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In Vitro and *In Vivo* Studies of Topical Delivery System of Gentisic Acid in Hairless Mice

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ABSTRACT–Gentisic acid is a skin-whitening agent which inhibits the tyrosinase activity, an essential enzyme in the process of biological synthesis of melanin. Since melanin is synthesized in melanocytes located between the viable epidermis and dermis layer, drug amount delivered into the epidermis/dermis layer can provide valuable information for the biological effect of skin-whitening agents. The purpose of this study was to prepare the gentisic acid patches with 2% dodecylamine as enhancer, and to observe the *in vitro* skin permeation and *in vivo* skin deposition of gentisic acid. Gentisic acid in Duro-Tak 87-2510 patch formulation permeated across hairless mouse skin at the rate of 40.79 μg/cm^2/hr. *In vivo* study showed that the gentisic acid amount in both the stratum corneum and the viable epidermis/dermis increased with the increase of application time. The amount of gentisic acid in the stratum corneum was higher than that in the epidermis/dermis layer, and was expected to provide a reservoir effect even after removing the patches. Thus, the patch formulation seems to be useful for the topical delivery of skin-whitening agent into the epidermis/dermis layer, the target site.

Key words–Gentisic acid, Topical delivery, Tape-stripping, Skin-whitening

Gentisic acid (Figure 1), a natural product found in the root of the *genus Gentiana*, can be applied in the treatment of skin pigmentary disorders.[1] It regulates the rate-limiting steps of mammalian melanin synthesis through inhibition of melanosomal tyrosinase activity, which catalyzes the conversions of L-tyrosine to L-dopa and L-dopa to L-dopaquinone.

Studies with corticosteroids, miconazole and ketoconazole have indicated that the topical drug concentration in the target site can be correlated to the pharmacodynamic response.[2] Since melanocytes lie in the interphase of the epidermis and dermis,[3] the topical concentration of gentisic acid in the epidermis and dermis layer may be a critical indicator for the skin-whitening effect.

In a recent study, we have observed the relationship between skin permeation of gentisic acid and its skin deposition after 12 hr permeation of gentisic acid patches in rats *in vitro*.[4] But these results were based on drug concentrations in the whole skin and were not able to provide data in various skin layers which is important for the evaluation of formulations. *In vivo* drug distribution in various skin layers can give more valuable information about the efficiency of topically applied formulations.

The tape stripping technique can be used to separate the stratum corneum from the epidermis/dermis layer and to measure the delivery efficiency of drug following topical application. It is a simple, easily performed as well as a well-controlled method.[5] This technique has already been successfully used in other molecules, especially in *in vivo* studies.[6-8]

In order to assess the matrix-type topical delivery system of gentisic acid, its distribution in the stratum corneum layer and the epidermis/dermis layer was separately determined after topical application for various time periods. Herein, we report the feasibility of the tape-stripping method to evaluate the skin deposition of the skin-whitening agent *in vivo* using hairless mouse.

Experimental

Materials

Gentisic acid and dodecylamine were purchased from Sigma Chemical Co.(St. Louis, MO, USA). The pressure-sensitive adhesive (DuroTak 87-2510) was kindly supplied by National Starch and Chemical Company (Bridgewater, NJ, USA).

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Release liner (Scotch® Tape MSX-378) and backing laminate (Scotchpad 1012 polyester film) were obtained from 3 M Co. (St. Paul, MN, USA). All the chemicals were of the purest grade available.

Stability of gentisic acid for skin permeation study

Extraction of hairless mouse skin was conducted in the Valia-Chien diffusion cells at 37°C for 24 hours as previously reported.[9] Briefly, a freshly-excised hairless mouse skin specimen was mounted between the two half-cells, with the stratum corneum facing the donor half-cell and the dermis facing the receptor half-cell. Both donor and receptor half-cells were filled with an isotonic phosphate buffer (IPB, pH 7.4) solution (3.5 mL). After 24 hr of extraction, the donor and receptor solutions from each pair of half-cells were separately combined together and stored in the freezer until used for the stability study.

