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# The Safety Assessment of Stable Type Neutral Vitamin C Derivative (MIRAVC)

**Masahiko NAKANO**[1)] ／ **Atsuyoshi MURAKAMI**[2)] ／ **Akio YAMAMOTO**[1)] ／ **Nobuo KUBO**[3)]

1) Dr's Choice Co., Ltd., Tokyo Japan
2) The Information of Biomedical Science and Ultimate Technology Co., Ltd., Tokyo Japan
3) The Kitahama TOWER Jibiinkoka Hifuka Clinic, Osaka, Japan

● **Abstract**

**Objective**: To examine the safety of a newly developed ascorbic acid derivative based on ascorbyl glucoside (MIRAVC), as a raw material for topical preparations.
**Methods**: A primary skin irritation test, an Ames test, a human patch test, a human stinging test, and an ocular mucosal irritation test were conducted to evaluate the safety of this compound. In addition, using a three-dimensional cultured skin model, living skin equivalent model (LSE-high), we also evaluated transdermal absorption using a Franz-type diffusion cell system.
**Results**: Together with the test results of stability and transdermal absorption, all safety tests showed negative results, and no events were observed that would be of concern when used topically by humans.

**Key words**: Safety assessment, Ascorbyl glucoside, Skin permeability, Topical

## *1. INTRODUCTION*

Ascorbic acid, also known as vitamin C, is a water-soluble vitamin. Although it is an essential nutrient, it cannot be biosynthesized by humans, so it must be ingested or applied topically. It has been widely used as a topical preparation for a long time, and is known to have various functions such as antioxidant, anti-inflammatory, whitening, and collagen production[1)2)]. Ascorbic acid is a widely used basic ingredient, but when made into an aqueous solution, it becomes strongly acidic, easily browns due to oxygen, heat, light, etc., and is not very stable. To solve these problems, various ascorbic acid derivatives have been developed, and their functions and safety have been reported[3)-5)]. Ascorbyl glucoside, which combines ascorbic acid with glucose, is widely used as a water-soluble derivative[6)]. We prepared MIRAVC, a stable neutral vitamin C derivative raw material, by adding the basic amino acid arginine to this existing derivative, ascorbyl glucoside. To use this compound as a topical preparation, it is necessary to confirm sufficient safety, but since it is a new compound, no safety evaluation has been performed on this compound to date. Therefore, in this study, we conducted basic safety tests to evaluate the safety of this compound, assuming that it would be used topically on the skin and eyes. In addition, permeability was evaluated using a Franz-type diffusion cell system[7)8)] using a living skin equivalent model (LSE-high), which is a three-dimensional cultured skin model.

Corresponding author; E-mail address: mnakano@drs-choice.co.jp
Address; Stage Bldg. 17F, 2-7-2 Fujimi, Chiyoda-ku, Tokyo 102-0071, JAPAN

## *2. MATERALS AND METHODS*

### 2.1. Test materials

Arginine powder was added to ascorbyl glucoside aqua solution, and the pH of the solution was adjusted to 6.4 with arginine. This solution was freeze-dried to obtain MIRAVC powder, which was used as a test product. HPLC analysis was performed by LC-2050C (Shimadzu, Japan) under the conditions shown in **Table 1**. HPLC purity was 100% in area%. As a result of measuring with an infrared moisture meter, the moisture content was 2%. In each test, control products were used as appropriate.

### 2.2. Safety assessment

2.2.1. Primary skin irritation test

Skin irritation is a skin reaction indexed by changes such as erythema, edema, and desquamation that occur when a test substance comes into direct contact with the skin. Skin reactions that are severe and cause irreversible tissue damage are called skin corrosive reactions. In addition, the irritation response when a test substance contacts the skin once is called primary skin irritation, and the irritation response when the test substance

**Table 1** HPLC analytical condition

| Condition | Description |
|---|---|
| Column | Inertsil NH2（5 μm, 250 × 4.6 mmI.D.）（GL Sciences Inc., Tokyo, Japan） |
| Guard column | Inertsil NH2（5 μm, 20 × 4 mmI.D.）（GL Sciences Inc., Tokyo, Japan） |
| Mobile phase | A）$CH_3CN$, B）$CH_3OH$, C）0.01M Sodium dihydrogen phosphate aqueous solution, A/B/C＝60/30/10 v/v/v |
| Flow rate | 2 mL/min |
| Column temperature | 40℃ |
| Detector | 270 nm |
| Injection volume | 10 μL |

comes into repeated contact with the skin is called continuous skin irritation.

