Enhancing Effect of *N*-Acetyl-L-cysteine or 2-Mercaptoethanol on the *in Vitro* Permeation of 5-Fluorouracil or Tolnaftate through the Human Nail Plate

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The enhancing effects of various vehicles on the *in vitro* permeation of a hydrophilic model drug, 5-fluorouracil (5-FU), or a lipophilic model drug, tolnaftate (TN), through human nail plates were investigated using a modified side-by-side diffusion cell. Tip pieces from the 5th finger-nail, clipped from healthy volunteers, were used in this permeation study. The swelling and softening properties of the nail pieces were also measured in each vehicle. The weights and stresses of the nail pieces were dramatically changed after immersion in aqueous solvents containing *N*-acetyl-L-cysteine (AC) or 2-mercaptoethanol (ME). However, no significant change in the physicochemical properties of the nail pieces was found in the lipophilic vehicles. Thus, the water content in the nail plates absorbed from vehicles may relate to their physicochemical properties. Although keratin-softening agents and new skin permeation enhancers did not significantly promote 5-FU permeation compared with water alone, the flux from solvent systems containing AC or ME was substantially higher. In addition, TN permeation from solvents containing AC or ME could be measured, whereas that from other solvents was undetectable. When the AC concentration was increased, the 5-FU permeation and the nail weight increased and the stress of each nail piece decreased. It is concluded from these experimental results that AC and ME may be useful as enhancers for increasing drug permeation through the human nail plate.

Key words nail; nail permeation; permeation enhancer; acetylcysteine; mercaptoethanol; 5-fluorouracil; tolnaftate

Recent research on fungal diseases has produced a variety of new antifungal agents, having a low minimum inhibitory concentration (MIC) on *Trichophyton rubrum* or *Trichophyton mentagrophytes*. Trichophytosis is a parasitic mycosis in keratinous tissues such as the stratum corneum of skin, hair and nails. Parasitic nail mycosis (onychomycosis trichophytica) is known as a particularly troublesome disease. Onychomycosis trichophytica has been treated mainly with oral antifungal medication.[1,2] This oral therapy, however, sometimes has severe systemic side-effects, such as liver dysfunction,[3] which interrupt the therapy. Although a lot of research has been published on the effect of clinical topical and systemic treatments of onychomycosis trichophytica, there are few *in vitro* nail plate penetration studies following topical drug exposure. Walters *et al.*[4] have suggested that the nail plate behaves like a hydrophilic gel membrane and that it has an additional lipophilic route from penetration data using homologous alcohols. They also inferred that solvents such as dimethylsulphoxide and isopropyl alcohol, which tend to promote drug diffusion through the stratum corneum of skin, had little effect on nail plate permeation.[5]

Since few investigations have appeared regarding the enhancement of nail plate permeation, we developed a modified side-by-side (2-chamber) diffusion cell to find out a penetration-enhancing system.[6] The barrier function of the nail plate may be closely related to changes in its physicochemical properties (swelling and softening). The changes in the weight and stress of the nail pieces were then investigated. Urea and sodium salicylate are well known to have a keratin-softening effect and we previously reported that a new hydrophilic multicomponent vehicle, consisting of l-menthol, ethanol and water (MEW system), markedly enhanced the permeation of morphine[7] and several cardiovascular agents[8] through hairless rat and human skin. We also reported that a

new lipophilic multicomponent vehicle, consisting of L-lactic acid, ethanol and isopropyl myristate (LEI system), greatly promoted the skin permeation of ketotifen and several other drugs.[9,10] In addition, chloramphenicol permeation from lipophilic vehicles or nail lacquers through the human nail plate or hoof membrane was compared with that from aqueous systems.[11] Also, a clinical trial showed that *N*-acetyl-L-cysteine (AC) increased oxiconazole levels in the nail.[12] Therefore, keratin-softening agents (urea and sodium salicylate), new skin permeation enhancers (MEW and LEI systems) and agents reducing the disulfide linkages in hard keratin (AC and 2-mercaptoethanol) were selected as additives for evaluation. Tolnaftate (TN) and 5-fluorouracil (5-FU) were used as lipophilic and hydrophilic model drugs, respectively.

