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Research paper

Application of tetra-isopalmitoyl ascorbic acid in cosmetic formulations: Stability studies and *in vivo* efficacy

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ABSTRACT

Liposoluble vitamin C derivatives, such as tetra-isopalmitoyl ascorbic acid (IPAA), are often used in dermocosmetic products due to their higher stability than vitamin C free form as well as its proposed effects in skin; however, there are no studies analyzing IPAA stability or its *in vivo* effects when present in dermocosmetic formulations. Thus, this study aimed to evaluate chemical stability and pre-clinical and clinical efficacy of dermocosmetic formulations containing IPAA in skin hydration and microrelief. Chemical stability of the formulations added with 1% IPAA was evaluated by heat stress during 35 days by HPLC. For pre-clinical evaluation, experimental formulations were topically applied on hairless skin mice during 5 days and animal skins were analyzed by non-invasive biophysic techniques (water content of stratum corneum, TEWL, viscoelasticity, and microrelief) and by histopathological studies. For clinical efficacy tests, the formulations were topically applied to the forearm and face of human volunteers, and 3 h and 15 days after applications, the skins were evaluated by the same non-invasive techniques mentioned before. Results showed that formulations containing IPAA had medium stability and had pronounced moisturizing effects on stratum corneum and on viable epidermis. These formulations also improved skin microrelief especially in relation to skin smoothness and roughness.

1. Introduction

The use of L-ascorbic acid (vitamin C) for topical application is not a new procedure. It has been used in pharmaceutical and cosmetic preparations for a long time on the basis of its many favorable effects on the skin [1]. However, free vitamin C is unstable, being easily oxidized and inactivated when exposed to air, and also it cannot penetrate into the skin because of its hydrophilicity [2].

Some marketed products containing unstable free vitamin forms need great investments in specific methods of packaging and containers and have short shelf-lives. Therefore, more stable and safer new delivery systems, such as microemulsions, microcapsules, nanospheres, liposomes [3] as well as the use of ester derivatives such as magnesium ascorbyl phosphate and sodium ascorbyl phosphate (water-soluble derivatives) and ascorbyl-6-palmitate and tetra-isopalmitoyl ascorbic acid (IPAA) (liposoluble derivatives) are an attempt to prolong formulation stability and are increasingly substituting the use of vitamin C free form [4,5]. Moreover, liposoluble derivatives such as IPAA are able to better penetrate the skin [1] and have increased stability when compared to vitamin C free form [6].

However, formulations supplemented with vitamins, even in the ester form, may present low shelf-life [7]. It is well known that the stability of all vitamin C derivatives is influenced also by structural properties of the formulations [1,8]. Thus, in the development of a cosmetic formulation, it is necessary to choose suitable vehicle raw materials because they must be compatible with the active substances selected to attend the indication for use of the product [9].

Cosmetic formulations containing IPAA have to be submitted to studies of chemical stability in order to evaluate their quality, since the integrity of the vitamin derivative must be kept constant during the formulation shelf-life.

Stability prediction is usually performed by accelerated storage conditions [7] such as temperature variation to induce rapid chemical alterations in formulations, which are usually detected by quantification of some components over time. Thus, in this study, a High Performance Liquid Chromatography (HPLC) method was developed to predict the chemical stability of formulations containing a liposoluble ester derivative of vitamin C [10].

Vitamin C, an essential skin component, has an important role in collagen production, being a co-factor in proline and lysine synthesis [11] and participates in connective tissue repair and cicatrization.

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The vitamin also captures free radicals in the skin and eliminates singlet oxygen species, superoxide, hydroxyl and peroxyl groups, and hypochlorous acid [2,8,12], thus preventing UV-induced damages and improving photo-aged skin [13]. However, on exposition to UV radiation, the levels of the antioxidant are sharply reduced [14].

Some studies are conducted to evaluate whether ascorbic acid properties may be extrapolated to its derivatives [12,15]. Maia Campos et al. [16] observed that liposoluble tetra-isopalmitoyl ascorbic acid (IPAA) and hydrosoluble magnesium ascorbyl phosphate were efficient in neutralizing free radicals and that the latter could have a hydration effect in the deeper epidermis layers.

