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Antibacterial activity of topical antifungal agent in the infected sites – Study

--- Skin permeability and absorption to horny materials ---

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The skin's permeability and retention of Butenafine hydrochloride (KP-363: N -4-tert-butylbenzyl-N-1-methyl-1-naphthalene-methylamine hydrochloride) were investigated using ^{14}C-KP-363. 1% 14C-KP-363 0.2 ml (2 mg) was hermetically attached to the dorsal skin of the guinea pig for 6 hours and when the distribution of the skin to each layer was examined, it was proved that KP - 363 was present at 50 μg / g or more in the epidermal layer including the stratum corneum, which is the habitat of ringworm and even after 24 hours of application (18 hours after the end of application), 10 μg / g or more of drug remained in the epidermis layer, and it was found that this drug was easy to be accumulated in the skin layer containing the barrier. On the other hand, when the adsorption property between the antifungal agent and horny substance was examined using human hair powder, KP-363, tolnaftate, clotrimazole and bifonazole were observed to be strongly adsorbed by the hair. The antimicrobial power of KP - 363, tolnaftate, clotrimazole and bifonazole adsorbed on the hair was 4.0, 15.6, 62.5 and 62.5 μg / g (hair), respectively and it was reduced compared to the antibacterial activity (0.0125, 0.05, 0.39 and 0.39 μg / ml) measured with Sabouraud liquid medium. However, it turned out that the concentration of KP - 363 in the stratum corneum obtained when 1% KP - 363 was applied was still sufficient to prevent the development of T. mentagrophytes.

Introduction

As factors determining the acceptability of external antifungal agents, A) having a strong in vitro antibacterial activity, B) little irritation, C) poor sensitization, D) it does not have strong skin permeability, Takahashi pointed out that it has affinity for the stratum corneum, which is a parasitic part of the fungus, and that it will stay there for a long time. Butenafine hydrochloride (KP - 363: N - 4 - tert - butylbenzyl - N - methyl - 1 - naphthalenemethylamine hydrochloride) shows a wide antifungal spectrum in many fungi including dermatophytes and excellent therapeutic effects in experimental guinea pig dorsal and tinea pedis with treatment once a day[2]; given that it completely protects infection of T.mentagrophytes in one external application before 24 or 48 hours, the in vivo effect of KP-363 is presumed to be due to the high skin retention as well as the strength of the antibacterial activity of this product[3)4)]. On the other hand, in the measurement of the medium skin concentration after application of KP - 363, the skin concentration in the skin after 24, 48 and 72 hours of application of 1% KP - 363 0.2 ml was 31.5, 18.2 and 8.8 μg / g respectively and it was 730 times higher than the bactericidal concentration (0.012 μg / ml) of T. mentagrophytes even after 72 hours of application. Despite the presence of high concentrations of drug in the skin, when T. mentagrophytes was infected 72 hours after application of KP-363, the observation of the presence of the specimen that developed it suggested that the antifungal activity of KP-363 may be decreased in the skin. In this study, we examined the penetrability into the skin of KP-363, using retention property 14C-KP-363, and compared with tolnaftate, clotrimazole and bifonazole which are widely used as an external antifungal agent and we examined the absorbability of KP-363 to horny matter and the reduction of antifungal activity when adsorbed with horny matter.

Materials and methods

1) Drug

KP-363 (Lot 2000), clotrimazole (Lot 840614) and bifonazole (Lot 870306) were synthesized in our laboratory. Tolnaftate (Lot 8509) was purchased from Sagami Chemical Industry. Each drug was dissolved in dimethylsulfoxide (DMSO; Wako Pure Chemical Industries) in such a way that it becomes 10mg/ml concentrated solution. After diluting the concentrated solution in DMSO two times, it was diluted with 0.1% Tween 80 distilled water to prepare a chemical solution of 1,000 to 0.03 ug / ml. ^{14}C-KP-363 (Lot 881111, specific activity concentration 44.3 μCi / mg) was labeled at our laboratory, and was dissolved in Macrogol 400:

ethanol: distilled water 30: 20: 50 mixture solution in such a way that it becomes 1%.

2) Study on percutaneous absorbability of KP-363

Hair of the back skin of the male Hartley type guinea pigs was depilated using electric hair clippers, an electric shaver and a depilatory cream to avoid irritation as much as possible. The next day, the ^{14}C - KP – 363 1% solutions, 0.2 ml (20 μCi / 2 mg / 0.2 ml) was applied to the gauze 2 × 2 cm, and it was hermetically sealed at the same site for 6 hours with parafilm and elastopore (Nichiban). The unabsorbed drug was removed with absorbent cotton and alcoholic cotton 6 hours after application. Animals were slaughtered 6 and 24 hours after application (18 hours after the end of application), the skin at the application site was cut out and 50 μm frozen segment were prepared parallel to the skin surface. Each section was solubilized with Soluene-100® (Packard), neutralized with acetic acid and radioactivity was measured with a liquid scintillation counter (Beckman LS 9000).

