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The Effect of Limonene on Skin Permeation and Localization of Ascorbic Acid

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ABSTRACT – Poloxamer-based hydrogel formulation for the topical delivery of ascorbic acid (AsA) was prepared and the effect of limonene on AsA skin permeation and localization was evaluated. In vitro rat skin permeation study, the AsA skin permeation of limonene-containing hydrogel was about 3 to 5 fold higher than control hydrogel. However the amount of permeated AsA was independent to the concentration of limonene. On the other hand the localized amount of AsA after 2 h increased proportion to the content of limonene. The increase in the ratio of localized AsA (Q_L) to permeated AsA (Q_P) was attributed to the limonene's ability of making polar pathway within stratum corneum by interacting on lipid domain of the skin and the AsA's hydration effect on the stratum corneum and effect on the protein domain of the skin.

Key words – Ascorbic acid, Limonene, Skin permeation, Skin localization

Ascorbic acid (AsA) has been introduced as an active ingredient for pharmaceutical and cosmetical products because of its anti-aging, anti-oxidant and skin whitening effect.[1,2] AsA stimulates human skin to produce collagen thereby retards the formation of wrinkles and assists to avoid a prematurely aged look of skin.[3] It also helps to prevent or minimize lipid oxidation, cellular damages resulting from prolonged exposure to the ultraviolet rays of the sun.[4,5] In addition, it inhibits the formation of melanin which leads to skin discoloration during the aging process and the release of histamine from cellular membranes reported to be responsible for many allergenic reactions.[6]

Generally, the skin permeation characteristics of hydrophilic drugs are less efficient than hydrophobic drugs because of their low affinity to the skin.[7] To enhance the delivery efficacy of hydrophilic AsA, both strategies of skin permeation and localization enhancement of AsA are required since AsA stimulates and regulates the biosynthesis of collagen by 4-hydroxylation of proline and lysine residue of collagen at viable skin layer.[8]

In 1998, Lee and Tojo have reported the enhanced skin permeation results of AsA, where the skin permeation of AsA was highly limited by the stratum corneum. In their study, AsA was able to be permeated greatly through the skin by the hydration of skin caused by AsA.[9] To date, many efforts have been centered to deliver AsA with high efficiency through the skin.[5,6,10] However, few studies have reported the strategies for increasing skin permeation and localization of AsA.

Terpenes have been known to increase the percutaneous absorption of hydrophilic drugs,[11] and believed to be a low irritant skin permeation enhancer.[12] The skin permeation enhancing mechanism of limonene, one of the terpenes, on hydrophilic drugs was known as its disrupting effect on intercellular lipid order of skin, increasing the opening of polar pathways in the stratum corneum.[13] In the present study, we intended to examine the effect of limonene on the skin permeation and localization of AsA.

Experimental

Materials

AsA was provided from Takeda. Co., Ltd (Chuo-ku, Osaka, Japan) and poloxamer 407 was obtained from BASF Korea (Chung-gu, Seoul, Korea). (R)-(+)-limonene was purchased from Sigma-Aldrich Company (St. Louis, MO, USA) and all other solvents reagents were of reagent grade and used as received.

Preparation of AsA formulations

Poloxamer hydrogel was prepared by traditional cold method. Briefly, 25 g of poloxamer 407 was added to 100 ml of distilled water and mixed for one day in cold chamber maintained at 4~8°C. AsA and various concentration of limonene were added to the hydrogel and mixed until skin permeation study. The concentration of AsA in the hydrogel formulation was 2 %.

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Skin permeation study

Male Sprague Dawley rats (12-14 weeks old, weighing 120 ± 10 g) were used for in vitro skin permeation study. The dorsal skin was removed carefully leaving the fat tissue and rinsed with PBS. Prepared skin samples were stored in −70°C deep freezer until skin permeation experiments. One gram of the hydrogel samples was loaded onto the donor compartment of Franz diffusion cells with a permeation area of 2.00 cm^2. A glycerin : phosphate buffer (pH 3.5) = 10 : 90 contains 0.1 mM EDTA was used as a receptor solution (11 ml) and the receptor solution was maintained at 37 ± 0.5°C.