A cytotoxicity test was conducted on the 3-dimentional cultured skin model LabCyte EPI-MODEL24 in accordance with the OECD Test Guidelines (OECD TG 439)[9]. The test substance (MIRAVC 30% aqueous solution), positive control (5% Sodium lauryl sulfate aqueous solution) and negative control (sterile distilled water) were exposed to the stratum corneum side of the 3-dimentional skin culture model LabCyte EPI-MODEL24 for 15 minutes, and then cultured for 42 hours. Cell viability was measured by MTT (3-(4,5-di-methyltiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, and primary skin irritation was classified based on the cell viability. This test was conducted at DRC Co., Ltd. (Osaka, Japan) from March 9 to 15, 2021.

2.2.2. Eye mucosal irritation test

The eye mucosal irritation test is a test to confirm the eye irritation (reversible inflammatory changes) and eye corrosion (irreversible tissue damage) of chemical substances. Ocular mucosal irritation was evaluated using the 3-dimentional cultured cornea model LabCyteCORNEA-MODEL24 EIT test method[10]. The test was conducted in accordance with OECD TG 492 SOP. The test substance (MIRAVC 30% aqueous solution), positive control (Ethanol), and negative control (sterile distilled water) were used. This study was conducted at DRC Co., Ltd. (Osaka, Japan) from April 18 to 20, 2023.

2.2.3. Ames Test

The Ames test[11] was conducted to determine the presence or absence of gene mutagenicity. The test was carried out by a pre-incubation method using S*almonella typhimurium* TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2uvrA under metabolic activation and non-metabolic activation conditions. Note that water for injection was used as the solvent for the test substance. To set the test dose, a dose-finding test was conducted with the test substance treatment doses of 1.22, 4.88, 19.5, 78.1, 313, 1250, and 5000 μg/plate, which were diluted in 6 stages with a common ratio of 4 as follows, with the highest dose at 5000 μg/plate. This study was conducted from April 6, 2021 to May 19, 2021 at Bozo Research Center Co., Ltd. (Tokyo, Japan).

2.2.4. Human patch test

A 24-hour occlusive human patch test[12][13] was conducted on 24 Japanese adults. Prior to conducting the study, approval was obtained from the Brain Care Clinic Ethics Review Committee (Tokyo, Japan). This study was conducted with consideration to protecting the human rights of the subjects, based on the ethical standards set forth in the Declaration of Helsinki (World Medical Association) and the ethical guidelines for medical research involving humans (Ministry of Education, Culture, Sports, Science and Technology, Ministry of Health, Labor and Welfare). Using a patch test unit, Finn Chamber on Scanpor Tape purchased from SmartPractice Japan (Kanagawa, Japan), each test article was occlusively applied to the upper back (paravertebral region) of the subject for 24 hours. The number of times it was pasted was once. MIRAVC 30% aqueous solution was used as the test substance. In addition, control substances were white vaseline, physiological saline, and distilled water for injection. The test judgement was observed on the application site one hour after removing the patch test unit, after the temporary erythema caused by removal had disappeared, and made a judgment after 14 hours based on the criteria. Furthermore, the application site was observed again 24 hours after removal, and judgment was made 48 hours later using the same criteria. Skin irritation index＝((sum of scores of the one with the stronger reaction after 24 hours and after 48 hours)/number of subjects) x 100. This study was conducted at DRC Co., Ltd. (Osaka, Japan) from April 15 to 17, 2021.