3) Anti-Trichophyton mentagrophytes activity of antifungal agent adsorbed to the hair

a) Preparation of used strain and bacterial solution

Trichophyton mentagrophytes strain KD - 04 was a clinical isolate and cultured in Sabouraud medium at 27 ° C for 2 weeks was used. Saline containing 0.1% Tween 80 was added dropwise, the surface was rubbed with a platinum loop, the conidia were suspended, gauze filtered, and small conidia were counted on a leukocyte calculation plate to prepare 10^5 cells / ml.

B) Preparation of crushed hair

Since it is difficult to obtain a large amount of normal horny layer, human hair was used. Human hair was degreased several times with ethanol • ether (50:50, vol / vol). After degreasing, it was thoroughly rinsed with distilled water. Subsequently, the hair frozen with liquid nitrogen was ground in a mortar to prepare crushed pieces.

C) Measurement of antimicrobial properties of hair with adsorbed drug

100 mg of crushed hair was taken in a test tube (inner diameter 18 mm), and a solution A (0.2% di-potassium phosphate, Wako Pure Chemical Industries, 0.005% magnesium sulfate, Wako Pure Chemical Industries, 0.005% calcium chloride, Hayashi Pure Chemicals, PH 6.0) was added, and autoclave sterilization was carried out. After sterilization, two times diluted KP-363, tolnaftate, clotrimazole or bifonazole solution 0.2ml (200,100, 50, …0.006 μg/100mg) (hair / tube) was added after 1 hour of shaking (120 rpm / min) at 30 ° C to absorb the agent and the hair in solution A, 0.2 ml (2 x 104 cells / tube) of T. mentagrophytes suspension was inoculated, and cultured at 30 ° C. for 7 days. In another experiment, drugs and hair were shaken for 1 hour at 30 ° C, unabsorbed drug was removed by centrifugation, 2 ml of solution A was added, and shake was carried out at 60 ° C for 3 hours. After shaking, the supernatant was removed by centrifugation; 1.8 ml of Solution A and 0.2 ml of bacteria were added and incubated at 30 for 7 days. Under this condition, T. mentagrophytes develops only hair fiber as a nutrient source. Presence or absence of bacterial growth was examined macroscopically or microscopically, and it was defined as the minimum inhibitory concentration (MIC) with the minimum concentration of the agent that does not show bacterial growth. As a control, MIC in sub-liquid medium (1% polypeptone, Japan Pharmaceutical, 3% Glucose, Wako Pure Chemical, PH 6.0) was examined by liquid medium dilution method.

Result

1. Investigation of drug permeability after applying KP-363 to guinea pig skin

To observe the distribution of KP-363 in the skin after 0.2 ml of 1% 14C-KP-363 solution was sealed and pasted for 6 hours and 18 hours after completion of the application, the skin specimen was sliced parallel to the skin surface at a thickness of 50 μm and the radioactivity of each section was measured with a liquid scintillation counter. As shown in Fig. 1, the results showed that after 6

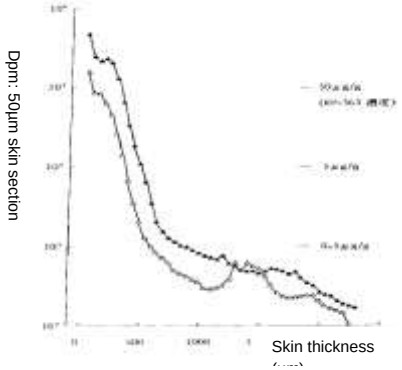

Figure 1 KP-363 Guinea pig skin concentration distribution after application

14C-KP-363 1% sealed and attached to guinea pig skin for 6 hours. Cut the skin at 6 hours of application and 24 hours after application (18 hours after application), prepare 50 am frozen sections parallel to the skin surface, and measure the radioactivity of each section with a liquid scintillation counter.

▲ - ▲ Skin KP - 363 distribution 6 hours after application

△ - △ distribution of skin KP - 363 after 24 hours of application

hours of application, drugs were present at several um of the surface layer at a high degree and next, after reserving storage up to 200 to 400 μm which seems to be a sebaceous gland existing site, it will spread rapidly but several peaks which appeared to be adsorbed in the hair were observed at the site of 1,000 to 2,000 μm as discussed in detail. A spread of 50 μg / g or more was found in terms of the concentration of KP - 363 in the total epidermis including the stratum cornea, which is the habitat of dermatophytes. The distribution pattern of KP-363 in the skin at 18 hours after the sealing and application of KP-363 for 6 hours was almost the same as after 6 hours of application, the whole concentration showed a moderate decrease, and suggesting high sustainability in the skin. In the epidermis containing the stratum corneum, a distribution of 10 μg / g or more in terms of the concentration of KP-363 was found, and a trend with high affinity was observed in the same site.