Experimental

Materials TN was supplied by Nissan Chemical Co., Ltd. (Tokyo, Japan). 5-FU and IPM were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Urea, sodium salicylate, L-lactic acid, ethanol, AC and 2-mercaptoethanol (ME) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *l*-Menthol was supplied by Toko Pharmaceutical Ind. Co., Ltd. (Tokyo, Japan). Other reagents were from commercial sources.

Preparation of the Nail Plate Tip nail pieces (8—25 mg) were obtained from the fingers of healthy volunteers (9 males; mean age 24 years, range 21—32) using nail clippers. Nail pieces, which had been allowed to grow for at least one month, were used in this study. Nine or seven nail pieces were obtained from the 5th finger of each volunteer, hydrated for a day and then used to compare the nail permeation from each solvent system. Nail pieces from the 2nd, 3rd and 4th fingers were used to measure physicochemical properties.

Solubilities and Partition Coefficients 5-FU- or TN-vehicle suspensions were mixed by a magnetic stirrer at 37 °C. After 24 h each suspension underwent filtration (Ekicrodisc 3 or 3CR; German Sciences Japan, Ltd., Tokyo). The filtrate was immediately diluted with methanol or acetonitrile to obtain samples for analysis. The octanol/vehicle partition coefficient of the drugs (K_{ov}) was defined as the solubility ratio in octanol/vehicle at 37 °C.

Measurement of Physicochemical Properties of Nail Pieces The

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weights and stresses of the finger nail pieces from healthy volunteers were measured before treatment (day 0) (Step 1). Each nail piece was immersed in 10 ml of each vehicle at 37 °C, and was subsequently measured on day 6 (Step 2). In the investigation to measure the effect of AC concentration on the physicochemical properties of the nail plate, each nail piece was immersed in 10 ml water for another 6 days to check the reversibility (Step 3). After this nail piece was wiped by Kimwipes® and weighed by an electronic balance (JL-200, Chyo Balance Corp.). The bending stress of each nail piece was measured by a rheometer (NMR-2000J, Fudo Kogyo Co., Ltd.) with an attachment guide (Fig. 1).[13] The resulting stress ($S_{[Step2\ or\ 3]}$) and weight ($W_{[Step2\ or\ 3]}$) product of each treated nail piece and its respective untreated nail piece ($S_{[Step1]} \cdot W_{[Step1]}$) were used as an index for nail softening and swelling ($S_{[Step2\ or\ 3]}/S_{[Step1]}$, $W_{[Step2\ or\ 3]}/W_{[Step1]}$).

Permeation Studies A piece of nail plate was sandwiched between 2 adapters made of polypropylene with an O-shaped ring (effective diffusion area, 0.049 cm²) and mounted in a side-by-side (2-chamber) diffusion cell with a water-jacket connected to a water-bath at 37 °C (Fig. 2) . The dorsal nail plate side was filled with 2.5 ml drug suspension in each solution, and the ventral nail plate side with the same volume of water or 40% polyethylene glycol 400. No preservative was added because the receiver solution was clear even at the end of the experiment. The drug permeation was measured by sampling the ventral nail plate side solution at predetermined times. The experimental period was 7 d (5-FU) or 18 d (TN), because of the low nail permeability of the drugs. To overcome the large variation in nail permeabilities due to individual differences in the barrier properties, two experimental schedules were maintained: (i) use of nail pieces from the same finger of the same volunteer and (ii) continuous use of the same nail piece. The effect of different concentrations of AC (0, 0.1, 0.5, 1, 3, 5, 10%) on the *in vitro* nail permeation of 5-FU was investigated according to the experimental schedule shown in Fig. 3.