**Table 2**　The basic information of the compound

| Item | Description |
|---|---|
| CAS RN | 718621-28-4 |
| CAS Index Name | L-Arginine, compd. with 2-O-α-D-glucopyranosyl-L-ascorbic acid (1:1) |
| INCI name | Ascorbyl Glucoside (and) Arginine |
| Molecular weight | 530.476 |
| Appearance | White to light yellow powder |
| pH | 6.43 (5% aqua. solution) |
| Solubility to water | ＞82.5% (room temperature) |

**Table 3**　The stability of the compound

| Storage condition | MIRAVC conc. % | Relative conc. % |
|---|---|---|
| 4℃ for 8 months | 31.11 | 100 |
| Room temperature for 8 months | 29.26 | 94.05 |
| 50℃ for 1 month | 29.64 | 95.27 |

2.2.5. Stinging test

Stinging test is a method to evaluate sensory stimulation such as stinging, itching, and hot flashes that occur when using cosmetics or cleaning products and is also called sensory stimulation test. A test was conducted on the stinging irritation of the test product on 10 adults who did not experience irritation from distilled water for injection (distilled water) but experienced irritation from 0.15% methylparaben aqueous solution (MP). Prior to conducting the study, approval was obtained from the Brain Care Clinic Ethics Review Committee (Tokyo, Japan). This study was conducted with consideration to protecting the human rights of the subjects, based on the ethical standards set forth in the Declaration of Helsinki (World Medical Association) and the ethical guidelines for medical research involving humans (Ministry of Education, Culture, Sports, Science and Technology, Ministry of Health, Labor and Welfare). A cotton pad soaked with 1 mL of MP was applied to the subject's skin over left and right cheekbones for 60 seconds. The cotton pad was removed, and the evaluation site was wiped with cotton soaked in distilled water, dried with a paper towel, and allowed to rest for 180 seconds. This process was repeated twice to select the subjects. A cotton pad soaked with 1 mL of the test product was applied near the skin over left and right cheekbones and left to stand for 60 seconds. The irritation felt within 31 to 60 seconds after the cotton was applied was scored on a five-point scale. The Sting scores (5 levels from 1 to 5) of the test product (MIRAVC 30% aqua solution) and the control product (distilled water or MP) were totaled, and comparisons between groups were performed using the Steel-Dwass test. The test was two-sided, and the significance level was 5%. This study was conducted on June 30, 2021 at DRC Co., Ltd. (Osaka, Japan).

2.2.6. Transdermal absorption test

Permeability to skin of this compound was evaluated using a Franz-type diffusion cell system using a living skin equivalent model (LSE-high), which is a three-dimensional cultured skin model[7)8)]. LSE-high consists of skin (Subcutaneous and viable epidermis from human keratinocytes) and a collagen matrix composed of human dermal fibroblasts and a polycarbonate membrane. 5% MIRAVC in PBS (phosphate buffer pH 7.4) solution or 30% MIRAVC in PBS. After applying 500 μL of the solution as a sample, the MIRAVC concentration in each receiver solution was analyzed by HPLC under the conditions in **Table 1** to evaluate the in vitro skin permeability of MIRAVC in each test solution. This test was conducted at Roman Skin Lab Co., Ltd. (Saitama, Japan) from May 31, 2021 to June 28, 2021.

## *3. RESULTS*

### 3.1. Physical properties

The basic physical properties are summarized in **Table 2**. When dissolved in water, the pH of a 5% aqueous solution was 6.43, which was within the neutral range. It was found that the solubility in water was over 82.5%, indicating that it could be dissolved very well in water.

### 3.2. Stability

A 30% aqueous solution of MIRAVC was prepared and stored at 4℃ and room temperature for 8 months or at 50℃ for 1 month, and then the MIRAVC concentration was measured by HPLC in the conditions shown in **Table 1**. As a result, the concentration after storage at 4℃ for 8

**Table 4** Primary skin irritation test

| Test sample | n | Cell viability （%） | | Classification |
|---|---|---|---|---|
| | | Mean | Standard deviation | |
| Negative control（Water*） | 3 | 100.0 | 3.8 | − |
| Positive control（5% SLS） | 3 | 1.6 | 0.1 | Category 2 |
| MIRAVC 30% aqua soln. | 3 | 97.1 | 3.2 | Not classified |

* Sterile distilled water.