Skin localization study

After skin permeation study, rat skin was removed from Franz diffusion cell. The hydrogel formulation of donor compartment was separated and the skin was washed with saline solution 3 times. The drug loaded area was cut off with scissor and stored in −70°C deep freezer until homogenization. The weight of the skin was measured and minced with Ultra-turrax homogenizer (Ultra-turrax T25 basic, IKA Inc., Germany) at 14,000×g for 5 min and centrifuged (Ecospin 314, Hanil R&D, Korea) at 4°C and 3,000×g for 10 min. One milliliter of supernatant was taken and filtered with 0.45 μm filter and analyzed with HPLC.

HPLC analysis condition

Li-NH_2 column (3.9×300 mm, 10 μm, Waters, USA.) was used to analyze AsA. The composition of mobile phase was methanol : phosphate buffer (0.02M, pH 3.5) = 70:30 with a flow rate of 1.0 ml/min. Detection wavelength was 255 nm and injection volume was 20 μl.

Data analysis

All data are represented as mean ± S.E. Statistical analysis of data sets for each groups of measurements was performed using the Student's t-Test. The levels of significance were taken as $P < 0.05$ except where stated otherwise.

Results and Discussion

To enhance skin absorption of hydrophilic drugs, chemical permeation enhancers, permeation-enhancing vehicle systems (e.g. microemulsion, liposome-based delivery system) and more complex physical enhancement strategies (e.g. iontophoresis, sonophoresis, and electroporation) have been studied.[14] In case of AsA, when considering its physico-chemical properties, it is easily expected that its skin permeation would be fairly difficult across the skin. However, it has been reported that the skin permeation of AsA was better than expected due to its hydration effect on the stratum corneum and changes in protein domain of the skin.[9] Thus, our primary aim was to test limonene which has been known to act on lipid domain of the skin so that the combined permeation effect of limonene and AsA itself may be expected. As the main action site of AsA has been known to be epidermis, a viable skin layer, the ability of penetrating across the skin would be necessary.

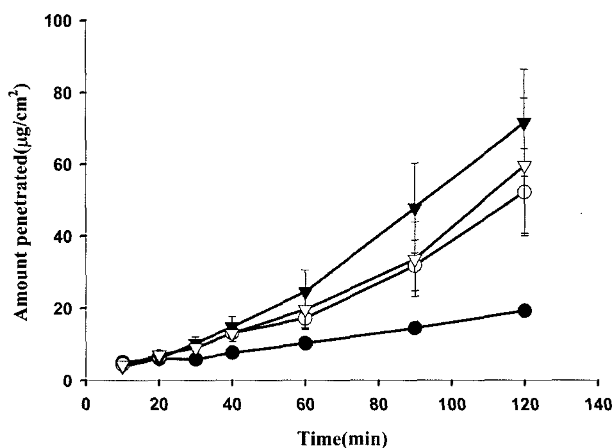

Figure 1–In vitro rat skin permeation profiles of ascorbic acid after applying 1.0 g of poloxamer hydrogel using Franz-diffusion cells (surface area of 2.00 cm^2) at 37°C (mean ± S.D., n = 3~4). Symbols are ●, control hydrogel; ○, 1 % limonene; ▼, 2 % limonene; ▽, 5 % limonene.

In vitro rat skin permeation study, we compared the skin permeation of limonene-containing hydrogel with control hydrogel. The flux values of AsA from limonene-containing hydrogels (1, 2, and 5 %) and control hydrogel were 28.99, 37.52, 25.25 and 7.93 μg/cm^2/h, respectively. The AsA skin permeation from limonene-containing hydrogels was about 3 to 5 fold higher than control hydrogel (Figure 1). The skin permeation of the hydrogel containing 5% limonene was lower than that obtained with the hydrogels containing 1 and 2% limonene but the difference was not statistically significant. The skin localization was evaluated after 2 h of experiments. The localized amount of AsA after 2 h in the skin was 85.37 (1 %), 111.01 (2 %), 146.22 (5 %), and 21.20 (control hydrogel) μg/g tissue, respectively. The localized amount of AsA after 2 h increased direct proportionally to the content of limonene (Figure 2). The ratio of localized AsA to AsA permeated linearly increased with increasing amount of limonene, indicating the increased accumulation of AsA with increasing amount of limonene (Figure 3).

