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Retention capacity of topical imidazole antifungal agents in the skin

Terushi Hashiguchi [a,*], Ai Kodama [a], Akie Ryu [a], Masaki Otagiri [b]

[a] *Hisamitsu Pharmaceutical, Tosu Laboratory, Tosu, Saga 841, Japan*
[b] *Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862, Japan*

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Abstract

We have studied the interaction between topical imidazole antifungal agents and keratin, of which the main protein in the stratum corneum is composed and which has a role in determining the retention capacity of the agents in skin. In 50% (v/v) aqueous methanol solution, omoconazole (OMZ) formed a precipitate in the presence of keratin. On Fourier-transform infrared (FTIR) spectroscopy of the precipitate, the peaks at about 1200–1500 cm^{-1} changed in pattern and it was thus thought that OMZ bound to keratin. In this case diffusion energy was measured and compared with other imidazole antifungal agents, bifonazole (BFZ) and clotrimazole (CTZ). The interaction energy obtained from the difference in diffusion energy between the presence and absence of keratin was the highest with OMZ (4.25 kcal/mol) and the lowest with BFZ (2.09 kcal/mol). Retention capacity of OMZ in excised hairless mouse skin was 10.0 $\mu g/cm^2$ at 24 h after application, whereas these values for BFZ and CTZ were 2.4 and 7.5 $\mu g/cm^2$, respectively. These results suggested that the magnitude of interaction with keratin is an important factor in determining the retention capacity of imidazole antifungal agents in skin. © 1998 Elsevier Science B.V.

Keywords: Omoconazole; Retention capacity; Diffusion energy; Keratin; Binding; Fourier-transform infrared

1. Introduction

Topical imidazole antifungal agents such as bifonazole (BFZ) are freely retained in the stratum corneum and are thus not easily taken up into the body (Schaefer and Stüttgen, 1976; Patzschke et al., 1983). Interestingly, even in oral administration of ketoconazole, it was accumulated in the skin for several days (Haneke, 1987). For topical antifungal efficacy, affinity of agents with the stratum corneum and retention capacity were important along with their intrinsic effects (Takahashi, 1988). Long-term efficacy of BFZ

* Corresponding author. Present address: Tsukuba Laboratories, Hisamitsu Pharmaceutical, 1-25-11, Kannondai, Tsukuba, Ibaraki 305, Japan. Tel.: +81 298 370701; fax: +81 298 370685.

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was based on its high retention capacity (Lüker et al., 1984; Tomura and Takahashi, 1995). These reports indicated that affinity to the stratum corneum is involved in retention capacity, and topically-applied agents were considered to be adsorbed on to keratin. The adsorption of antifungal agents on human hair as a source of horny material has been demonstrated with a bioassay (Freedman et al., 1962; Takahashi, 1972; Arika et al., 1990; Niwano et al., 1996). The adsorption of terbinafine, an allyl amine antifungal agent, on keratin was examined, and the adsorption was weak and reversible (Uchida and Yamaguchi, 1993). Therefore, keratin is a good reserving material for antifungal agents and it helps to maintain the efficacy of the agents for long periods.

In this study, we examined the potency of keratin as a reservoir for antifungal agents in detail by physicochemical and in vitro skin adsorption methods. Moreover, the influence of adsorption potency on affinity and retention capacity in the skin was investigated. Omoconazole (OMZ) is an imidazole antifungal agent which is chemically known as (*Z*)-1-{2-[2-(4-chlorophenoxy)-ethoxy]-2(2,4-dichlorophenyl)-1-methyl-vinyl}-1*H*-imidazole (Fig. 1). It has a wide antifungal spectrum (Itoyama et al., 1993), and has high efficacy and lipophilicity (Zirngibl et al., 1988). It was synthesized in 1976 and developed by Siegfried in Europe (Zirngibl et al., 1988). The affinity and retention potency of keratin for BFZ and clotrimazole (CTZ), as reference agents, both of which are well-known as imidazole antifungal agents, were also examined.