**Table 5** Eye mucosal irritation test

| Test sample | n | Cell viability （%） | | Classification |
|---|---|---|---|---|
| | | Mean | Standard deviation | |
| Negative control（water*） | 3 | 100.0 | 7.1 | − |
| Positive control（Ethanol） | 3 | 3.4 | 0.9 | Can not predict |
| MIRAVC 30% aqua soln. | 3 | 90.4 | 1.8 | Not classified |

* Sterile distilled water.

months was 29.26%, which was a relative concentration of 94.05% compared to the initial concentration. The concentration after storage at 50℃ for one month was 29.64%, which was a relative concentration of 95.27% compared to the initial concentration (**Table 3**). From this, it was considered that the stability of this compound was sufficiently enough for topical application.

### 3.3. Safety

3.3.1. Primary skin irritation test

The cell survival rates for the test substance, positive control, and negative control were 97.1 ± 3.2, 1.6 ± 0.1, and 100.0 ± 3.8%, respectively. The cell viability of the positive control was less than 40%, the measured values of the negative control were 0.7 or more and 2.5 or less, and the standard deviations of the cell viability were all 18 or less. It was confirmed that the test met the test acceptance criteria. Based on the UN GHS (United Nation Globally Harmonized System of Classification and Labeling of Chemicals) classification of RhE (reconstructed human epidermis) method prediction model, the cell viability relative to the negative control is greater than 50% and outside the classification. The substance was determined to be non-irritating to the skin (**Table 4**).

3.3.2. Eye mucosal irritation test

As a result of this test, the cell viability of the positive control was 3.4%, which was less than 40%, and the measured value of the negative control was 0.940, which was 0.5 or more and 1.6 or less. The standard deviations were 1.8 for the test substance, 0.9 for the positive control, and 7.1 for the negative control, all of which were below 18, confirming that the test acceptance criteria were met. The cell viability of the test substance was 90.4%, which was higher than 40%, so the ocular mucosal irritation of the test substance was classified as not classified (**Table 5**).

3.3.3. Ames Test

Since no precipitation or growth inhibition was observed in the dose-finding test, this test was conducted at five doses ranging from 313-5000 μg/plate for all strains, regardless of the presence or absence of metabolic activation. No precipitation on the plate due to the test substance was observed at any dose, regardless of the presence or absence of metabolic activation. As a result of observing bacterial growth inhibition, no growth inhibition was observed in any bacterial strain, regardless of the presence or absence of metabolic activation. With this test substance, regardless of the presence or absence of metabolic activation, no increase in the number of revertant colonies, which was more than twice that of the negative control, was observed in any of the strains, and no dose response was observed (**Table 6**). From the above test results, it was determined that MIRAVC does not have the ability to induce genetic mutations in bacteria (negative) under the test conditions.

3.3.4. Human patch test

The subjects were 3 men and 21 women, aged 23-58 years (mean age 44 years). **Table 7** shows the skin irritation index of each test product. MIRAVC 30% aqueous solution had a skin irritation index of 0.0, and no irritation was observed.

