

Percutaneous absorption test – A case of effectiveness evaluation of skin-whitening cosmetics

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Abstract

To evaluate the effect of skin-whitening cosmetics, there is currently available a method to evaluate the degree of the disappearance of pigmented areas produced by ultraviolet irradiation by applying a sample to the skin. This method is convenient for evaluating a cosmetic formula. In animal models, this method shows much less individual differences, and due to the easiness in preparing animals to be tested, it is easier to obtain the number of experiments necessary for statistical analysis, providing an advantage in making an objective evaluation. However, fixing a live animal for irradiating with ultraviolet rays etc. may cause pain to the animal. By adopting an alternative method, we believe that "reduction in pain for testing animals" will become possible. In evaluating the effectiveness of a skin-whitening cosmetic, we produced a Percutaneous Absorption Test, in addition to the blackening reduction test in humans. This enabled us to predict the difference in the skin-whitening effect of a formula more clearly. This case proved the effectiveness of a formula of a skin-whitening cosmetic without use of a testing animal, and we described the evaluation of the skin penetration.

Keywords: skin whitening, percutaneous absorption, skin penetration, ultraviolet irradiation, riboflavin

Introduction

To date, various methods have been proposed to assess the skin-whitening effects of cosmetic products to deal with skin spots, and other blemishes. They included an assessment of melanin suppression *in vitro*, monitoring of humans, and assessment of the effects against spots artificially induced by ultraviolet (UV) rays in experimental animals. For cosmetic preparations using oil as a vehicle, assessment of their skin-whitening effects in cells *in vitro* is difficult because of the cytotoxicity of oil. When assessment is made in humans, it is expected to obtain highly practical data through assessment on live skin, reflecting the real skin conditions (temperature, texture irregularities, permeability, etc.). However, assessment in humans can be affected greatly by inter-individual differences in skin condition, etc. Furthermore, it is difficult to collect subjects in a number large enough to allow valid statistical analysis. It is therefore difficult to obtain consistent data from multiple studies in humans. It is particularly difficult to assess delicate differences

in efficacy depending on the formulation of cosmetic preparations even when the preparations are based on the same active ingredient. Inter-individual differences are smaller in animal studies than in human studies and it is relatively easy to collect sufficient data from a number of animals. However, animal studies inevitably involve some pain to the animals and are thus against the 3R principle "the reduction of pain in laboratory animals". It is therefore necessary to reduce the frequency of animal studies. So, to evaluate the effectiveness of skin-whitening cosmetic formulations, we made a percutaneous absorption test, in addition to a blackening reduction test for humans. This enabled us to predict the difference in the skin-whitening effect of a formula more clearly.

Material and methods

Blackening reduction test

UV irradiation with an exposure of 1.8 MED was applied continuously on the upper arm of six panels using a solar simulator (Koyo Co., Ltd, Japan), and an erythematous patch 1 cm in diameter was created.

Table.1 Formulation sample containing a given amount of kojic acid

Formulation Type	Concentration of target ingredient (%)	Form of formulation	Remarks
A	1	emulsion	Containing 1% micro emulsion
B	1	emulsion	-

Different formulation samples A and B containing a given amount of kojic acid as a whitening active ingredient were prepared, and they were applied on the erythematous sites twice a day, in the morning and at night for 10 days. In addition, the skin color was measured with a spectrophotometer (CR-2002, Minolta, Japan) before the application and one day, four days, ten days after the application.

Measuring the amount of permeated kojic acid

A sample containing a given amount of kojic acid was applied on a human forearm in a fixed quantity, and left to rest for 10 minutes. Then, after the remaining sample on the skin surface was wiped off with a given pressure, a tape strip was performed at a certain range with adhesive tape. (Serotape, Nichiban, Japan.) Next, the tape containing stratum corneum was immersed in a 0.1 mol/l citric acid solution. After 24 H, a constant amount was taken as a sample from the solution for measuring. Then, for every sample, the amount of kojic acid was measured by HPLC (Waters, Japan). BEHC18 1.7 (Column Size: 2.1 mm ×100 mm) was used as a column and 0.1 mol/l citric acid was used for the mobile phase. Detection limits were set at 270 nm with a 10 µl injection.