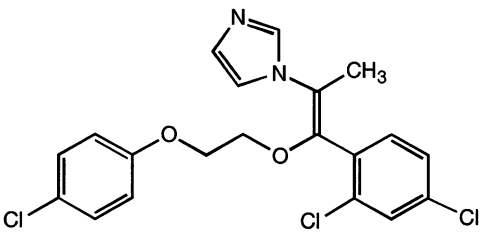
2. Materials and methods

2.1. Materials

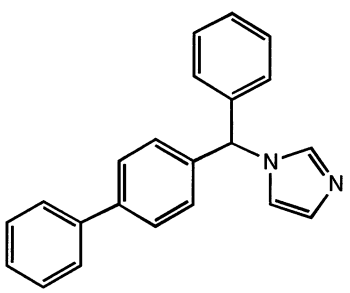
OMZ was obtained from Siegfried Pharma (Zofinge, Switzerland). BFZ and CTZ were purchased from Sigma (St. Louis, MO). Keratin powder and isopropyl myristate (IPM) were obtained from Nacalai Tesque (Kyoto, Japan) and Nikko (Tokyo, Japan), respectively. Other chemicals and reagents were of analytical grade. Hairless mice were obtained from Kyudo (Tosu, Japan).

2.2. Analytical method

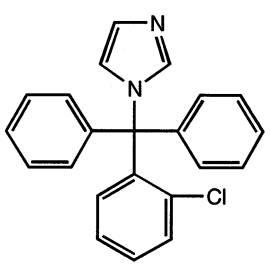
The antifungal agents in the test solutions were detected by HPLC with an ODS-120T® column



Omoconazole(OMZ)



Bifonazole (BFZ)



Clotrimazole (CTZ)

Fig. 1. Structure of antifungal agents.

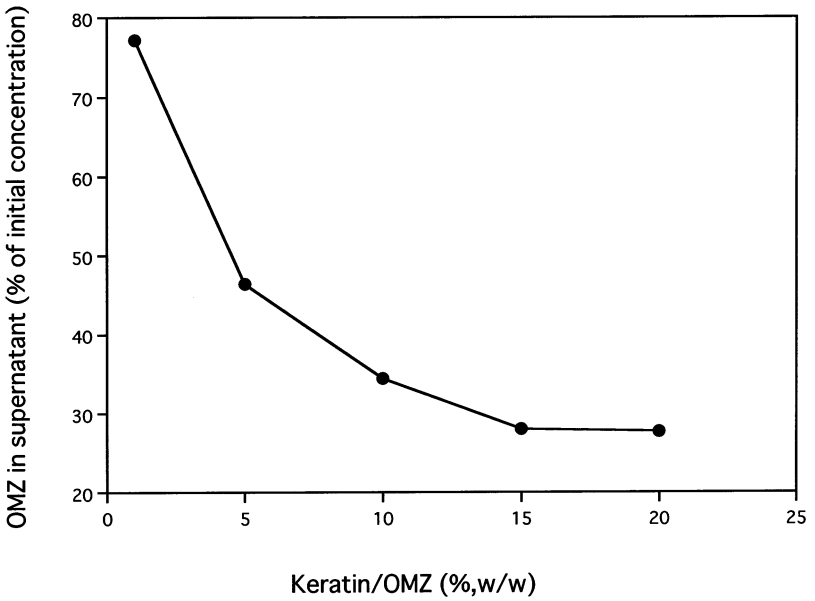


Fig. 2. Interaction between OMZ and keratin by adsorption equilibrium method in solution. Each value represents the mean of three determinations.

(i.d. 4.6 mm and length 150 mm; Tosoh, Japan). The mobile phase consisted of 80% (v/v) methanol and 20% (v/v) 10 mM phosphate buffer (pH 7.4). Aliquots of 20 μl of the test solutions were injected with a 712-WISP autoinjector (Waters, Milford, MA). A flow rate of 1.0 ml/min was maintained with a Waters Model 510 pump, and temperature was maintained at 40°C with a Waters TCM column oven. The eluent was monitored at a wavelength of 210 nm (BFZ, CTZ) or 254 nm (OMZ) with an SPD-6AV (Shimadzu, Kyoto, Japan). The retention times of OMZ, BFZ, CTZ and benzophenone (internal standard) were 7.6, 5.5, 5.3 and 3.4 min, respectively. Standard calibration curves were linear for each agent over the range of 1–100 μg/ml in the solution. Concentrations of each agent were calculated using a Shimadzu C-R3A integrator.