Dermal and epidermal extraction of hairless mouse skin containing 10 μg/mL of gentisic acid was placed in a shaking incubator at 37°C. At predetermined time intervals, samples were taken and the concentration of the gentisic acid was analyzed by HPLC. The relative percentage of remaining gentisic acid was calculated as a function of time when the initial concentration was considered as 100%.

Preparation of gentisic acid patches

Gentisic acid (0.2 g) was first dissolved in the minimum volume of acetone, and then was mixed with 10 g of pressure-sensitive adhesive and 200 mg dodecylamine using a mechanical stirrer at 800 rpm for 15 min under occluded condition. The mixture was sonicated for an additional 10 min. The solution was cast on the release liner with a micrometer adjustable casting knife (R.K. Coat Instruments LTD., U.K.) set at 100 μm, and was dried at 80°C for 25 min. The patches were covered with backing laminate and cut into appropriate sizes.

***In vitro* skin permeation study of gentisic acid patches**

Male hairless mice (20–30 g) were obtained from Dae-Han Laboratory Animal Research Center Co. (Daejeon, Korea), and were humanely sacrificed in a CO_2 chamber. A full-thickness of skin was surgically removed from the dorsal region, and was carefully cleaned with normal saline, then cut into 1.6×1.6 cm^2 pieces for the permeation experiment.

In vitro skin permeation was conducted using Valia-Chien diffusion cells at 37°C. Freshly excised mouse skin was mounted between the two half-cells, then a gentisic acid patch was applied on the stratum corneum side of the skin. The other half-cell was also mounted with the skin and a patch, then two half-cells were clipped together. The receptor half-cells were filled with 3.5 mL of IPB solution containing 40% (v/v) propylene glycol (PG). At predetermined time intervals, 400 μL of receptor solution was taken to determine the amount of gentisic acid permeated, and refilled with the same volume of fresh receptor solution. Samples were kept in the freezer until analyzed by HPLC.

***In vivo* skin deposition of gentisic acid**

In order to investigate the skin deposition of gentisic acid *in vivo*, a topical patch was applied on the dorsal skin of hairless mouse after the animals were anesthetized by urethane. At predetermined time interval (3, 6, and 12 hours), mice were sacrificed in a CO_2 chamber. The patch was removed and the stratum corneum of the patch-treated site was collected using the tape-stripping method.[6] Ten individual Scotch tapes (1.8×1.8 cm^2, 3 M Company, USA) were utilized while each piece of tape was used in two successive strips, thus the total strip number was 20 times. Then, every piece of tape was extracted with 1.0 mL of methanol by shaking overnight. The stripped skin was surgically peeled and was cut into small pieces, and homo- genated with 2 mL of methanol for 2 min at 10,000 rpm. Then, the suspension was centrifuged for 20 min at 5000 rpm. The supernatant was filtered through micropore filters (0.45 μm, Sartorius Co., Germany). Concentration of gentisic acid in all samples was analyzed by HPLC.

HPLC analysis of gentisic acid

The concentrations of gentisic acid were determined by a HPLC system (Gilson Model 306) equipped with an automatic injector (Gilson Model 234). A Merck C_{18} column (LiChrospher 125×4 mm, 5 μm particle size, Merck RP-18, Darmstadt, Germany) was used at ambient temperature. The mobile phase was a mixture of methanol and water (28:72, v/v) containing 1% phosphoric acid at a flow rate of 1.0 mL/min. The variable wavelength ultraviolet detector (Gilson Model 118) was set at 333 nm. Injections of 20 μL were made for all samples to be analyzed. The retention time of gentisic acid was about 5.7 min.

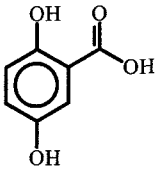

Figure 1–Chemical structure of gentisic acid.