Analytical Methods 5-FU and TN were determined by HPLC. The absolute calibration method was applied for 5-FU. The sample containing TN was added to a methanol solution containing diphenyl phthalate as an internal standard. The solut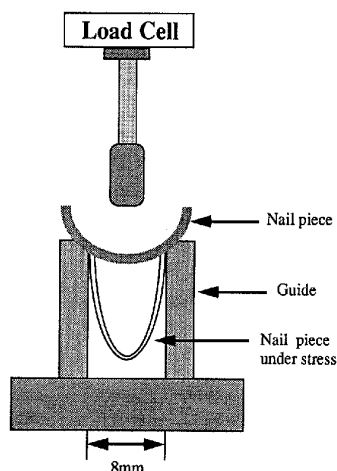ion was injected into an HPLC consisting of a pump system (LC-6A, Shimadzu Seisakusho, Kyoto), an UV detector (SPD-6A, Shimadzu), a chromatopack (C-R6A, Shimadzu), a system controller (SCL-6B, Shimadzu), an auto injector (SIL-6B, Shimadzu) , and a reverse phase column (Inertsil ODS 250 mm×4.6 mm i.d., GL Sciences Inc., Tokyo). The mobile phase used for measuring TN was water : methanol (3 : 7) at a flow rate of 1 ml/min, and detection at $\lambda = 260$ nm. The mobile phase for 5-FU was 0.1% phosphoric acid:acetonitrile (98 : 2) at a flow rate of 1 ml/min, and detection at $\lambda = 270$ nm.

Results and Discussion

Effect of Each Vehicle on the Physicochemical Properties of the Nail Plate Table 1 summarizes the composition of the various solvent systems and the solubility of 5-FU and TN in the solvent systems used in these experiments. The effect of each vehicle on the physicochemical properties (swelling and softening) of the nail plate was studied to compare the drug permeation from each vehicle through each human nail plate. Water was selected as a control vehicle.

Figure 4 shows the weight and stress ratios ($W_{[Step2]}/W_{[Step1]}$ and $S_{[Step2]}/S_{[Step1]}$) 6 d after immersion of the nail pieces in several solvent systems. In comparison with non-treated nail pieces, the nail piece weight ratio of the control
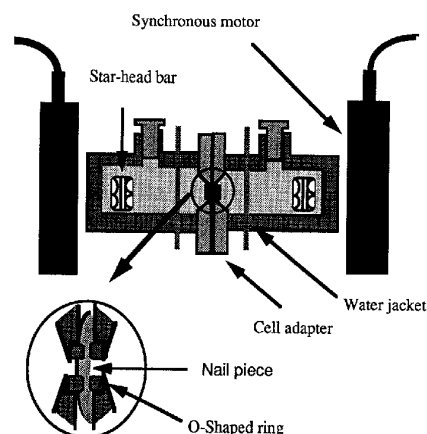

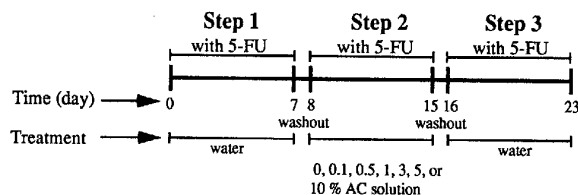Fig. 2. Schematic Diagram of the Modified Side-by-Side Diffusion Cell