2.3. Equilibrium method for adsorption

OMZ (10 mg/ml) was dissolved in 50% (v/v) aqueous methanol solution. Keratin solutions (0–200 mg/ml) were prepared in the same manner and aliquots were added to OMZ solutions. The obtained mixtures were incubated for 3 h at room temperature. The precipitate was filtered and OMZ in the filtrate was determined by HPLC. The precipitate obtained after addition of keratin, which was produced at 200 mg/ml, was dried and then its Fourier-transform infrared (FTIR) spectrum was measured. Spectra were also measured for OMZ and keratin powders for comparison.

Spectra were measured with an FT/IR-7000 FTIR spectrometer (Nihonbunko, Tokyo, Japan) by the KBr method.

2.4. Determination of diffusion constant

Each agent was dissolved in 50% (v/v) aqueous methanol solution to a concentration of 500 μg/ml. These solutions were incubated at room temperature for a few minutes, filtered through membrane filters and the filtrates were used in the experiment. Keratin powder was dissolved in the same solution to a final concentration of 10 mg/ml. After centrifugation at 3000 rpm for 10 min, supernatant was used. The filtrate and superna-

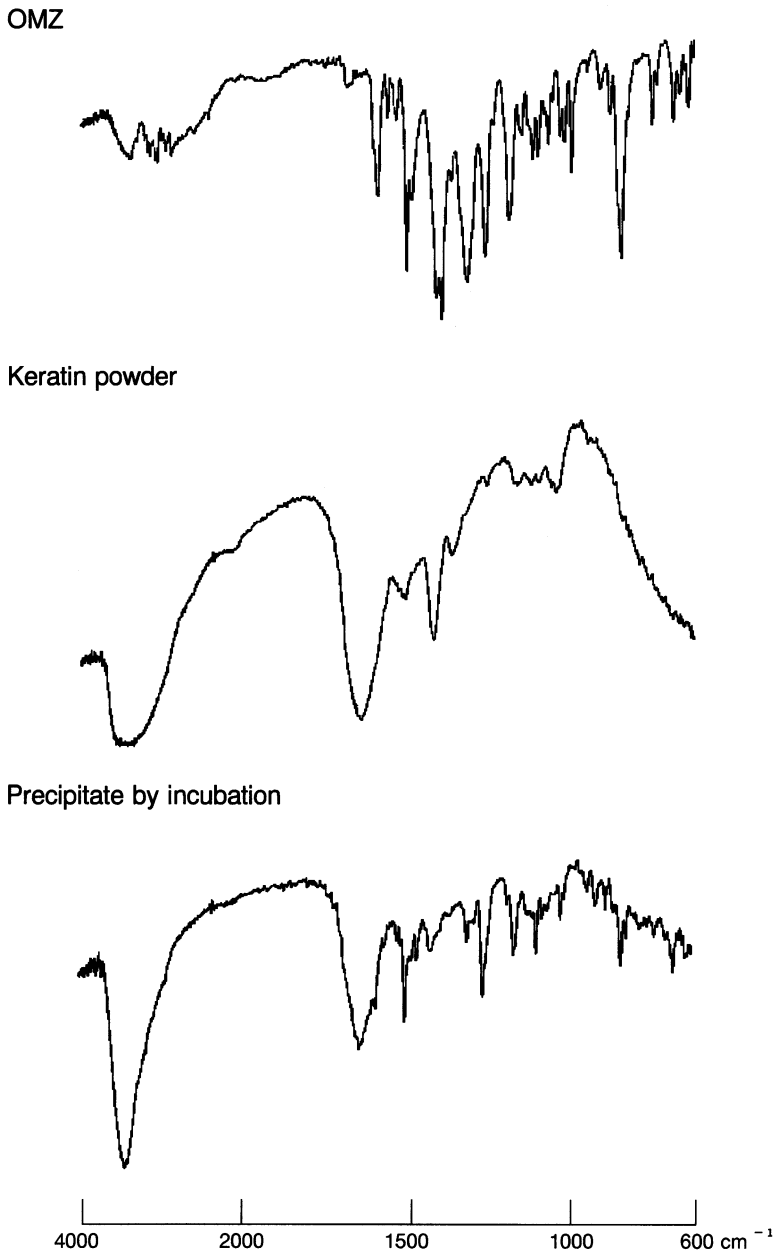


Fig. 3. Interaction between OMZ and keratin by IR spectra.

tant were combined at the same volume, and this mixture solution was used as a test solution. Diffusion experiments were carried out with a diffusion cell of which the receptor volume was 50 cm^3 and effective diffusion area was 19.625 cm^2, and which is similar to that of Loftsson and Bodor (1981). A cellulose membrane (Viskase Sales, Chicago, IL) was mounted between donor and receptor compartments. Test solutions were added to the donor compartment, and then agitation of the receptor solution (50% (v/v) aqueous methanol) and a diffusion examination were car-

Table 1
Physicochemical properties of antifungal agents

Agent	m.w.[a]	m.p.[a] (°C)	Solubility[a] (mg/ml)		log P[b]
			Methanol	Chloroform	
OMZ	423.7	89	38	—	5.76
BFZ	310.4	149	37	345	4.95
CTZ	344.8	143	—	500	4.91

[a] Data of OMZ was obtained by Manako et al. (1997), BFZ and CTZ by Takahashi et al. (1984) and Büchel et al. (1972), respectively.
[b] log P represents partition coefficient between n-octanol and water at 25°C.

ried out at 5, 20 and 30°C. Receptors were serially collected and the concentration was determined by the analytical method. As a control, 50% (v/v) aqueous methanol solution containing only the agent was also investigated.

