, Tokyo, Japan), the vertical cross section of the skin was prepared with a microtome. The boundary line between the outermost lipid layer and the lower

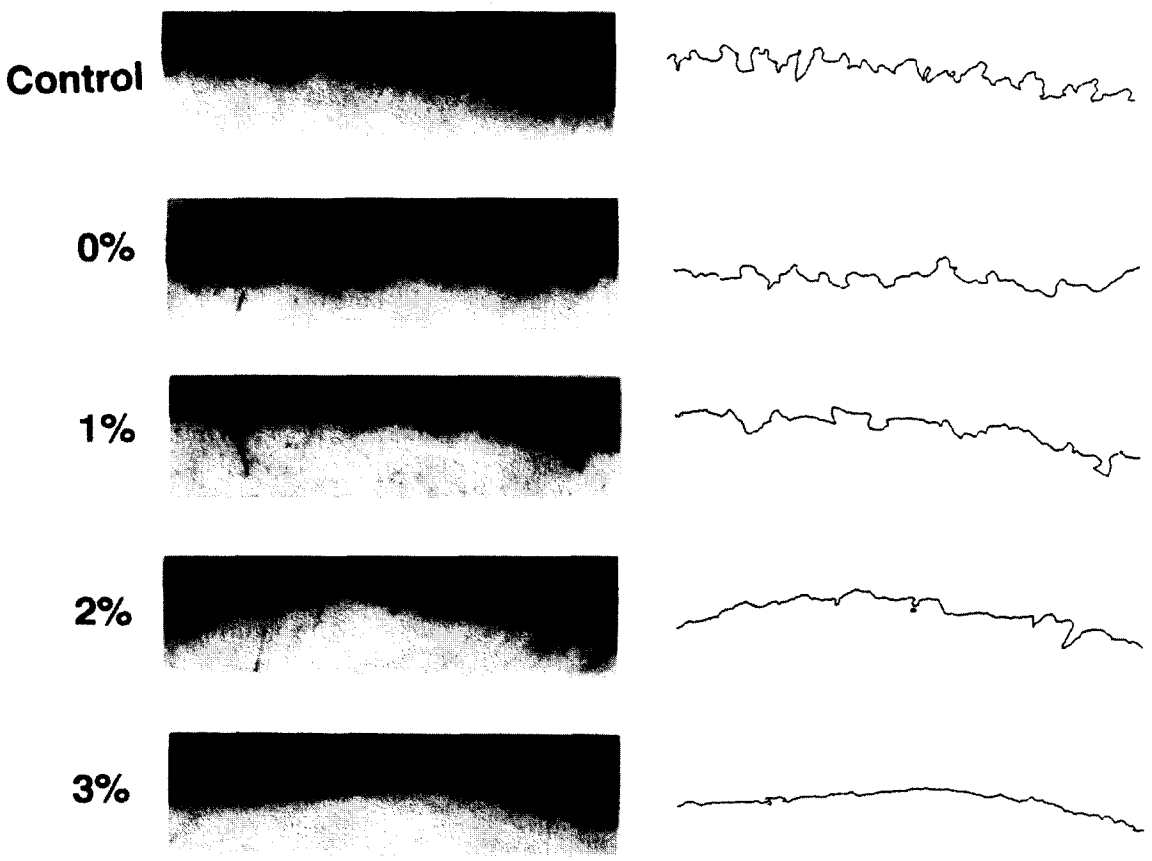

Fig. 2. Microphotographs and boundary images of vertical section of hairless rat abdominal skin at 6 h after application of indomethacin hydrogels containing 0–3% *d*-limonene. Control means untreated skin.

protein part of the skin cross section was monitored with a CCD camera. Image data taken by the CCD camera were fed into a desk-top digital computer (PC-9801 BA2, NEC Corp., Tokyo, Japan) with a scanner (Image Scanner HS60F, OMRON, Tokyo, Japan) and then analyzed by a self-made computer program developed for the data processing.