**Table 6**　Ames test

| Group | | Dose（μg/plate） | Base pair substitution type | | | Frame shift type | |
|---|---|---|---|---|---|---|---|
| | | | TA100 | TA1535 | WP2uvr | TA98 | TA1537 |
| S9Mix（−） | | Negative control | 106, 121 (114) | 8, 12 (10) | 24, 22 (23) | 18, 22 (20) | 8, 11 (10) |
| | | 313 | 105, 121 (113) | 14, 8 (11) | 17, 14 (16) | 24, 23 (24) | |
| | | 625 | 146, 147 (147) | 8, 9 (9) | 17, 18 (18) | 17, 19 (18) | |
| | | 1250 | 130, 132 (131) | 7, 6 (7) | 20, 27 (24) | 14, 10 (12) | |
| | | 2500 | 130, 122 (126) | 7, 9 (8) | 26, 20 (23) | 14, 26 (20) | |
| | | 5000 | 126, 133 (130) | 10, 6 (8) | 14, 19 (17) | 23, 20 (22) | |
| S9Mix（＋） | | Negative control | 119, 119 (119) | 11, 11 (11) | 27, 28 (28) | 25, 34 (30) | |
| | | 313 | 123, 117 (120) | 13, 13 (13) | 19, 18 (19) | | |
| | | 625 | 124, 124 (124) | 8, 6 (7) | 23, 167 (20) | | |
| | | 1250 | 123, 109 (116) | 11, 9 (10) | 28, 17 (23) | | |
| | | 2500 | 134, 131 (133) | 13, 10 (12) | 30, 20 (25) | | |
| | | 5000 | 134, 122 (128) | 7, 10 (9) | 22, 19 (21) | | |
| Positive Control | S9Mix（−） | Compound | AF-2 | SAZ | AF-2 | AF-2 | ICR-191 |
| | | Dose | 0.01 | 0.5 | 0.01 | 0.1 | 1.0 |
| | | Colony count/plate | 605, 621 (613) | 200, 212 (206) | 113, 122 (118) | 423, 444 (434) | 1765, 1778 (1772) |
| | S9Mix（＋） | Compound | B［*a*］P | 2AA | 2AA | B［*a*］P | B［*a*］P |
| | | Dose | 5.0 | 2.0 | 10.0 | 5.0 | 5.0 |
| | | Colony count/plate | 999, 1003 (1001) | 187, 178 (183) | 750, 778 (764) | 256, 267 (262) | 88, 87 (88) |

The values in brackets show the mean values（n＝2）.

**Table 7**　Human patch test

| Sample | MIRAVC 30% aqua soln. | | White vaseline | | Saline | | Distilled water for injection | |
|---|---|---|---|---|---|---|---|---|
| Judgement time / Reaction | 24-hr | 48-hr | 24-hr | 48-hr | 24-hr | 48-hr | 24-hr | 48-hr |
| − | 24/24 | 24/24 | 24/24 | 24/24 | 24/24 | 24/24 | 24/24 | 24/24 |
| ± | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 |
| ＋ | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 |
| ＋＋ | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 |
| ＋＋＋ | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 | 0/24 |
| Skin irritation index | 0.0 | | 0.0 | | 0.0 | | 0.0 | |

3.3.5. Stinging test

The subjects were 10 adult men and women, 2 men and 8 women, aged 25-56 years (average age 43 years). Since MP had a significantly higher Sting score than distilled water, it was confirmed that it was valid as a Sting stimulation evaluation test. A significant difference test of the Stinging score revealed that the test product had a significantly higher Stinging score than distilled water and a significantly lower Stinging score than MP (**Table 8**).

### 3.4. Transdermal absorption test

For both 5% and 30% test specimen, the Q value, which is the cumulative permeation amount per unit area, increased over time. After 12 hours, the Q value of 5% and 30% of aqua solution was 72.7 ± 46.0 μg/cm$^2$, 1117 ± 360 μg/cm$^2$, respectively (**Figure 1**).