IPAA is the first lipoidic-liquiform vitamin C derivative by itself and has been one of the frequently used ascorbic acid derivatives in cosmetic c formulations [6]. Ochiai et al. [17] demonstrated 84% liberation of vitamin C by the derivative in reconstructed skin. Studying the effects of IPAA on keratinocyte cultures, the authors demonstrated reduction of oxidative stress and of interleucin-1 and prostaglandin E_2 production induced by UVB. Secretion of melanocyte proliferation factors was also suppressed, indicating that the hyperpigmentation effect induced by UVB may be alleviated.

Application of IPAA may also alleviate skin damage following physiologic doses of UVA irradiation and should be adopted in the preventative and protective strategies against UVA injuries [6].

IPAA, which has already been widely used as an additive in cosmetic formulations [6], has also been approved as "quasi drugs" by Japanese authorities. These "quasi drugs" are only approved based on equivalency evaluation and examination conducted by the Pharmaceutical and Medical Device Agency and thus they are products with proven efficacy in a specific claim category, in this case for skin whitening, recognized by the Ministry of Health and Welfare [18].

Considering that IPAA is a liposoluble vitamin C derivative more stable than the free form, it could have vitamin C skin effects and also provide other skin benefits such as hydration, smoothness, and reduction of wrinkles. However, it still needs to be analyzed by *in vivo* studies, mainly clinical studies, which are necessary to confirm the effects of formulations added with IPAA on human skin in actual conditions of use since most studies involving the derivative were performed *in vitro*.

Regarding *in vivo* efficacy testing, histopathological and histometrical studies determine epithelial thickness and the number of cell layers in the viable epidermis and are able to show alterations produced on the skin by a specific treatment and may have a role in the understanding of the effects of topical substances [19]. Since these are invasive techniques, hairless mouse could be an appropriate model for preliminary studies on anti-aging properties of topical formulations.

On the other hand, modern non-invasive skin biophysical techniques are often used since they allow evaluation of cosmetic products under actual conditions of use [16]. The skin biophysical methods typically measure selected properties that depend on the measuring principle. Hydration, skin viscoelasticity and microrelief, barrier function, and skin thickness are examples of parameters that can be quantified non-invasively using these techniques [16,20].

Thus, the aim of this study was to evaluate chemical stability and pre-clinical and clinical efficacy of dermocosmetic formulations containing IPAA by investigating possible alterations caused by these formulations in the epidermis. There are not studies analyzing the stability of this liposoluble vitamin C derivative when vehiculated in topical formulations and their efficacy by pre-clinical and clinical parameters, mainly using biopsies and objective measurements, which are an important tools in efficacy studies.

2. Materials and methods

2.1. Formulations

Experimental formulations contained acrylic polymer, methylphenyl polysiloxane, and octyl octanoate added or not of 1% ascorbyl tetra-isopalmitate at pH 5.5, the active principle optimal pH.

2.2. Chemical stability

2.2.1. Stability in thermal stress

Formulation samples were maintained at room temperature or in controlled temperature incubators set at 37 °C or 45 °C and also having humidity and photoperiod controls. At 7 day intervals during 35 days, aliquots were analyzed for IPAA concentrations (short term stability). Determinations conducted 90 and 180 days after storage at room temperature (long term stability) were meant to confirm results obtained in the accelerated stability studies [7].

2.2.2. Determination of IPAA and validation of the method

The concentration of the vitamin C derivative was determined by HPLC in a Shimadzu equipment, connected with two pumps, a C-18 reverse column, and a UV/visible light detector. Optimal chromatographic conditions were the following: 45:55 (V/V) methanol/isopropanol as the mobile phase, 0.8 mL/min. flux, and detection at 235 nm. The efficiency of IPAA extraction from the formulation and the precision and accuracy of the method (intra and inter-assay) were determined. For precision assays, samples of formulations were analyzed six times. Inter-assay precision was determined by analyzing the samples in three different days. The intra and inter-assay accuracy was also evaluated, by assessing the agreement between the measured and nominal concentrations of the analytes. In addition, the efficiency of the extraction method ($n = 6$) was estimated from the formulations under study. Vitamin K1 was the internal standard in the determination [7].

2.2.3. Calculation of shelf-life through chemical kinetics equations

Data were treated graphically and analytically for the calculation of the IPAA degradation constant, K, in the different periods of time and temperatures evaluated. The values were used in 1st order chemical kinetics equations, and 85% remaining IPAA concentrations were considered in the determination of shelf-life periods [7,21,22].