2.6. Determination of skin color reflectance

The color change on the skin surface after the application of promoters was measured by a Minolta chromameter (CR-200, Minolta, Tokyo, Japan). A Minolta chromameter has recently been used for the quantification of surfactant-induced erythema (Agner and Serup, 1990; Wilhelm et al., 1994) and the erythema in a soap chamber test (Babulak et al., 1986). Color is expressed in a 3-dimensional space, L*a*b* as recommended by Commission Internationale de l'Eclairage, CIE (Robertson, 1976). The L* value (luminance) expresses the relative brightness of the color, ranging from completely black (L* = 0) to white (L* = 100). The a* value gives the balance between red and green (+ 100 to − 100) and b* represents the color spectrum from blue (+ 100) to yellow (− 100). Before starting the experiment, the instrument was calibrated to a standard white plate. The change of skin color reflectance brought about by the application of hydrogels containing

each promoter was defined by the following equation: $\Delta E^*ab = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$, where Δ means the difference between colorimetric values (L*, a* and b*) of the abdominal skin before and after the application of hydrogels.

3. Results and discussion

3.1. Pathological findings

In general, the skin is described in term of three tissue layers: the stratified, avascular, cellular epidermis, the underlying dermis of connective tissue and the subcutaneous fat layer. In addition, the highly vascularized dermis and the epidermis support several skin appendages: eccrine, apocrine, sebaceous glands and hair follicles. The pathological findings of the above-mentioned three tissue layers were discussed. For example, Fig. 1 shows a microscopic photo of hairless rat skin at 6 h after application of indomethacin hydrogels containing *d*-limonene or Azone. The three layers of skin tissue (epidermis, dermis and hypodermis) showed almost no change at 6 h after application of hydrogels not containing promoters (Fig. 1a). However, the hairless rat skin treated with hydrogel containing 2% *d*-limonene, showed moderate liquefaction in the epidermis and collagen fiber swelling in the dermis. Furthermore, collagen fiber swelling and inflammatory cell infiltration were observed in the hypodermis (Fig. 1b). As did *d*-limonene, 2% Azone in the hydrogel also caused skin damages such as liquefaction, edema and collagen fiber swelling (Fig. 1c). Pathologically, the skin section was divided into several parts; epidermis, dermis, hypodermis and appendages. As Table 1 and Table 2 show, the damage to each part evoked by *d*-limonene and Azone was classified into five levels, i.e., no change (0), very slight (1), slight (2), moderate (3) and marked (4). The total irritation score (TIS) was defined as the summation of damage levels in every part. The extent of skin damage increased with increasing concentration of promoters although each promoter exhibited a different tendency. The skin irritation of Azone was relatively stronger than that of *d*-limonene. In both promoters, the increase of the applied concentration led to an increase of TIS values.

3.2. Fractal dimension

Fig. 2 shows microphotographs and boundary images of the vertical cross section of hairless rat abdominal skin at 6 h after application of indomethacin hydrogels containing 0 ~ 3% *d*-limonene. The boundary line of untreated hairless rat skin was observed to be extremely rough. However, the roughness of the boundary line was gradually decreased with an increasing concentration of *d*-limonene. A similar result was obtained with Azone. Fig. 3 shows a double logarithmic plot of the number of squares, *N*, entered as a function of square size, r, on the boundary line of untreated hairless rat skin (control), based on the box-counting algorithm. The excellent linearity with a significantly high correlation coefficient ($r = -0.999$) suggested that the skin cross section characterized by the boundary line was a typical fractal. The fractal dimension, D, for the control skin was estimated to be 1.120. Thus, the irregularity of cross sectional skin structure was quantified as a non-integral fractal dimension. Fig. 4 shows the fractal dimension of hairless rat skin at 6 h after application of indomethacin

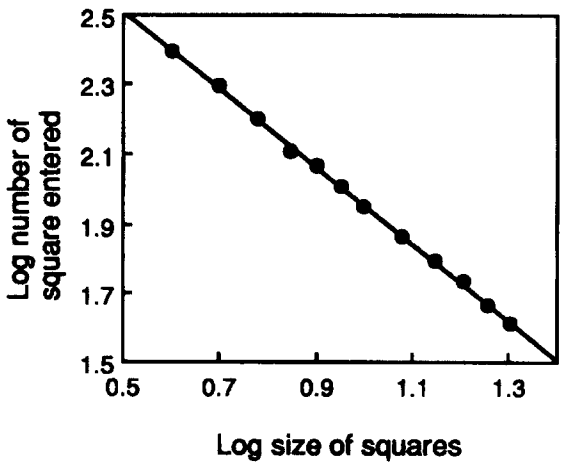

Fig. 3. Double logarithmic plot of the number of squares entered as a function of square size on the boundary line of untreated hairless rat abdominal skin. $r = -0.999$. D = 1.120.