2.5. Skin permeability experiment with antifungal agents by using excised hairless mouse skin

The dorsal skin of female hairless mice (8 weeks old) was excised immediately before in vitro absorption experiments. Absorption was determined with a diffusion cell whose receptor volume was 5 cm^3 and effective diffusion area was 0.785 cm^2, using the method of Gummer et al. (1987). Excised hairless mouse skin was mounted between donor and receptor compartments. After suspending 50-mg aliquots of agents in 5 ml of IPM, they were shaken for 24 h at room temperature. Aliquots 200 μl of the supernatant were added to the stratum corneum side of excised hairless mouse skin. The receptor solution, 10 mM phosphate buffer solution (pH 7.4) containing 3% Tween 80 as a solubilizer, and 0.025% sodium azide, was circulated at 32°C. The perfusate was collected at intervals to assay the drug content by HPLC. The skin was removed from the cell after 24 h, wiped three times with dry gauze and then three times with gauze soaked in ethanol. After wiping with dry gauze again, the skin was washed with 50% (v/v) aqueous methanol solution and the solution wiped thereafter. Subsequently, the skin was cut into small pieces with scissors, and then homogenized after adding methanol. The homogenized solution was centrifuged at 3000 rpm for 10 min. The agents in the supernatant were determined by HPLC.

3. Results and discussion

Keratin is the main protein of the stratum corneum, and its binding to many drugs is a major factor of its role as a drug reservoir (Vickers, 1963; Baker et al., 1977; Walter and Kurz, 1988; Sasaki et al., 1990). Interaction of OMZ with keratin was determined by the adsorption equilibrium method. The results obtained are shown in Fig. 2. When OMZ and keratin were added together to the solution, a precipitate was formed immediately at high keratin concentrations, while at low concentration precipitation occurred slowly. With increasing ratio of keratin to OMZ, this precipitation increased and as a result OMZ concentration in the supernatant decreased. Above 15% in weight ratio between keratin and OMZ, the supernatant concentration was a constant, therefore this suggests that the binding reached a steady state equilibrium above this point, and the binding was reversible.