2.3. Pre-clinical efficacy of formulations

This study was approved by the Committee on Ethics in the Use of Animals of the Ribeirao Preto Campus of the University of Sao Paulo (Protocol CEP-FCFRP no. 05.1.320.53.2). Formulations (5 mg/cm^2) containing or not (vehicle) 1% IPAA were applied daily, for 5 days, on the dorsal skin of two groups ($n = 5$) of hairless mice. A third group (control) with the same number of animals did not receive any treatment. Measurements of stratum corneum water content, erythematic index, transepidermal water loss (TEWL), and viscoelasticity and skin microrelief were made by non-invasive methods using the Corneometer CM 825, Mexameter MX16, Tewameter TM 210, Cutometer SEM 575 and Visoscan VC 98, respectively, [22] as described in item 3.2. Mice were euthanized by CO_2 inhalation, and skin fragments were obtained and immediately immersed in a fixing solution consisting of 85 mL of 80% alcohol, 10 mL formaldehyde, and 5 mL acetic acid. After 24 h, the fixed fragments were dehydrated, cleared, and embedded in paraffin. Semiserial 6 mm-thick sections were then obtained and each section corresponded to an interval of 50 sections, that is, 10 sections were obtained from the 2 mm biopsy. The sections were stained

with hematoxylin and eosin and evaluated as to qualitative and quantitative skin alterations including thickness measurements of viable skin and determination of the number of cell layers though an optical microscope and the software Image J [16,22]. Data were statistically analyzed by the nonparametric Kruskal–Wallis test according to the sampling distribution.

3. Clinical studies

3.1. Casuistic and methods

3.1.1. Study design

The study was approved by the Committee on Ethics in Research with Human Beings of the Faculty of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo (Protocol CEP-FCFRP no. 45). Twenty volunteers, aged 25–55 years, with skin types II–IV [23] were selected and signed the Term of Informed Consent after being oriented as to the objectives and research methods.

Formulations containing or not 1% IPAA were applied to the face and forearm of each volunteer, daily, for 15 days. Before starting the measurements, the subjects stayed during 30 min in a temperature and humidity controlled environment (20–22 °C and 45–55%, respectively) for acclimatization [16,24]. Measurements were made before application (basal values), 3 h after (immediate effect) and 15 days later (long term effect).

3.2. Measuring instruments

Clinical efficacy of the formulations was evaluated by biophysical techniques and skin image analyses according to the following parameters: stratum corneum water content, TEWL, visco-elastic characteristics (U_a/U_f, gross elasticity; U_r/U_e, neto-elasticity of the skin; U_r/U_f, biological elasticity and U_v/U_e, the ratio of viscoelastic to elastic distension) and skin microrelief (roughness – R_t, number and width of the wrinkles – SE_w, skin smoothness – SE_{sm}) [16].

The stratum corneum moisture content was determined with a non-invasive, skin capacitance meter (Corneometer® CM 825, Courage + Khazaka, Cologne, Germany), which measures capacitance and is entirely dependent on the water content in the skin. Different capacitance changes are converted into a digital measured value (arbitrary units), which is proportional to the skin humidity [25].

Barrier function was evaluated by measuring the TEWL (g/cm^2 h) using the Tewameter TM 210 (Courage & Khazaka, Cologne, Germany). TEWL is considered an important measure of epidermal barrier function. Evaporimetry consists of applying a probe with two sensors directly to the skin, with one sensor pair measuring humidity and the other temperature. The acquired data are used by integrated microcomputer to compute the water vapor partial pressures at the two parallel levels of each sensor pair and, via the partial pressure gradient, the rate of evaporation. To minimize outside interference, the measurements were carried out in an open-top chamber with closed sides [25,26].

Skin microrelief parameters were evaluated using Visio Scan® VC98, which is a special UV-A light video camera with high resolution developed especially to study the skin surface directly and the SELS (Surface Evaluation of the Living Skin) method. The images show the structure of the skin, and the level of dryness and the gray level distribution of the image is used to evaluate the following skin roughness parameters: skin roughness (R_t), skin smoothness (SE_{sm} – proportional to width and form of the wrinkles), and number and width of the wrinkles (SE_w) [27].

The viscoelastic properties of the skin were investigated with a Cutometer SEM 575 (Courage und Khazaka, Germany). The measuring principle is suction/elongation. An optical system detects the decrease in infrared light intensity depending on the distance the skin is being sucked into the probe. In this study, the strain-time-mode was applied. A probe with a 2 mm opening was used, and a pressure of 450 mbar was applied in order to suck the skin into the probe. Each measurement consisted of five suction cycles (3 s of suction followed by 3 s of relaxation). The parameters U_a/U_f (gross elasticity), U_r/U_e (neto-elasticity of the skin), U_r/U_f (biological elasticity), and U_v/U_e (the ratio of viscoelastic to elastic distension) were evaluated [27,28].