Skin permeation enhancers would act by one or more of three main mechanisms as followings i) disruption of highly

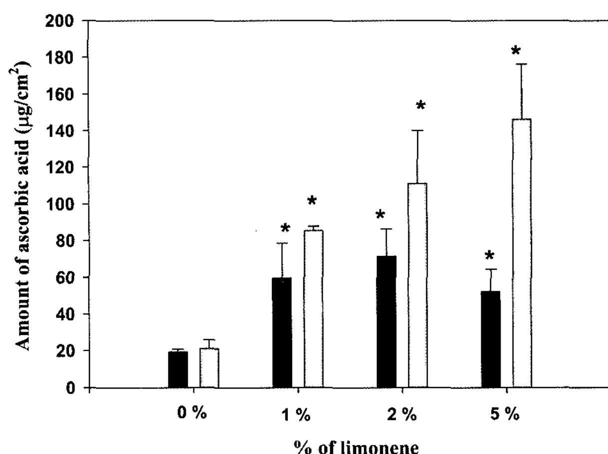

Figure 2–Comparison of permeated ascorbic acid with localized ascorbic acid with the function of the content of limonene after 2 h. Black, permeated ascorbic acid; gray, localized ascorbic acid. *P<0.05, compared to the control (0 % limonene).

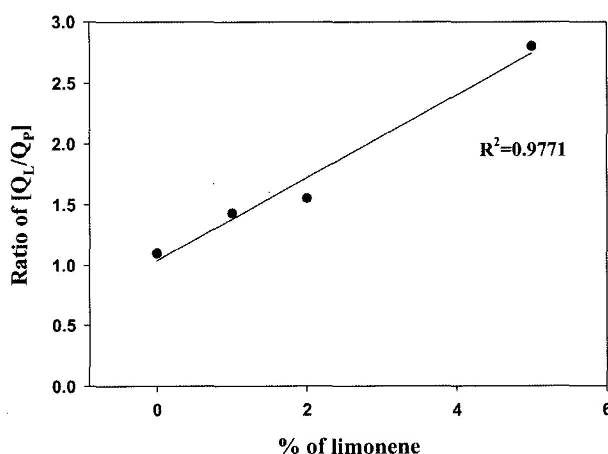

Figure 3–Correlation between content of limonene and Q_L/Q_P ratio. Q_L is the localized ascorbic acid and Q_P is permeated ascorbic acid after 2 h.

ordered structure of stratum corneum (SC) lipid, ii) interaction with intercellular protein and iii) improvement of partitioning of a drug into the SC.[13] Many evidences regarding the effect of limonene on physico-chemical changes in the skin have been reported. For example in the differential scanning calorimetric analysis, limonene decreased the transition temperature associated with the stratum corneum lipids and the drug diffusivity was increased.[15] The FT-IR study has also shown that limonene produced a decrease in peak heights and the areas for both asymmetric and symmetric C-H stretching absorbance. The decrease in peak height suggested an overall extraction of the SC lipids.[16]

Because an octanol/water partition coefficient of AsA was known as 0.02 ± 0.002,[9] there was little possibility for AsA to be transported across the skin. However, the solubility (mg/cm^3) of AsA within stratum corneum, viable skin and whole skin were reported as 21.34, 56.0 and 56.5 respectively,[9] thus if stratum corneum layer was disrupted by limonene, probably the amount of AsA within stratum corneum would be increased.

Takahashi *et al.* reported that the corneocyte could be effectively solubilized by limonene-added aqueous micellar solution of N,N-dimethyldodecylamine oxide and the cornoeocyte extraction effect was maximized within limonene saturated solution.[17]

From these facts, we could consider how the skin permeation of AsA could further be enhanced from the permeability made by AsA itself. AsA was able to hydrate the stratum corneum and change the barrier property of the protein domain so that the skin permeation of AsA alone could be made.[9] In this study we observed the permeation enhancing effect of limonene that can act on the lipid domain of the permeation barrier. When limonene was combined with AsA formulation, the permeation rate and the localization of AsA was increased. Increased skin localization of AsA may be explained by the fact that increased transport of AsA resulted from limonene caused an increase in skin deposition and this deposited AsA molecules were probably further transported through the paracellular pathway to demonstrate an increased permeation. Thus, increased permeation of AsA led to the increased deposition of AsA into the skin and the AsA deposited resulted in an increased permeation.

In conclusion, AsA incorporated in the limonene-containing poloxamer hydrogel formulation enhanced the skin permeation and localization of hydrophilic drug, AsA. Limonene gradually increased skin localization of AsA, along with an increase in the permeation of AsA.

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