The dried precipitate was investigated by FTIR (Fig. 3). The IR spectrum of the precipitate was slightly different from those of keratin and OMZ. A differential spectrum was determined from the IR spectrum of the resulting precipitate and the IR spectrum of keratin, to compare the differential spectrum with the OMZ spectrum and examine the modification thereof. Consequently, the elimination of the peak possibly derived from the methyl group in OMZ around 1390 cm^{-1} was

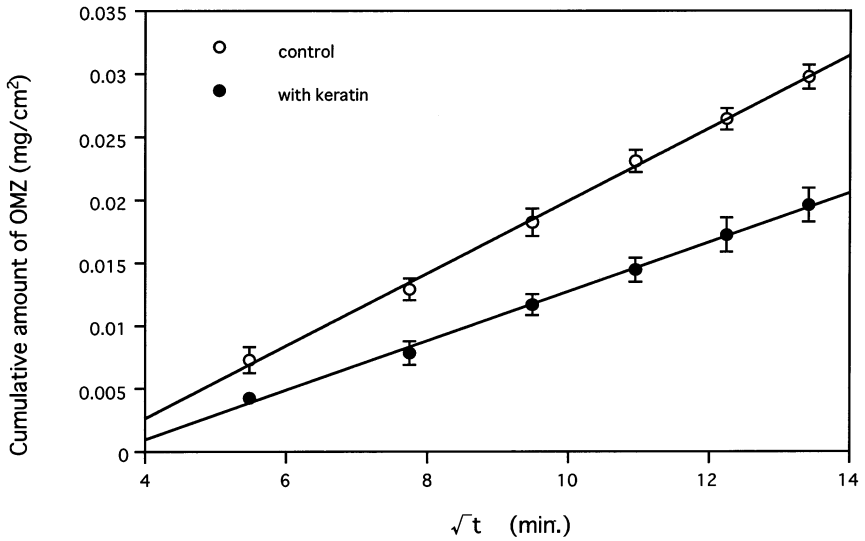


Fig. 4. Influence of keratin on release profiles of OMZ with cellulose membranes at 5°C. The same receptor solution, 50% (v/v) aqueous methanol, was used in all the experiments. Each symbol and bar represent the mean ± S.D. of four determinations.

observed. It was also observed that the absorption intensities of the peaks derived from the ether bond at 1306 and 1176 cm^{-1} were attenuated. It was thus indicated that the methyl group in the OMZ molecule was inserted into the space of keratin through the hydrophobic interaction, so that the oxygen atom in the ether bond in the OMZ might acquire a hydrogen bonding with a polar group in the keratin molecule. Therefore, it was supposed that a complex formed as a result of hydrophobic interaction or hydrogen bonding between OMZ and keratin. It is known that when keratin has a disordered water molecule in its matrix chain, the disordered water molecule possesses strong hydrogen-bonding capacity, therefore keratin binds to drugs with hydrogen bond and maintains the bonding (Blank, 1952). In this study, however, the commercial keratin used was of considerably low molecular weight, easily dissolved in water, and it was difficult to maintain tertiary configuration. Hence the keratin in this study was thought to be denatured so that its disulfide bond derived from cysteine was reduced, and as a result, keratin side groups capable of bonding were available to the drug. It has also been reported that the binding capacity of denatured keratin is of a higher degree (Freedman et al., 1962; Stüttgen, 1989). Because OMZ has some binding sites for hydrogen bonding, hydrophobic bonding or van der Waal's bonding in its molecular structure, it was supposed that it bound to side-chain groups in the keratin by non-covalent bonding to produce an insoluble precipitate.

Subsequently, to examine the strength of this type of binding, the binding energy was investigated from a diffusion coefficient at a designated temperature.