3.3. Statistical analysis

According to the sampling distribution of clinical evaluation data, the parametric Analysis of Variance was used to interpret the results.

4. Results and discussion

4.1. Chemical stability

Considering the involvement of procedures as extraction, filtration and dilution, the precision and accuracy of the analytical methods were acceptable with values of intra/inter-assay of 2.8 and 2.42, and of 9.92 and 9.37, respectively. Chromatographic screening of IPAA-free formulations did not show components with elution times coinciding with the active principle (Fig. 1).

Data referring to remaining IPAA concentrations measured by HPLC, after different times and storage conditions, were applied in 1st order chemical kinetics equations to calculate formulation shelf-life (Fig. 2). The value obtained was 173 days or approximately 6 months.

The long term stability studies (more than 6 months, at room temperature) confirmed the period estimated in accelerated stability determinations, since less than 15% of IPAA was lost after 6 months. In addition, it was also observed that less than 15% of IPAA was lost, when the formulations were stored at 37 °C for 3 months. Thus, according to Pope [29], this shelf-life could probably be extended to a medium value of 6–12 months, because the formulation did not show physical, physicochemical, and organoleptic alterations after 6 months at room temperature or for 3 months at 37 °C, loosing no more than 15% of the active principle. Other results from this laboratory show that addition of vitamin E to formulations considerably increases IPAA chemical stability and consequently the shelf-life, which is of 12 months or more [30].

Other authors observed that the water soluble vitamin C derivative, magnesium ascorbyl phosphate, was more stable than ascorbyl palmitate (lipid soluble derivative), which was considered very unstable, due to the lipophilic ester in position 6 that does not protect this vitamin from degradation [31]. Another study showed that magnesium ascorbyl phosphate had a medium shelf-life (7 months) [32], which confirmed the capability of the phosphoric group to protect the enediol system from hydrolysis, even when it is included in cosmetic formulations. In addition, Segall and Moyano [33] showed that ascorbyl esters, sodium ascorbyl phosphate, and magnesium ascorbyl phosphate are more stable than ascorbyl palmitate, since after 6 months, they found a remaining concentration of 20% of ascorbyl palmitate in comparison with 70–80% of the other derivatives.

However, in the present study, it was observed that tetra-isopalmitoyl ascorbic acid, a lipid soluble vitamin C derivative showed good stability. It is suggested that the chemical structure of IPAA improves its stability due to its tetra substitution that may act as a steric protection to the molecule against degradation. In addition, IPAA optimal pH (4.0–6.0) is compatible to skin.

It is concluded that tetra-isopalmitoyl ascorbic acid (IPAA) the active compound of the formulations under study is degraded