Fig. 1. Measurement of Stress on Nail Pieces

Fig. 3. Experimental Schedule (ii) for the Nail Plate Permeation Study

Table 1. Solubilities of 5-FU and TN in Each Solution

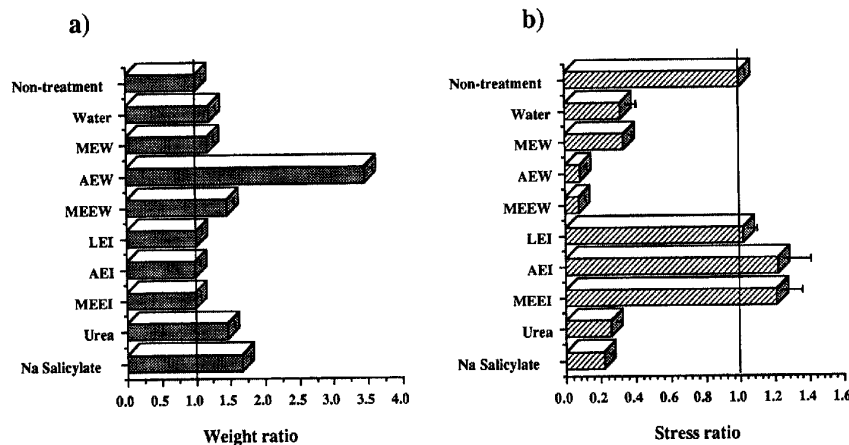| Composition of solvent systems	5-FU (mg/ml)	TN (μg/ml)
Water	17.1	0.391
MEW (3% l-menthol–40% ethanol–water)	19.6	373.8
AEW (3% acetylcysteine–40% ethanol–water)	20.2	118.1
MEEW (3% mercaptoethanol–40% ethanol–water)	24.6	107.1
LEI (1% lactic acid–10% ethanol–isopropyl myristate)	0.228	29610
AEI (0.5% acetylcysteine–10% ethanol–isopropyl myristate)	0.528	24681
MEEI (3% mercaptoethanol–10% ethanol–isopropyl myristate)	0.266	28009
Urea (8 M urea)	5.11	0.853
Na salicylate (40% sodium salicylate)	38.4	39.82

Fig. 4.   Weight and Stress Ratio 6 days after Treatment with Several Solvent Systems

Each value represents the mean±S.E. (n=6).

vehicle (water) was increased by about 0.2 points and its stress ratio was decreased by about 0.7 points. In addition, the nail piece weight was increased and its stress was decreased in water-containing solvent systems. Compared with the water control, 8 M urea (Urea) and 40% sodium salicylate (Na salicylate) increased the nail piece weight ratio by 0.25—0.47 points and lowered the nail piece stress ratio by 0.06—0.10 points. In contrast, the MEW system had approximately the same nail weight and stress ratio as those of the control vehicle. AEW (3% acetylcysteine–40% ethanol–water) and MEEW (3% mercaptoethanol–40% ethanol–water) systems containing agents reducing the disulfide linkages increased the nail weight ratio by 2.45 and 0.45 points, respectively. The AEW and MEEW systems decreased the nail stress ratio by about 0.915 and 0.926 points, respectively. In contrast with the non-treated nail pieces, the LEI system, a water free system, had little effect on the weight and stress ratio of each nail piece. Although the AEI (0.5% acetylcysteine–10% ethanol–isopropyl myristate) and MEEI (3% mercaptoethanol–10% ethanol–isopropyl myristate) systems also had little effect on nail swelling and softening, AC and ME in aqueous solvent systems had a significant effect. These results suggest that water may physically change the nail plate keratin. In addition, urea, sodium salicylate, AC and ME in aqueous solvent systems may denature the nail keratin, whereas this denaturation may not take place in water-free solvents. Urea and ME can be used to extract keratin from nails or wool.[14,15] Therefore, those additives are likely to increase drug permeation through the human nail plate.

Effect of Each Vehicle on Drug Permeation through the Nail Plate   The steady-state flux (J) of drugs through the human nail plate can be represented by Fick's first law of diffusion, as follows:

$$J = D \cdot K_{N/V} \cdot C_V / L \tag{1}$$

where D, $K_{N/V}$, C_V and L are the diffusion coefficient in the human nail plate, the human nail plate/vehicle partition coefficient, the concentration in the donor vehicle of the drug and the thickness of the human nail plate, respectively. The permeability coefficient (P) is represented by

Table 2.   Effect of Several Solvent Systems on the Flux of 5-FU and TN through the Human Nail Plate