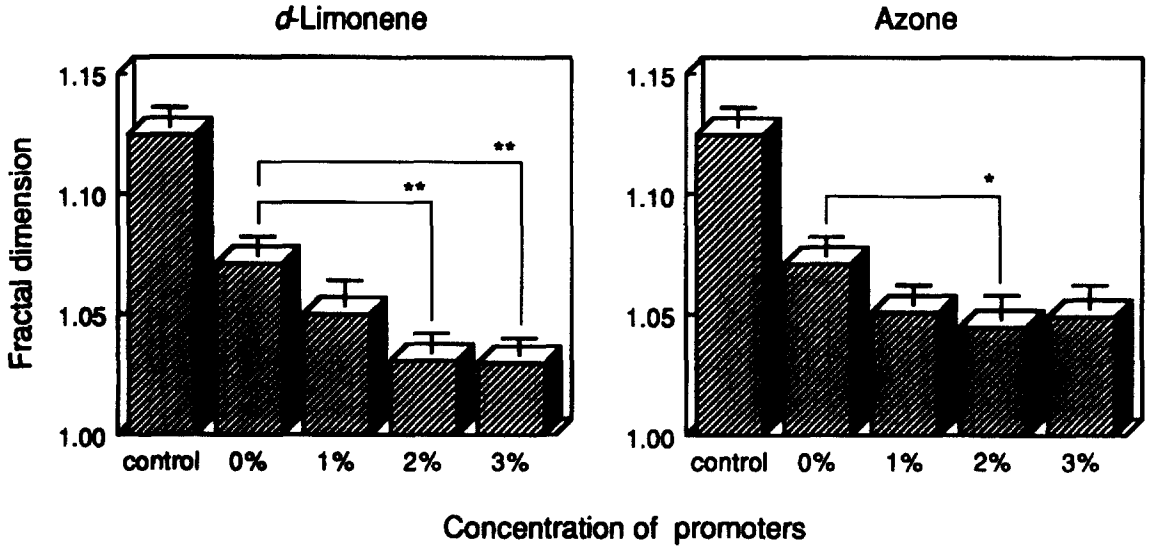

Fig. 4. Fractal dimension of hairless rat abdominal skin at 6 h after application of indomethacin hydrogels. Each column represents the mean ± S.E. (n = 11 ~ 17). Control means untreated skin. *P < 0.05, **P < 0.01.

hydrogels containing d-limonene and Azone. The fractal dimension markedly decreased when the skin was treated with hydrogels not containing promoters. Then the fractal dimension significantly decreased with increasing d-limonene concentration, compared to the hydrogel without d-limonene ($p < 0.01$). However, the degree of decrease in fractal dimension was poor when Azone was applied to the skin. Only in the case of 2% Azone, was fractal dimension significantly decreased, compared to the hydrogel without Azone ($P < 0.05$). Fig. 5 shows the relationship between the fractal dimension and the TIS values. A fairly good linear relation was observed with d-limonene with a significantly high correlation coefficient, but, the relation was rather poor with Azone. The decrease of fractal dimension may be attributed to the smoothing of cross sectional structure, which will be caused by the hydration and/or edema formation in the shallow part of the skin. This suggests that primary damage evoked by d-limonene is the hydration and/or edema formation and, therefore, well quantified as a change of fractal dimension of the skin.

3.3. Skin color reflectance

Skin color reflectance measured with a chromameter using the CIE system has been reported to be useful for the quantification of erythema induced by several surfactants (Agner and Serup, 1990; Wilhelm et al., 1994). As we described above, the fractal dimension is effective to evaluate the hydration and edema formation of skin, however, it may not be applicable to the evaluation of the erythema of the skin. Therefore, the skin color reflectance (ΔE*ab) was applied to the quantitative evaluation of the erythema formation evoked by d-limonene and Azone (Fig. 6). The ΔE*ab value increased with increasing concentrations of each promoter in the hydrogels. At 3% of d-limonene, the erythema formation was clearly observed with the naked eyes and the ΔE*ab value significantly increased, compared with the values measured at 0 ~ 2% d-limonene ($P < 0.01$). In the case of Azone, the erythema formation was partly observed even at the lower concentration (1%) and the ΔE*ab value markedly increased, compared with the control not containing Azone ($P < 0.01$). Fig. 7 shows the relationship between the ΔE*ab and TIS values. No linear relation was observed with d-limonene. On