Measuring the amount of permeated riboflavin

A sample containing a given amount of riboflavin was applied on a human forearm in a fixed quantity, and left at rest for 10 minutes. Then, after the remaining sample on the skin surface was wiped off with a given pressure, a tape strip was performed for a certain range with adhesive tape (Serotape, Nichiban, Japan.). Next, the tape containing stratum corneum was immersed in a 1% sodium lauryl sulfate solution. After 24 H, a constant amount was taken as a sample from the solution for measuring. Then, for every Scary sample, the amount of riboflavin was measured by fluorescence spectrophotometer (Power scan, DS Pharma Biomedical, Japan.) The excitation wavelength was 400 nm, and the fluorescence wavelength was 460 nm. Different formulation samples, C and D and E and F containing a given amount of target ingredient were prepared (detail is shown in Table.1.), and the amount of riboflavin was measured the same way.

Measuring the amount of exfoliated stratum corneum

Protein assay was done to measure the exfoliated stratum corneum (SC) by tape-stripping. First, the tape attached SC was immersed in 1%TrironX in 0.1M

phosphoric acid buffer and incubated 30 minutes. Then, BCA protein Assay Reagent (PIRCE, Rockford, IL) was added to the sample, and absorbance at 562 nm was measured. From the fluorescence intensity the amount of exfoliated SC was calculated, and used for the compensation of the attached SC.

Results

Blackening reduction test

Fig. 1 shows the mean skin brightness level after exposure to UV rays. This figure shows a change in skin brightness after UV ray exposure relative to the pre-exposure brightness. Percent elevation in skin brightness 4 days or more following exposure to UV rays was higher in the sample-treated area than in the untreated area (Fig. 2). This increase was particularly marked in the area treated with Formulation A.

Measuring the amount of permeated kojic acid

Fig. 3 shows the results of a test in which the amount of kojic acid permeating across the horny layer was measured by tape-stripping and compared between human forearms treated with two samples (Formulation A and B). As shown in this figure, the amount of permeation of kojic acid (a major ingredient in skin-whitening preparations) was greater with Formulation A than with Formulation B at each depth of the horny layer evaluated, consistent with