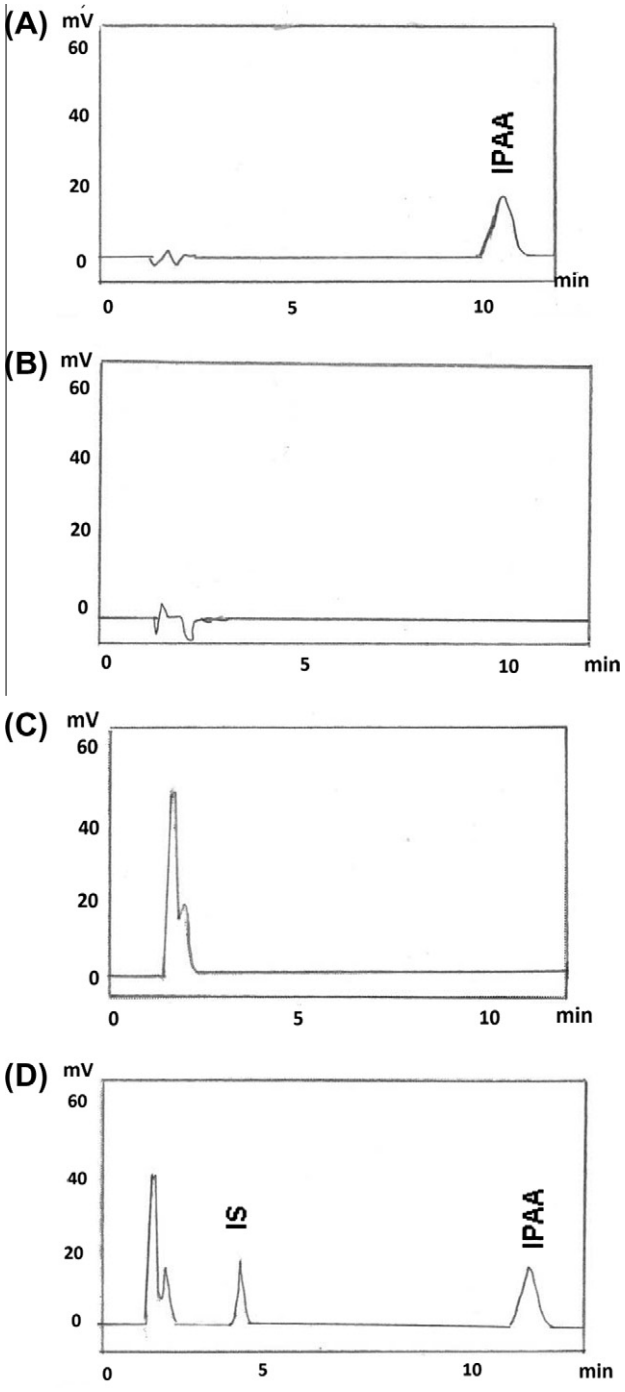

Fig. 1. Tracings in HPLC chromatograms of formulations and standard. (A) Vitamin derivative, ascorbyl tetra-isopalmitate (IPAA). (B) Reagent control (isopropanol). (C) Formulation IPAA-free. (D) Formulation containing IPAA and the internal standard vitamin K1 (IS).

according to first order kinetics. The results suggest a shelf-life of 6–12 months for formulations containing this ascorbic acid derivative, which may be easily used in cosmetic products.

4.2. Pre-clinical efficacy of formulations

Results showed that the increased thickness of viable epidermis in hairless mice induced by IPAA-containing formulations compared to controls (Fig. 3A), as determined in histopathological and histometric procedures, suggest intra and extracellular

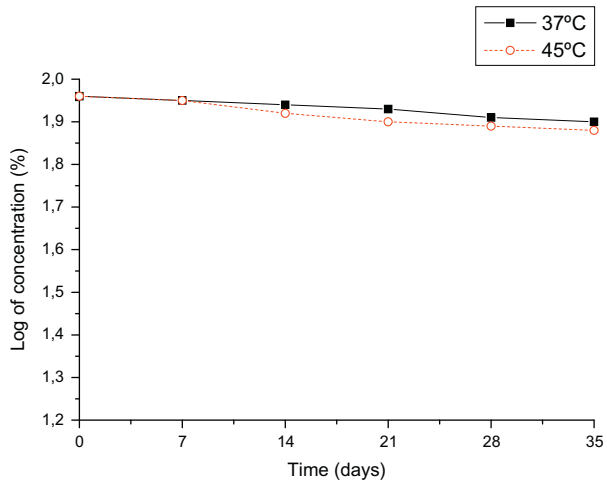

Fig. 2. Log of remaining IPAA concentrations in formulations stored at 37 °C or 45 °C during 0–35 days. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hydration (Fig. 4). Histopathological studies in experimental animals reported by other authors showed similar results when magnesium ascorbyl phosphate, a water soluble derivative of ascorbic acid, was evaluated [19].

The non-invasive methods (biophysical techniques and skin image analyses) showed that formulations containing IPAA promoted a statistically significant increase ($p < 0.01$) of stratum corneum water content in mice skin when compared to control and vehicle (Fig. 3B). However, the skin barrier function was not altered (Fig. 3C).

The relation U_v/U_e (viscoelastic-to-elastic ratio) was altered by topical application of formulations containing IPAA when compared to control and vehicle (Fig. 3D) mainly due to increased U_v, which according to the histometric studies (Fig. 3A), may be related to increased water content in viable epidermis. Dobrev [27] observed that the relationship viscoelasticity/elasticity is linked to the hydration of epidermis and superficial dermis deeper layers. The parameter U_v/U_e, according to the author, is the most sensitive in the evaluation of moisturizers efficacy and may be related to the reduced viscosity of the interstitial fluid resulting from increased amounts of water in the epidermis. Neither formulations altered the erythema index, suggesting that the IPAA effect was not inflammatory but of hydration.