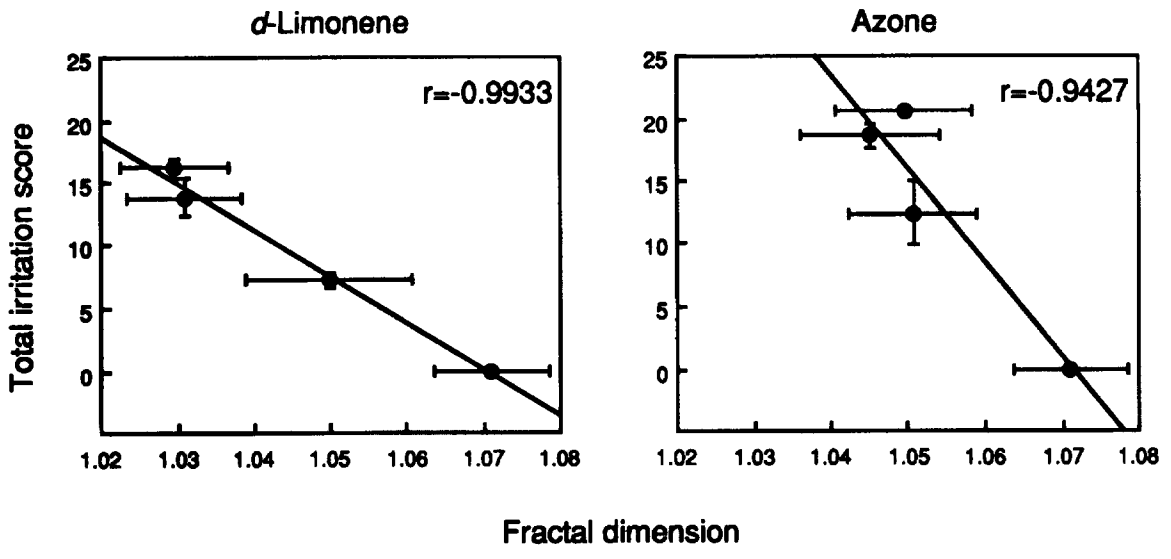

Fig. 5. Relationship between fractal dimension and total irritation score at 6 h after application of indomethacin hydrogels.

the other hand, an excellent linearity was obtained when the skin was treated with Azone. As a result, measurement of the skin color reflectance was quite useful to evaluate the erythema formation caused by Azone.

In conclusion, the skin damages such as edema and erythema are objectively quantified by means of the fractal dimension of the skin cross sectional structure and the change of color reflectance on the skin surface. The difference between the skin damages caused by *d*-limonene and Azone was quantitatively discriminated by the application of fractal geometry and color reflectance measurement.