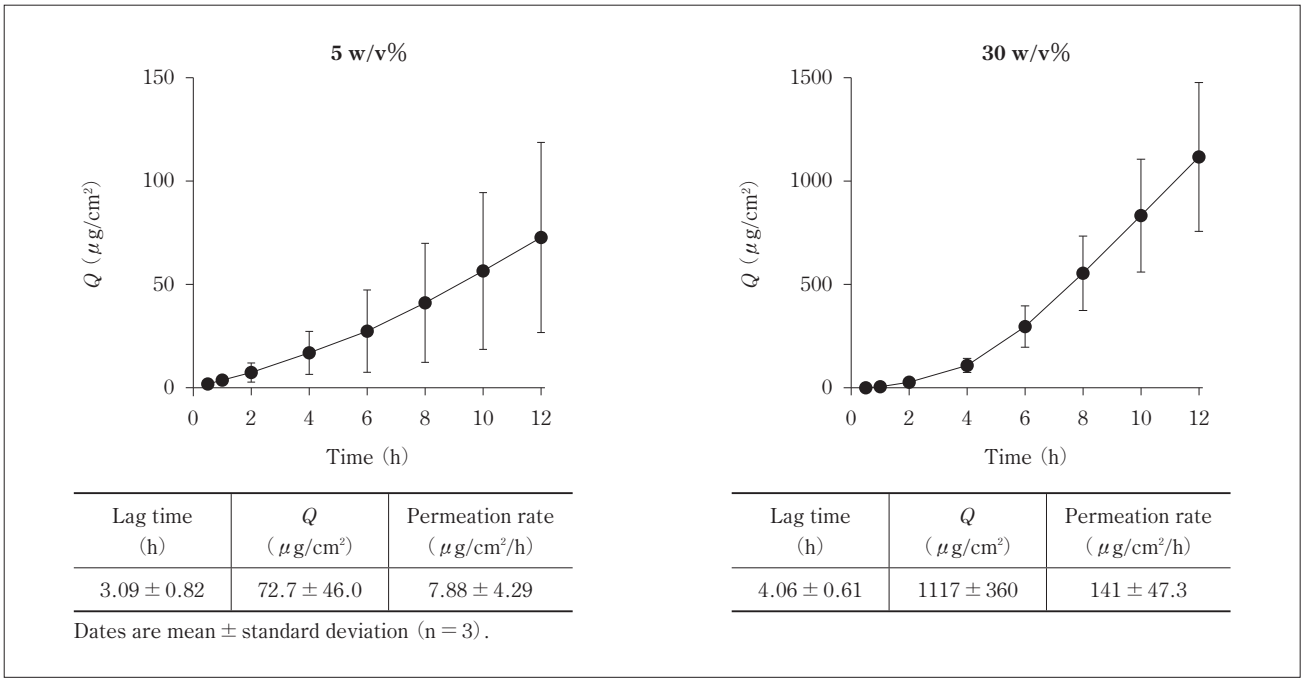
## *4. DISCUSSION*

We evaluated the safety of a stable neutral vitamin C derivative (MIRAVC) for topical use. In the primary skin irritation test, the cell survival rate was 97.1 ± 3.2%, indicating that there was almost no primary skin irritation compared to the negative control. In addition, MIRAVC was judged to be a substance that does not show irritation to the skin based on the out-of-classification evaluation. The cell survival rate in the eye mucosal irritation test

**Table 8** Stinging test

| No. | Sex | Age | Distilled water | MP* | MIRAVC 30% aqua soln. |
|---|---|---|---|---|---|
| 1 | F | 56 | 1 | 2 | 1 |
| 2 | F | 26 | 1 | 2 | 2 |
| 3 | F | 36 | 1 | 3 | 1 |
| 4 | F | 50 | 1 | 3 | 1 |
| 5 | F | 49 | 1 | 2 | 2 |
| 6 | F | 49 | 1 | 3 | 2 |
| 7 | F | 50 | 1 | 2 | 2 |
| 8 | M | 51 | 1 | 4 | 2 |
| 9 | F | 25 | 1 | 3 | 2 |
| 10 | M | 34 | 1 | 2 | 1 |
| Average | | | 1.0 | 2.6 | 1.6 |
| *p* value against distilled water | | | | < 0.001 | 0.014 |
| *p* value against MP | | | | | 0.011 |

* 0.15% Methyl Paraben aqua. solution.




| Lag time (h) | *Q* (μg/cm²) | Permeation rate (μg/cm²/h) |
|---|---|---|
| 3.09 ± 0.82 | 72.7 ± 46.0 | 7.88 ± 4.29 |

| Lag time (h) | *Q* (μg/cm²) | Permeation rate (μg/cm²/h) |
|---|---|---|
| 4.06 ± 0.61 | 1117 ± 360 | 141 ± 47.3 |

Dates are mean ± standard deviation（n = 3）.