2. Anti-T. Mentagrophytes Activity of Antifungal Agents Adsorbed on the Hair

The anti- T. mentagrophytes activity of KP-363, tolnaftate, clotrimazole and bifonazole was investigated and the results are shown in Table 1. As shown in C of Table 1, KP-363 had the strongest antibacterial activity in the Sabouraud medium, and inhibited the growth of the fungus at 0.0125 μg / ml. Its antibacterial activity was 4 to 32 times stronger than that of the control drug. Then, when examining the antimicrobial effect of the drug adsorbed on the hair in a medium using the hair as a nutrient source, as shown in A in Table 1, KP-363 had a MIC of 4 μg / g (hair) on the hair follicle and a 320-fold increase compared to MIC in the Sabouraud medium. A similar tendency was observed for the control drugs, and tolnaftate, clotrimazole and bifonazole showed increases of 312 times, 160 times and 160 times, respectively. Even when treating the hair and the absorbed drug for 3 hours at 60°C, its antibacterial activity remained unchanged and its adsorption was considered to be strong (B in Table 1).

Table 1 Effect of Milled Hair on Anti-Trichophyton mentagrophytes Activity of KP-363, tolnaftate, clotrimazole and bifonazole

Medicine	MIC (μg/g hair) (μg/ml) (Sabouraud medium)		
	(medium)		Hair grinding
	A*	B**	C***
zKP-363	4.0	4.0	0.0125
Tolnaftate	15.6	15.6	0.05
Clotrimazole	62.5	62.5	0.39
Bifonazole	62.5	62.5	0.39

*: Add 1- step dilution series of medicinal solution to the crushed piece, add T. mentagrophytes 2×10^4 cells after 30 minutes at 30°C, incubate for 7 days at 30°C, then MIC measurement from the presence or absence of bacterial growth

**: Add chemical solution of two-step dilution series to crushed pieces and shake for 60 minutes at 30°C. Removed unapplied medicine in hair, further inoculated at 60°C, shake for 3 hours, inoculated with 2×10^4 T. mentagrophytes, and judged after culturing for 7 days

***: Add 2-step dilution series of drug solution to Sabouraud liquid medium, shake for 60 minutes at 30°C, then contact 2×10^4 T. mentagrophytes

Concept

Regarding the adsorption ability of horny and antifungal agents, Freedman et al.[6] tested the adsorption between human hair and griseofulvin and when griseofulvin was added at a concentration of 16.5 μg / ml, it was reported that it can absorb from 20 to 30% and recover 70 to 80% by extraction with 60 °C of distilled water. In the study of the anti - T. mentagrophytes activity of the drug adsorbed to the hair and the hair's ability to absorb thiomersal, an organic mercurial agent, halogen phenolic pentachlorphenol soda (PCP - Na) and antiseptic soap benzethonium chloride, Takahashi has reported that there is a difference in adsorption of these drugs to the hair, 15% by thiomersal, 79% by PCP - Na, 80% by benzethonium chloride is adsorbed to the hair, and thiomersal has a low adsorption rate but the binding rate is high. The antimicrobial effect of the drug adsorbed on the hair was 64 μg / g (hair) of thiomersal, 500 μg / g of PCP - Na, 3,600 μg / g of penicillium chloride, and the MIC of the above - 10 and 25 μg / ml, and it was revealed that the antimicrobial concentration per unit hair amount of medicine adsorbed to hair decreased by 42 times, 50 times and 144 times respectively compared with those in liquid state and in order to expect a reliable antimicrobial effect, it is suggested that the intracellular degree showing growth inhibition in in vivo experiments on hair rather than in vitro solution must be obtained by application of an antibacterial agent. When KP-363 1% solution 0.2 ml (2 mg) was hermetically attached to the back skin of the guinea pig for 6 hours, the presence of KP-363 of 50 μg / g or more was observed on the surface skin (FIG. 1). As with tolnaftate, clotrimazole and bifonazole, KP-363 decreased its antimicrobial activity by adsorbing with keratin (Table 1) but nevertheless, considering the inhibition of the development of T. mentagrophytes at 4 μg / g, it was revealed that the 1% solution of KP-363 had sufficient concentration to show antimicrobial effect in keratin after application. In addition, since KP - 363 adsorbed by hair does not change antibacterial effect even with

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