Evaluating skin microrelief, it was observed that roughness values (R_t) were significantly reduced by application of IPAA-containing formulations when compared to control and vehicle (Fig. 3E), that is, an improvement of hairless mouse skin microrelief was observed. These results confirm Sato et al. [34] who observed that moisturizers significantly reduce skin roughness in hairless mice. Concerning skin smoothness, there was not a significant difference between vehicle and the IPAA-containing formulation (Fig. 3F).

In summary, pre-clinical studies indicated that IPAA-containing formulations were efficient in increasing the thickness of viable epidermis and the content of water in the stratum corneum. The viscoelastic-to-elastic ratio was also increased and skin roughness improved (R_t reduction) when compared to vehicle.

4.3. Clinical evaluation of formulation

Results obtained by biophysical techniques and skin image analyses are shown in Fig. 5, indicating an improvement in skin microrelief in relation to the smoothness parameter (SE_{sm}) in the forearm and face of volunteers when IPAA-containing formulations

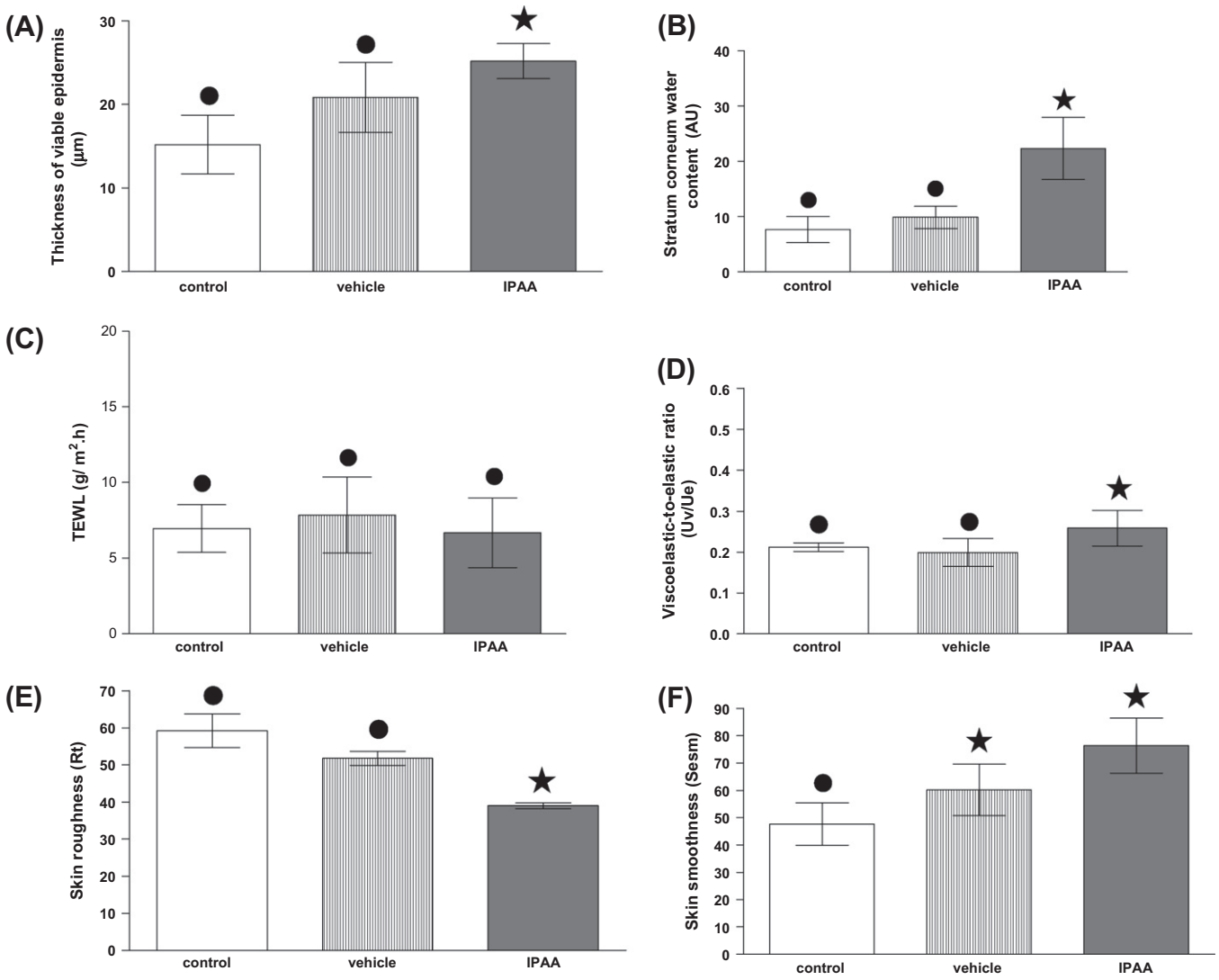

Fig. 3. Effects of topical application of formulations containing IPAA, control, and vehicle on the dorsal skin of hairless mice. (A) Thickness of viable epidermis (m). (B) Stratum corneum water content. (C) TEWL. (D) Viscoelastic-to-elastic ratio. (E) Skin roughness (R_t). (F) Skin smoothness (SE_{sm}). Different symbols signal statistically different means ($p < 0.01$). Median and confidence interval of 95%.

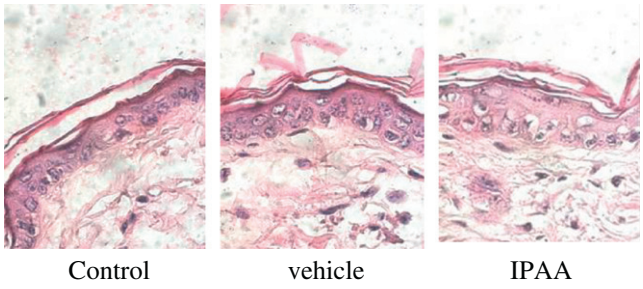

Fig. 4. Photomicrographs of hairless mice skin. Effects of topical application of formulations containing IPAA, control and vehicle on the dorsal skin of hairless mice. Initial magnification 1000×, HE.

were applied topically and in contrast to vehicle effects and control.

Evaluating stratum corneum water content, significantly increased values were observed when formulations containing or not IPAA were applied to the forearm and measured 3 h or 15 days later when compared to basal values determined at zero time (Fig. 5A). However, no significant differences were encountered when the application was on the face of volunteers, since basal values already indicated optimal hydration conditions, according to specifications in the instrument used for the measurements [35].

The skin barrier function was not altered when treated by the experimental formulations in comparison with basal values both in the forearm (Fig. 5B) and the face of volunteers [16]. The same results were obtained when formulation effects on the viscoelastic-to-elastic ratio in forearm and face were compared to basal values (Fig. 5C).