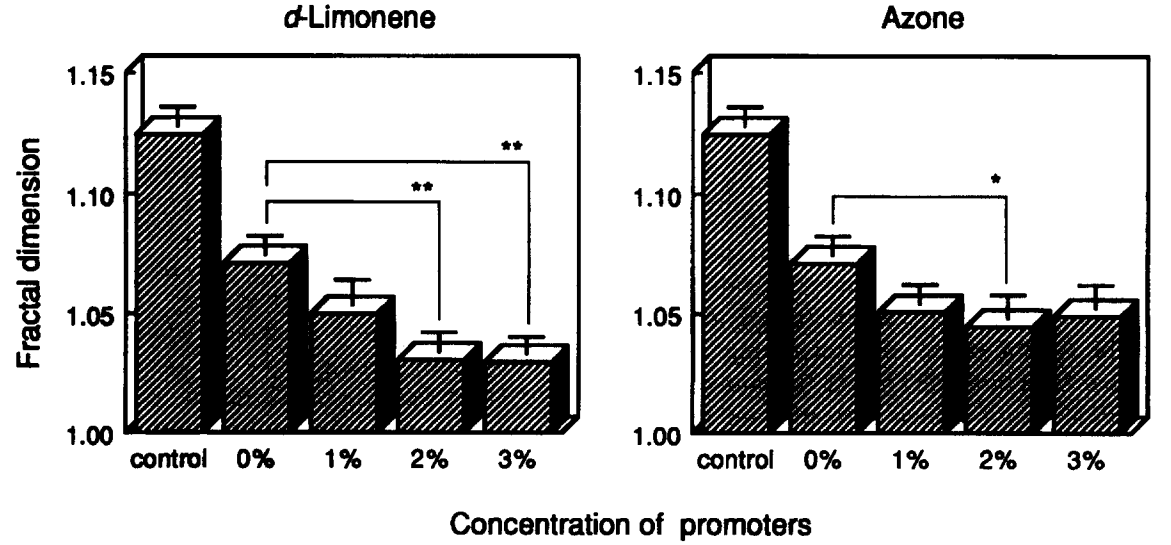

Fig. 6. ΔE*ab of hairless rat abdominal skin at 6 h after application of indomethacin hydrogels. Each column represents mean ± S.E. ($n = 6 \sim 10$). *P < 0.05, **P < 0.01.

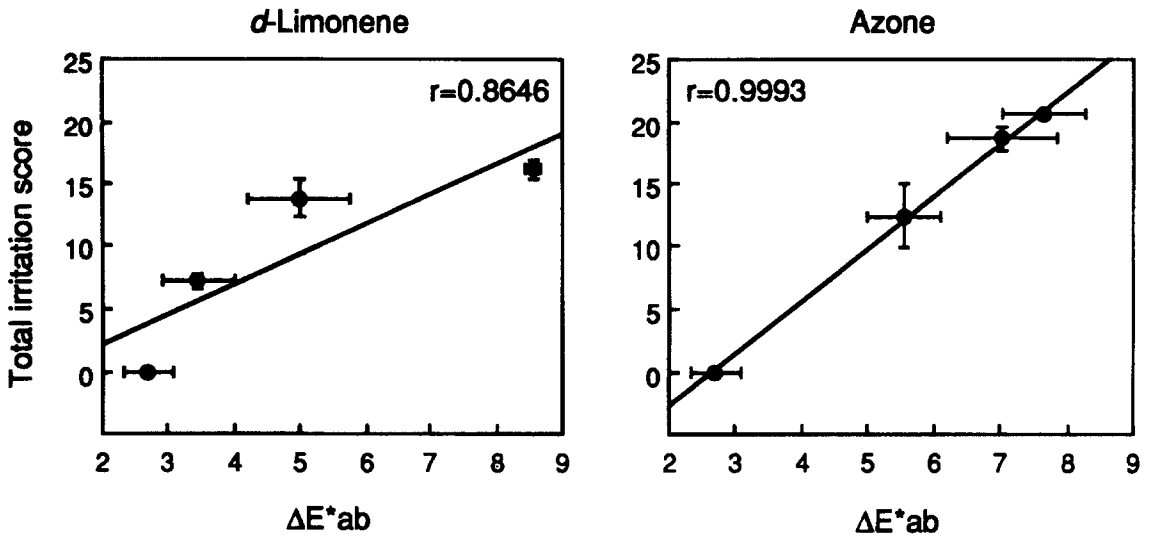

Fig. 7. Relationship between ΔE*ab and total irritation score at 6 h after application of indomethacin hydrogels.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research, from the Ministry of Education, Science and Culture, Japan 06672156 and 07672329. The authors are grateful to Mr. K. Kobayashi and Miss H. Takahashi for assistance in the experimental work.

References

Agner, T. and Serup, J., Sodium lauryl sulphate for irritant patch testing: a dose-response study using bioengineering methods for determination of skin irritation. *J. Invest. Dermatol.*, 95 (1990) 543–547.

Babulak, S.W., Rhein, L.D., Scala, D.D., Simion, F.A. and Grove, G.L., Quantitation of erythema in a soap chamber test using the Minolta Chroma (Reflectance) Meter: comparison of instrumental results with visual assessments. *J. Soc. Cosmet. Chem.*, 37 (1986) 475–479.

Bonny, J.D. and Leuenberger, H., Determination of fractal dimensions of matrix-type solid dosage forms and their relation with drug dissolution kinetics. *Eur. J. Pharm. Biopharm.*, 39 (1993) 31–37.