Solvent system	5-FU (μg/cm^2/h)	TN (μg/cm^2/h)
Water	17.4±4.3	N.D.[a]
MEW	16.3±3.3	N.D.
AEW	228.8±52.3	0.137±0.080
MEEW	275.1±125.8	0.058±0.017
LEI	20.5±5.2	N.D.
AEI	116.4±6.8	0.053±0.009
MEEI	146.8±23	0.223±0.032
Urea	3.7±0.2	N.D.
Na salicylate	6.6±0.3	N.D.

a) Not determined. Each value represents the mean±S.E. (n=3).

$$P = (D \cdot K_{N/V}) / L \tag{2}$$

In the present study, both TN and 5-FU were suspended in all the donor vehicles. Therefore, their thermodynamic activities in each donor vehicle are at a maximum and the same value. In the case of equal diffusion coefficients or equal activity coefficients, it is thought that the permeation fluxes of the drugs from each vehicle are the same. On the other hand, the permeability coefficient reflects the activity coefficient of the drugs in the human nail plate and the change in their solubility in vehicle. Thus, it is possible to evaluate drug diffusion in the membrane and drug partition to the membrane.

Table 2 shows the steady-state flux of TN and 5-FU from several solvent systems through the human nail plate. The 5-FU flux from the MEW system was not increased, in comparison with that from the water (control) vehicle. In spite of other changes in the physicochemical properties of the nail plate, aqueous solvent systems containing keratin-softening agents (urea and Na salicylate) decreased the 5-FU flux. The LEI system (a lipophilic system) slightly enhanced the permeation of 5-FU through the human nail plate. It has been reported that the dissociation of benzoic acid and pyridine leads to a reduction in their penetration rate through hoof membrane.[16] When an acidic drug, 5-FU (pK_a=8.0, 13.0), is suspended in aqueous solutions of keratin-softening agents (urea: pH=7.2, Na salicylate: pH=6.2), the pH in the nail plate would be shifted to a more basic value, compared with
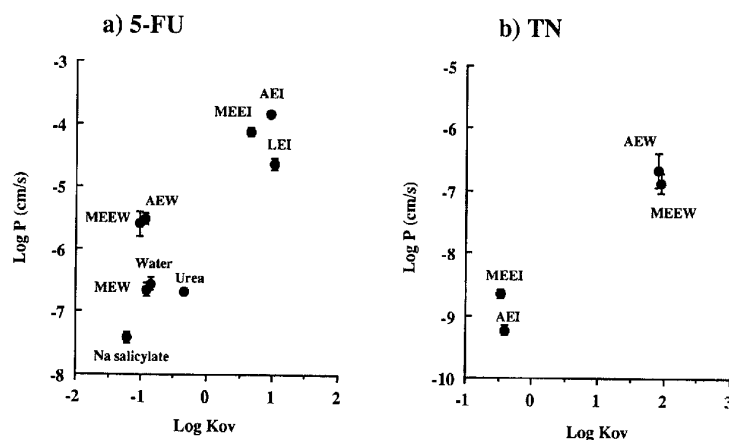
Fig. 5. Relationship between log K_{ov} and log P of 5-FU and TN
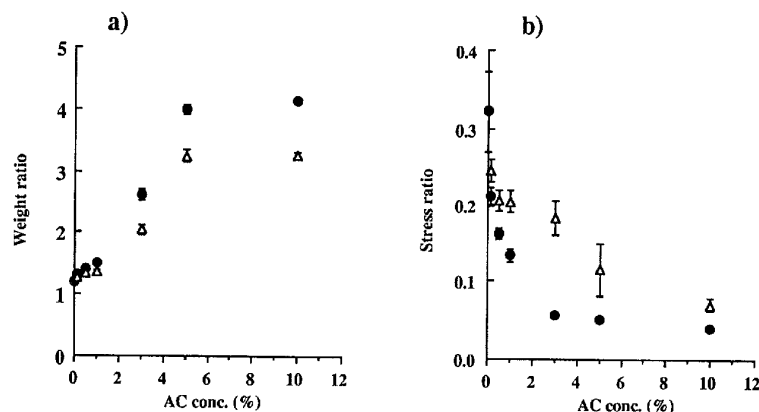
Each value represents the mean±S.E. ($n=3$).