After preparation of a keratin dispersion, the insoluble part was discarded by filtration and OMZ, BFZ or CTZ were added to the filtrate. Using this solution, a diffusion experiment was carried out with a cellulose membrane. BFZ or CTZ are widely used in therapy and their lipophilicity is different from that of OMZ (Table 1). The cumulative penetration amount of the agent against time at 5°C is shown in Fig. 4. Because the cumulative amount of agent is in proportion to $t^{1/2}$ in Fig. 4, it is suggested that the release is controlled in the donor solution (Higuchi, 1960; Bottari et al., 1974). Therefore, the diffusion coefficient of the agent in the donor solution was estimated by the following equation (Bottari et al., 1974): $Q = 2C_0 (D_t/\pi)^{1/2}$, where Q is the cumulative amount of agent in the receptor

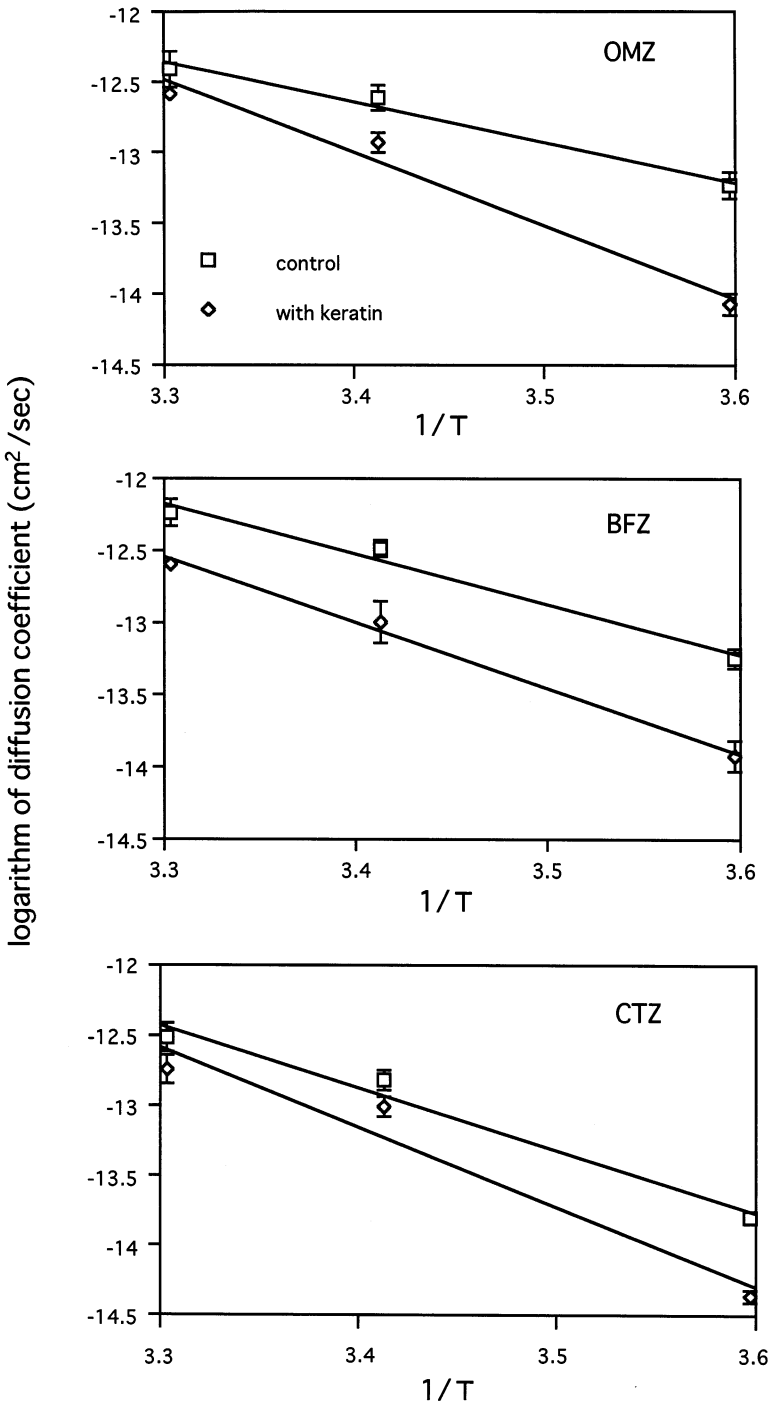


Fig. 5. Relation between diffusion coefficients and temperature. Each symbol and bar represent the mean ± S.D. of four determinations.