Cooper, E.R., Increased skin permeability for lipophilic molecules. *J. Pharm. Sci.*, 73 (1984) 1153–1156.

El-Arini, S.K., Thibert, R. and Tawashi, R., Use of fractal dimension in the study of fractured surfaces of insulin zinc crystals. *J. Pharm. Sci.*, 82 (1993) 844–846.

Koch, H.P., The concept of fractals in the pharmaceutical sciences. *Pharmazie*, 48 (1993) 643–659.

Lahti, A., Kopola, H., Harila, A., Myllyla, R. and Hannuksela, M., Assessment of skin erythema by eye, laser Doppler flowmeter, spectroradiometer, two-channel erythema meter and Minolta chromameter. *Arch. Dermatol. Res.*, 285 (1993) 278–282.

Mandelbrot, B.B., *The Fractal Geometry of Nature*, Freedman, New York, 1983.

Michniak, B.B., Player, M.R., Chapman, J.M. and Sowell, J.W.Sr., In vitro evaluation of a series of Azone analogs as dermal penetration enhancers. *Int. J. Pharm.*, 91 (1993) 85–93.

Obata, Y., Takayama, K., Okabe, H. and Nagai, T., Effect of cyclic monoterpenes on percutaneous absorption in the case of a water soluble drug (diclofenac sodium). *Drug Des. Delivery*, 6 (1990) 319–328.

Okabe, H., Takayama, K., Ogura, A. and Nagai, T., Effect of limonene and related compounds on the percutaneous absorption of indomethacin. *Drug Des. Delivery*, 4 (1989) 313–321.

Okabe, H., Takayama, K. and Nagai, T., Percutaneous absorption of ketoprofen from acrylic gel patches containing *d*-limonene and ethanol as absorption enhancers. *Chem. Pharm. Bull.*, 40 (1992) 1906–1910.

Okabe, H., Obata, Y., Takayama, K. and Nagai, T., Percutaneous absorption enhancing effect and skin irritation of monocyclic monoterpenes. *Drug Des. Delivery*, 6 (1990) 229–238.

Okamoto, H, Hashida, M. and Sezaki, H., Structure-activity relationship of 1-alkyl- or 1-alkenylazacycloalkanone derivatives as percutaneous penetration enhancers. *J. Pharm. Sci.*, 77 (1988) 418–424.

Okamoto, H., Muta, K., Hashida, M. and Sezaki, H., Percutaneous penetration of acyclovir through excised hairless mouse and rat skin: effect of vehicle and percutaneous penetration enhancer. *Pharm. Res.*, 7 (1990) 64–68.

Quan, D., Takayama, K., Mitsuzono, T., Isowa, K. and Nagai, T., Influence of novel percutaneous absorption enhancers, cyclohexanone and piperidone derivatives, on histopathology of rat skin. *Int. J Pharm.*, 68 (1991) 239–253.

Robertson, A.R., The CIE 1976 color difference formulas. *Color Research and Application*, 2 (1977) 7–11.

Santus, G.C. and Baker, R.W., Transdermal enhancer patent literature. *J. Contr. Rel.*, 25 (1993) 1–20.

Serup, J. and Agner, T., Colorimetric quantification of erythema – a comparison of two colorimeters (Lange Micro Color and Minolta Chromameter CR-200) with a clinical scoring scheme and Laser Doppler flowmetry. *Clin. Exp. Dermatol.*, 15 (1990) 267–272.

Takayama, K. and Nagai, T., Simultaneous optimization for several characteristics concerning percutaneous absorption and skin damage of ketoprofen hydrogels containing *d*-limonene. *Int. J. Pharm.*, 74 (1991) 115–126.

Wilhelm, K.P., Surber, C. and Maibach, H.I., Quantification of sodium lauryl sulfate irritant dermatitis in man: comparison of four techniques: skin color reflectance, transepidermal water loss, laser Doppler flow measurement and visual scores. *Arch. Dermatol. Res.*, 281 (1989) 293–295.

Wilhelm, K.P., Freitag, G. and Wolff, H.H., Surfactant-induced skin irritation and skin repair. *J. Am. Acad. Dermatol.*, 30 (1994) 944–949.