Fig. 6. Weight and Stress Ratio 6 d after Treatment with a Variety of AC Concentrations

●: Step2/Step1, △: Step3/Step1. Each value represents the mean±S.E. ($n=6$).

that of the control vehicle (pH=4.7). In contrast, the pH would be lowered by L-lactic acid in the LEI system. Therefore, a pH change in the nail plate can account for these observations. A combination with urea effectively increased bifonazole penetration into the nail plate in clinical trials.[17] If the penetrant is a basic drug, such as bifonazole, then urea and sodium salicylate may exhibit increased permeation through the nail plate.

On the other hand, aqueous solvent systems (AEW or MEEW systems) containing AC or ME had about 13 and 16 times higher 5-FU fluxes than those obtained in the control vehicle. In addition, lipophilic systems (AEI and MEEI systems) containing AC or ME had about 6.7 and 8.4 times higher 5-FU fluxes than in the control, respectively. Only the permeation of TN from AEW, MEEW, AEI and MEEI systems could be measured, while that from other solvent systems was undetectable. It was assumed that TN had a low nail permeation because of its high molecular weight and low solubility in water, compared with 5-FU. The TN flux levels, through the human nail plate from several solvent systems containing AC or ME, exhibited the following pattern: AEI<MEEW<AEW<MEEI. AC and ME also increased the permeation flux of TN through the nail plate.

The octanol/vehicle partition coefficient (K_{ov}) has been used extensively as an index of biological membrane/vehicle partition. We thus examined the relationship between the logarithm of the octanol/vehicle partition coefficient for TN and 5-FU and the logarithm of the permeability coefficient (Fig. 5). TN showed high permeability coefficients from aqueous solvent systems, whereas 5-FU had high values from lipophilic systems. Solvent systems containing AC or ME increased the permeability of 5-FU and TN, but they were not dependent on log K_{ov}. The lipid content in the human nail plate is much lower than that in the stratum corneum of skin.[18,19] Lipophilic additives such as *l*-menthol and isopropyl myristate, which increase the skin permeation of drugs, had no effect on drug permeation through the nail plate. Ethanol was not able to promote drug diffusion through the nail plate, which agrees with the findings of Walters *et al.*[5] The nail plate of a healthy volunteer is usually hydrated and the water vapor loss normally amounts to about 1.6 mg/cm²/h.[20] If the nail plate has swelled as is the case *in vitro*, a hydrophilic drug such as 5-FU would require a lipophilic vehicle to retain the high thermodynamic activity of the drug. Consequently, aqueous solvent systems are found to be best for lipophilic drugs like TN.

Effect of AC Concentration The effect of different concentrations of AC on the physicochemical properties of nail pieces and the nail plate permeation of 5-FU (Step 2/Step 1) were investigated in more detail. The reversibility of any changes was also tested (Step 3/Step 1). The flux ratio for Step 2/Step 1 was used as an index for the enhancing effect of AC, and the flux ratio for Step 3/Step 1 was used as an index for the disappearance of the enhancing effect of AC.

Figure 6 shows the weight and stress ratio of nail pieces on day 6 (Step 2/Step 1) and day 12 (Step 3/Step 1) at each concentration of AC. The weight and stress ratio of nail pieces increased and decreased, respectively, with increasing AC concentration. In high concentrations of AC, the weight ratio of nail pieces increased marginally. A marked effect on the stress ratio of nail pieces was found at low concentrations of AC. Although low reversibility was observed for the physicochemical changes in nail pieces, the weight and stress ratio of the nail, were not similar to those of the control (water).