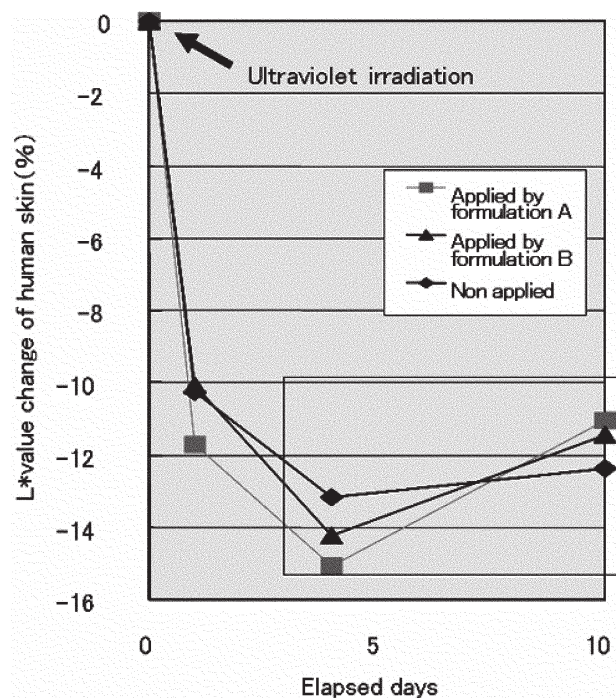


Fig. 1. L*value change of human skin after ultraviolet irradiation

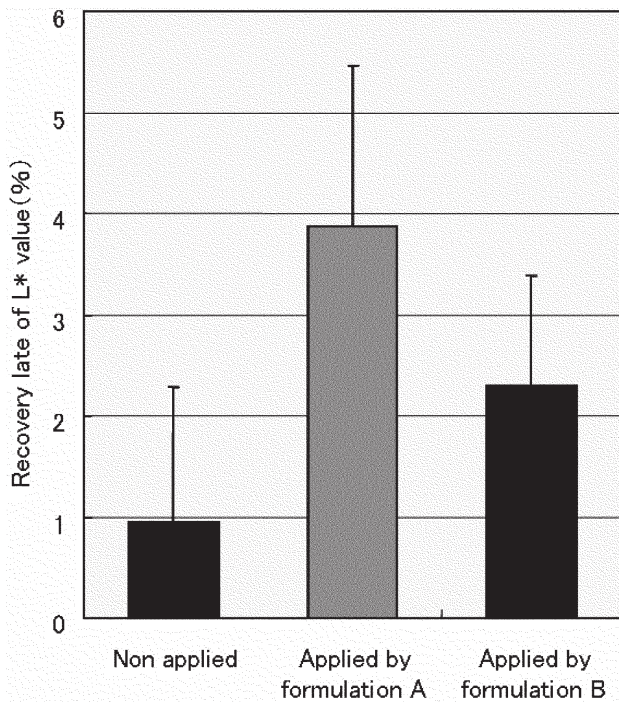


Fig. 2. Comparison of lightening effect of the control and formulation A and formulation B. (From day 4 to day 10)

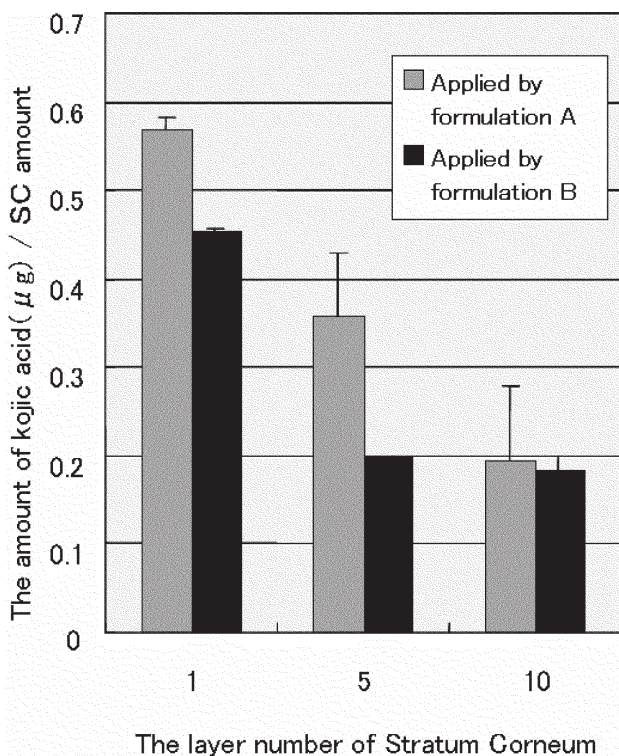


Fig. 3. Comparison of the amount of kojic acid with formulation A and formulation B at different depths of the stratum corneum.

the results of the blackening reduction test designed to evaluate skin-whitening effects.

Measuring the amount of permeated riboflavin

Fig. 4 shows the results of a test in which the amount of riboflavin permeating across the horny layer was measured by tape-stripping and compared

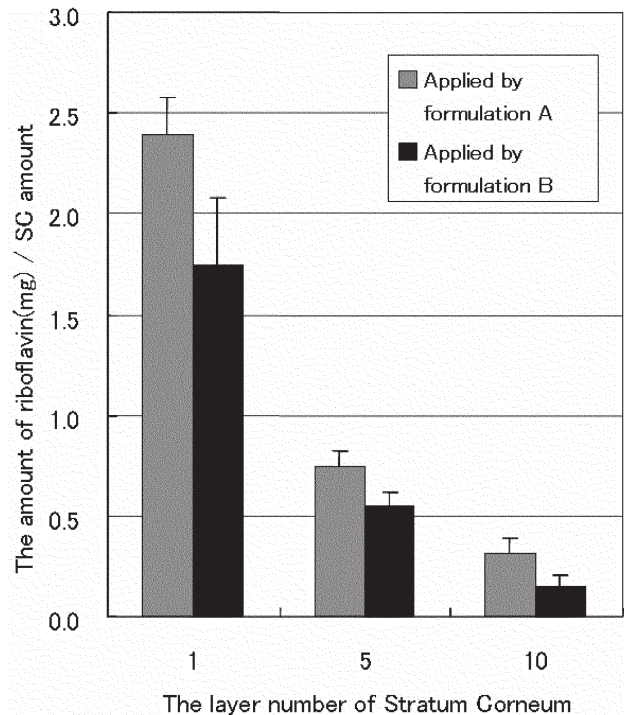


Fig. 4. Comparison of the amount of riboflavin with formulation A and formulation B at different depths of the stratum corneum.