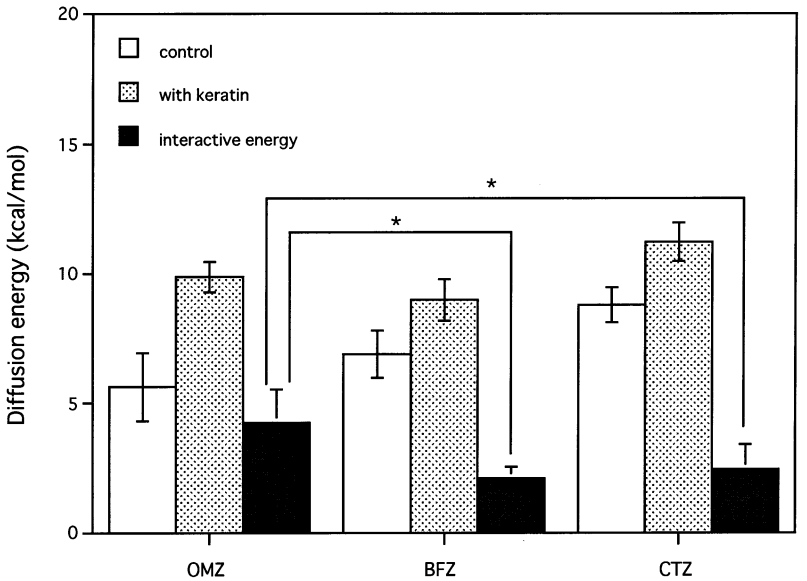


Fig. 6. Interactive energy between antifungal agents and keratin. Each symbol and bar represent the mean ± S.D. of four determinations. Significant differences were calculated using Student's t-test (* $P < 0.05$).

solution. Penetrants diffuse in the donor solution with a diffusion coefficient of D. C_0 expresses the initial concentration of the test agent in the donor solution. The results indicated that when keratin was added to OMZ, the diffusion coefficient in keratin solution decreased to half that in the controls. Those of BFZ and CTZ decreased similarly. These decreases were thought to be due to interactions of the antifungal agents with keratin.

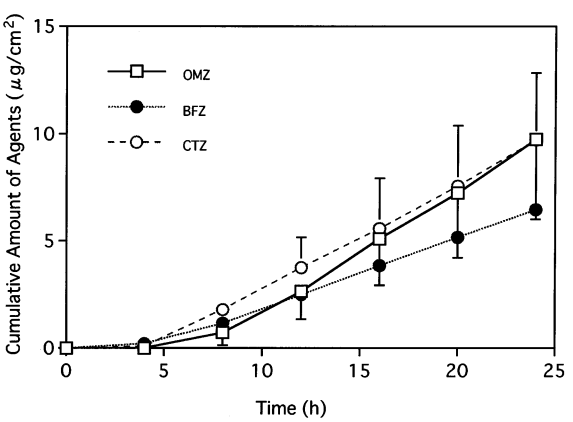


Fig. 7. Percutaneous absorption profiles of antifungal agents in IPM through the excised hairless mouse skin. Each symbol and bar represents the mean ± S.D. of three experiments.

The viscosity of the donor solutions presently used is as low as negligible, and it is therefore thought that the viscosity possibly has no influence over the diffusion of the drug.