**Figure 1** Transdermal absorption test

was 90.4%, and it was classified as “Not classified”. The positive control survival rate in this test was less than 40%, confirming the reliability of the test. In the Ames test, MIRAVC was judged to be negative because it does not have the ability to induce gene mutagenesis. Furthermore, no dose response or growth inhibition was observed. In a 24-hour occlusive human patch test, the skin irritation index was low in 24 adults. In addition, its score in the stinging test was significantly lower than that of methylparaben. The results of the transdermal absorption test confirmed that MIRAVC absorbs into the skin, and it was confirmed that it is not less irritating because it does not penetrate the skin.

MIRAVC has a molecular weight of 530, is relatively small, and contains amino acids, which are biocomponents, so it is thought to have high biocompatibility. When glucose is added, the time required for permeation becomes longer, so it is thought that MIRAVC permeates slowly and has sustained release properties. Although the test conditions are not completely the same as this study, there is a report on ascorbyl glucoside using LSE-high[8]. It has been reported

that ascorbyl glucoside has strong hydrophilicity, so its penetration is relatively slow[6]. The penetration rate when using 1% ascorbyl glucoside solution is $0.91 \pm 0.15$ $\mu g/cm^2/h$, while $7.88 \pm 4.29\,\mu g/cm^2/h$, which is the penetration rate for 5% MIRAVC in this study. If the concentration is reduced to 1/5, it is calculated as 1.576 $\mu g/cm^2/h$, which suggests that MIRAVC has a higher penetration rate than that of ascorbyl glucoside.

All the results of the safety tests were negative, and no adverse events were observed in all the tests conducted. From these facts, there are no safety concerns for human topical use. Even in the eye mucosal irritation test, which is thought to be easily irritating, there was no irritation at all even at a high concentration of 30%, so this compound is considered to be extremely non-irritating. This is because the pH of the aqueous solution of this compound is extremely close to the pH of the epidermis or dermis of the skin, and it is presumed that irritation is extremely suppressed compared to ascorbic acid or ascorbyl glucoside.

This compound has arginine added to ascorbyl glucoside, in which glucose is bound to the 2nd-position of ascorbic acid. Arginine is probably bound to the 3rd-position of ascorbic acid. More specifically, it is considered necessary to determine the structure of this compound by NMR (Nuclear Magnetic Resonance) or the like. HPLC analysis of transdermally absorbed receiver fluid detected glucose and arginine in a bound state. In this three-dimensional skin model, the enzyme system, etc. may be slightly different from that of a living body. It is assumed that the chemical form is stable even within the skin tissue, and this is thought to contribute to its low irritation. Future research will be needed to further investigate the dynamics and chemical forms within the skin.

These results suggest that MIRAVC has low irritation to the skin and eyes, and moreover has no ability to induce genetic mutations. It was confirmed that there is almost no skin irritation in humans, and the irritation is low. This indicates that MIRAVC can be safely used for topical applications such as on the skin and eyes. In this study, we confirmed the basic safety of MIRAVC through several safety tests, but we did not examine sensitization, phototoxicity tests, or the safety of repeated or long-term use. It is likely that future research will accumulate even more data. Through future research and application, it is expected that MIRAVC will be widely used around the world and make a major contribution to society.

## *5. CONCLUSION*

In this study, we conducted a comprehensive test on the safety of a stable neutral vitamin C derivative (MIRAVC). As a result, MIRAVC was negative for primary skin irritation, ocular mucosal irritation, and gene mutagenicity, and no significant irritation was observed in human patch tests or stinging tests. Judging from the results of these safety tests, MIRAVC was confirmed to be safe for human topical use even at a concentration as high as 30%, and to have low irritation to the skin and eyes.

Therefore, MIRAVC is a safe and promising compound, and is expected to be used in future applications and development of various topical products. As further research and clinical trials progress, it is believed that our understanding of its safety and utility will further be examined.

### *CONFLICT OF INTEREST STATEMENT*

Dr's Choice Co., Ltd. provided the funding for this study. There was no particular conflict of interest.

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