Figure 7 shows the permeation flux ratio of 5-FU through the nail plate from vehicles containing different concentrations of AC, for each step. The flux ratio of 5-FU for Step 2/Step 1 increased with an increase in AC concentration. The Step 3/Step 1 ratios were similar to the Step 2/Step 1 ratios, although they were a little lower than the Step 2/Step 1 ratios, especially at high concentrations, suggesting low reversibility

of the enhancing effect by AC. A long period may be necessary until the barrier function of the nail plate recovers. In 3% AC, water flux was about 3 times higher than that of the control (data not shown), showing that AC increased drug diffusion across the nail plate.

Figure 8 shows the relationships between the nail weight or stress ratio and the flux ratio of 5-FU. The flux ratio increased with an increase in the weight ratio and a decrease in the stress ratio. This suggests that the penetration-enhancing effect of 5-FU by AC is closely depending on the swelling and softening of the nail pieces. A marked swelling and softening of the nail plate takes place, presumably due to cleavage of S–S bonds in the nail plate. As a result, the barrier to drug permeation across the nail plate is low.

A lot of cystines, having stable disulfide linkages, are present in human keratinized nail plate.[21] Since cysteine has a reducing action on cystine in the human nail plate, AC may cleave the S–S bonds in keratin. Cleavage of these bonds may be associated with the increase in weight and decrease in stress of the nail pieces. In addition, drug permeation through the human nail plate may also be enhanced as a result of increased diffusion through the affected keratinized nail plate.

Conclusion

The penetration of 5-FU and TN through the human nail plate was studied *in vitro*. A penetration-enhancing effect was observed in all solvent systems containing AC or ME. The permeation flux of 5-FU was directly proportional to the AC concentration. However, the reversibility of the nail plate barrier function was insignificant. The penetration-enhancing effect by AC was closely dependent on the swelling and softening of the nail plate. AC and ME cleave the S–S bond in the hard keratin in human nail plate. Such additives are thus able to enhance drug permeation through the human nail plate.

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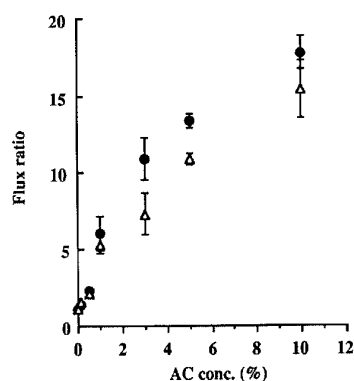

Fig. 7. Enhancing Effect of Various AC Concentrations on the Nail Plate Permeation of 5-FU

●: Step2/Step1, △: Step3/Step1. Each value represents the mean±S.E. (*n*=3).
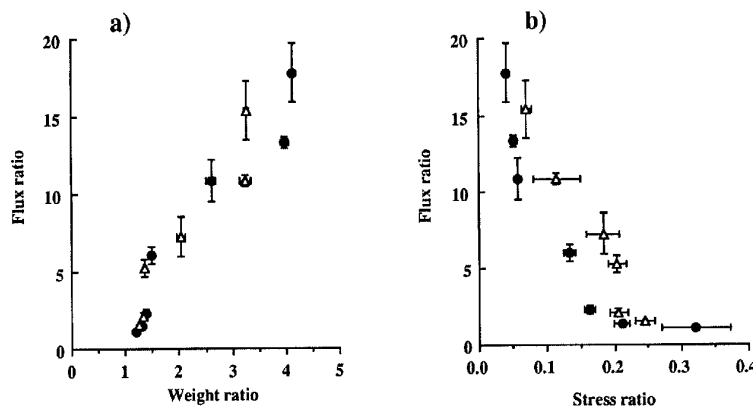

Fig. 8. Relationship between the Weight Ratio (a) or the Stress Ratio (b) and the Flux Ratio of 5-FU

●: Step2/Step1, △: Step3/Step1. Each value represents the mean±S.E. (*n*=3—6).

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