The interaction energy for each antifungal agent was therefore investigated. In general, the relation between diffusion coefficient and diffusion energy (E_a) follows Arrhenius' equation: $\ln D = -(E_a/R)(1/T) + \ln D_0$, where R is the gas constant. The results for OMZ are shown in Fig. 5. E_a of drugs was obtained from the slope by the least squares method, and the results are shown in Fig. 6. E_a of OMZ was higher in the presence of keratin relative to the controls. BFZ and CTZ showed the same trend; their diffusion needed more energy in the presence than in the absence of keratin. It was supposed that the difference in E_a between the presence and absence of keratin was employed for diffusion, and was considered as an interaction energy. The interaction energies had the highest value in OMZ, 4.25 kcal/mol. In CTZ and BFZ it was 2.44 and 2.09 kcal/mol, respectively. The interaction energies of ionic or covalent bonds are generally 50–100 kcal/mol, and those of hydrogen or hydrophobic bonds are generally 3–7 kcal/mol. Therefore, the interaction of

Table 2
Percutaneous absorption parameters of antifungal agents through hairless mouse skin

Agent	Initial concentration in IPM (μg/ml)	Flux (μg/cm^2/h)	K_p (cm/h)	Concentration of agent in the skin (μg/cm^2)*
OMZ	9654.4	0.6589	6.825×10^{-5}	9.95 ± 2.75
BFZ	1407.5	0.3386	24.059×10^{-5}	2.40 ± 0.17
CTZ	5624.0	0.5084	9.041×10^{-5}	7.62 ± 0.16

* These values represent the mean $\pm$ S.D. for three examinations which were determined after 24 h in permeation study.

agents with keratin also seems to occur by formation of hydrogen or hydrophobic bonds, and so is probably reversible. In binding to keratin, it was reported that drug lipophilicity is a significant factor (Freedman et al., 1962; Sasaki et al., 1990). It was considered in this study, therefore, that the enhancement of binding capacity derives from affinity of the drug for the binding site of keratin and its lipophilicity. The lipophilicities of CTZ, BFZ and OMZ were determined by the partition coefficient ($\log P$) between n-octanol and water. The $\log P$ values of CTZ, BFZ and OMZ were 4.91, 4.95, and 5.76, respectively (Table 1). The increase in lipophilicity of agents was in the order of OMZ > BFZ $\geqq$ CTZ, so enhancement of binding was roughly related to the lipophilicity of the agents. Consequently it was presumed that one of the factors in the affinity of the agent for the binding site of keratin was derived from the lipophilicity of the agent, and this influenced the enhancement of binding capacity at the same time. It was therefore expected that OMZ has a higher retention capacity than the other two antifungal agents because of the high degree of interaction energy, and high lipophilicity.

Subsequently, to confirm the correlation between interaction with keratin and retention capacity, an in vitro percutaneous absorption test was carried out by using excised hairless mouse. The results are shown in Fig. 7. IPM used as a solubilizer is a general base component of topical preparation, and the thermodynamic activity of each agent in IPM suspension can be made at maximum to reduce the influence of IPM as less as possible. According to Komatsu et al. (Komatsu et al., 1986), the flux and permeation parameter (K_p) of each agent was determined on the cumulative permeation curve through fitting by non-linear least square method, as shown in Table 2. The flux value of each antifungal agent was in proportion to the concentration of the agent in the saturated IPM solution, and OMZ with the highest solubility in IPM had a higher flux value than the flux values of the remaining agents. Alternatively, their K_p values increased in the order BFZ > CTZ > OMZ. It is therefore concluded that OMZ is retained intradermally for a prolonged time, compared with CTZ and BFZ. The amount of each agent in the skin was determined after the skin permeation test. The amount of OMZ partitioned into the skin was 9.95 μg/cm^2 after application for 24 h. This value decreased in the order OMZ > CTZ > BFZ (Table 2), which correlated well with interaction energy with keratin. Thus, affinity for keratin appears to be one of the factors affecting storage of antifungal agents in skin.

4. Conclusions

Affinity for keratin is important for retention of antifungal agents in skin. Binding of antifungal agents to keratin decreases their efficacy in in vitro antifungal activity (Takahashi, 1994; Tomura and Takahashi, 1995), but this binding is weak and reversible, and so can result in long-term retention in skin (Uchida and Yamaguchi, 1993).

The results of the present research works indicate that an antifungal agent with a larger keratin binding energy has a higher intradermal retentivity.

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