Results and discussion

Stability of gentisic acid for skin permeation study

The degradation of gentisic acid during the course of *in vitro* skin permeation study was investigated using the skin extracts. As shown in Figure 2, gentisic acid was stable in both the dermis and epidermis extracts of hairless mouse skin. Thus, no degradation of gentisic acid was anticipated during the *in vitro* skin permeation study. In order to provide better sink condition, 40% PG/IPB (v/v) solution was chosen as receptor solution.

In vitro skin permeation of gentisic acid

In our previous study,[4] dodecylamine was the most potent among the enhancers tested. Thus, Duro-Tak 87-2510 patch with 2% dodecylamine and 2% gentisic acid was chosen as the formulation for the *in vitro* skin permeation study in hairless mice. As shown in Figure 3, the skin permeation rate of gentisic acid decreased after 9 hours, probably due to the depletion of the gentisic acid and/or dodecylamine thereby decreasing the driving force. The permeation rate of gentisic acid in the initial stage was 40.79 (±2.43) μg/cm^2/hr with 0.75 (±0.23) hours of lag time.

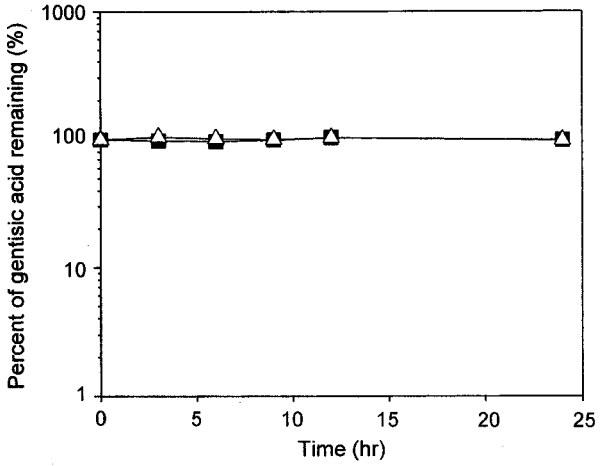

Figure 2–Stability of gentisic acid in dermal extraction of hairless mouse skin at 37°C. Key : ■; epidermal extract and △; dermal extract of hairless mouse skin.

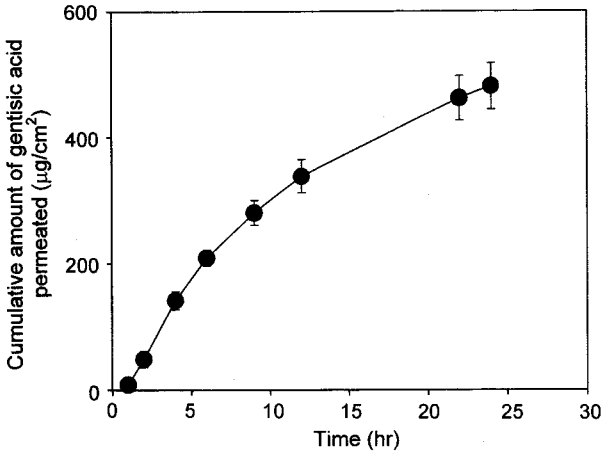

Figure 3–Hairless mouse skin permeation profile of gentisic acid from Duro-Tak 87-2510 topical delivery system containing 2% gentisic acid and 2% dodecylamine as an enhancer.