between human forearms treated with two samples (Formulation A and B). Because riboflavin is dispersed mostly in the aqueous phase of the formulations, the amount of permeation determined in this test can be viewed as reflecting the amount of dispersion of the bulk drug. As shown in this figure, the amount of permeation of riboflavin was greater with Formulation A than with Formulation B at each depth of the horny layer evaluated, indicating that the tendency in riboflavin dispersion was consistent with that of kojic acid dispersion.

Discussion

As shown above, the results of the blackening reduction test (designed to evaluate skin-whitening effects) of Formulations A and B were identical to the results of the percutaneous absorption test (designed to measure permeation across the horny layer) of the same formulations. Formulations A and B contained the same active ingredient (kojic acid) which was expected to exert skin-whitening effects, and the concentration of this ingredient was the same for the two formulations. The results suggested that the difference in permeability associated with the composition of the formulations resulted in a difference in skin-whitening effects between these two formulations. When the frequency of animal studies needs to be reduced, they are usually replaced with in vitro tests or studies in humans. For cosmetic preparations using oil as a vehicle, assessment of their skin-whitening effects in cells in vitro is difficult because of the cytotoxicity of

the oil. When assessment is made in humans, it is expected to produce highly practical data through assessment in live skin, reflecting the real skin conditions (temperature, texture irregularities, permeability, etc.). However, assessment in humans can be affected greatly by inter-individual differences in skin condition, etc. Furthermore, it is difficult to collect subjects in a number large enough to allow valid statistical analysis. It is particularly difficult to assess delicate differences of efficacy in studies with humans. The only difference between Formulations A and B was the presence/absence of a small amount of 1% emulsion. Although Fig. 1 and Fig. 2 shows a difference in the blackening reduction test (an indicator of skin-whitening effects) between the two formulations, the variance in results among different individuals was too large to allow the results to be accepted as reliable data. For this reason, we additionally conducted a test designed to measure the skin permeability of the formulations in humans. First, we measured the amount of permeated kojic acid that was the whitening agent of the formulations by using HPLC. From Fig. 3, we can see the difference in permeability between the two formulations. Next, we measured the amount of permeated riboflavin. The permeability of riboflavin reflects the barrier function of human skin (Minehiro, 1999), and it is generally well known that the skin barrier function has a high correlation with skin absorption rates (Andre, 2005). So we performed a skin absorption test for riboflavin using another method to distinguish the difference between the two formulations. As a result, we can see the same tendency of penetration from Fig. 3 and Fig. 4. The reason was that riboflavin and kojic acid have close IOB (Inorganic Organic Balance) in addition they are soluble in water, they locate about the same in a formulations. When skin permeability of cosmetic preparations in humans is evaluated, the target ingredient is usually quantified by HPLC, etc. However, the results from the measurement of Bulk permeation using riboflavin were similar to

the results from quantification with HPLC. The use of riboflavin as an indicator of skin permeability for cosmetic preparations is advantageous in that the manipulations required for the experiment are simple. When HPLC is used, it is necessary to select a column and a mobile phase optimal to the samples to be tested. In case of riboflavin measurement, color development is possible with a minimum amount of the sample if the sample is soluble, and assessment can be done simply just by measuring the intensity of fluorescence from the eluate. On the whole, to use percutaneous absorption tests in addition to the blackening suppression test makes it possible to determine the merits and demerits of formulations more clearly, even in human clinical trials. Therefore, it can also support the goal of "the reduction of pain in laboratory animals", since the UV irradiation test using animals become unnecessary. Also the permeability politic of pair formulation estimated by riboflavin fluorescence and measured by water-soluble active ingredients was the same.

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