In vivo skin deposition of gentisic acid

Figure 4 shows the cumulative amounts of gentisic acid in the tape-strips following the application of the patches for 6, 9 and 12 hr, respectively. The amount of gentisic acid in the

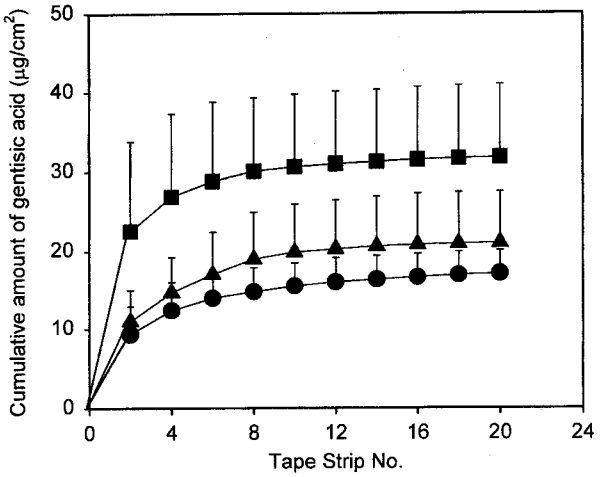

Figure 4–Cumulative amount of gentisic acid within tape-stripped stratum corneum after applying the topical delivery system for various durations. Key : ●; 6 hr, ▲; 9 hr, and ■; 12 hr application.

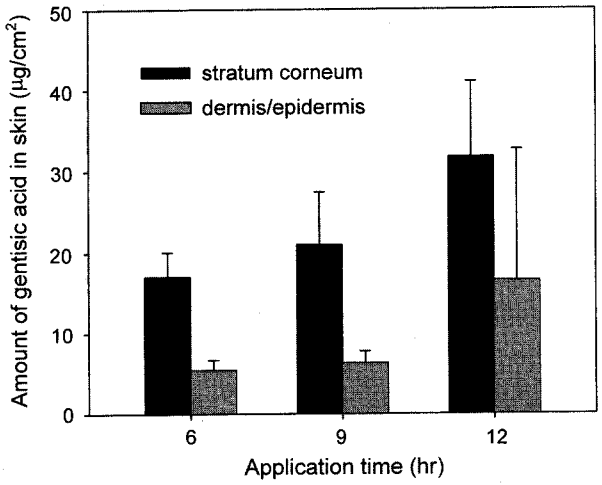

Figure 5–Amount of gentisic acid in stratum corneum and viable epidermis/dermis layer following application of the topical delivery system on hairless mice *in vivo* for various durations.

stratum corneum increased as the application time of the patches increased. Also, cumulative amount of gentisic acid reached the plateau level after 20 times of tape strips. It is interesting to note that cumulative amounts of gentisic acid rapidly increased in the beginning, but did not linearly increase with the tape strip number. It might be due to the localization of gentisic acid near the surface of the stratum corneum.

The amounts of gentisic acid in the stratum corneum and the viable epidermis/dermis layer after various application times of patches are shown in Figure 5. The amount of gentisic acid in both stratum corneum and viable epidermis/dermis layer increased with increasing the application time. Gentisic acid content in the stratum corneum was higher than that in the epidermis/dermis layer. However, as the reservoir effect of the stratum corneum has been well established,[10] gentisic acid in the stratum corneum is expected to diffuse slowly to the direction of deeper layers of skin, the target site responsible for the skin-whitening, even after the termination of the application and could show a sustained effect.

Nowadays, the use of transdermal drug delivery technology employing skin patches has been applied to the local delivery of cosmetically active ingredients.[11] Applying the patch technology may further improve the product performance by providing longer duration of action and deeper penetration of the active ingredients.[12] Meanwhile the patch-type formulation shows several advantages compared with other topical dosage forms, such as ointment, lotion, cream: (1) avoidance of contamination, (2) ease of discontinuing the administration, and (3) prevention of wash-out. Compared to the *in vitro* skin permeation study, the *in vivo* study can well imitate the situation of application of the patches on human skin because the microenvironment under mammalian skin is quite different from the receptor solution of the *in vitro* studies.

In conclusion, 2% gentisic acid patches with 2% dodecylamine as enhancer could effectively deliver gentisic acid into the epidermis/dermis layer, the target site of the skin-whitening effect. Also, the *in vivo* skin deposition study seems to be more useful to evaluate the topical delivery systems than the *in vitro* skin permeation study.

Acknowledgements

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