Microrelief in the skin volar forearms of volunteers was improved after 3 h (immediate effect) and 15 days (long term effect) of the application of formulations under study due to reduced R_t values (skin roughness), when compared to basal values (Fig. 5D). The results confirm other reports showing the effect of moisturizers in significantly reducing R_t values[36]. However, this effect was not present when formulations were applied to the face showing R_t values not very different from the basal ones, since the place of application and time of measurement influence the parameters of the skin microrelief.

In relation to other skin microrelief parameters, such as skin smoothness (SE_{sm}), our results showed that the formulations studied produced a significant improvement in the forearm 3 h after application, when compared to basal values; after 15 days, however, only the IPAA containing formulation confirmed the improvement showing greater and more sustained effects than the vehicle alone on skin smoothness (Fig. 5E). Face applications only showed improvements by IPAA 3 h after application; after 15 days, no difference was found. Dobrev [37] evaluating moisturizers effects on dry skin arrived at the same conclusions by utilizing a subjective visual analysis and parameters related to skin microrelief such as SE_{sm} and R_t.

The clinical results in this report are relevant and confirm observations by other authors showing the efficacy of anti-ageing formulations containing anti-oxidants, such as vitamins and flavonoids and an improvement in skin microrelief seen objectively or in a subjective manner as a perception of efficacy [13,16,38]. In addition, several studies indicate that these effects greatly contribute to improve the appearance of photo-aged skin leaving it more exuberant and healthy [16,24,32,35,36].

The differences in efficacy related to the skin viscoelasticity and microrelief observed when comparing the results obtained in the pre-clinical and clinical trials could be due to the higher sensitivity and smaller thickness of hairless mice stratum corneum when compared to human skin [38]. Thus, a complete efficacy study of cosmetic formulations containing vitamins and/or other active substances is of fundamental importance in order to obtain conclusive results and as an indicator of adequate components to be included in formulations of stable and efficient products.

According to results obtained in efficacy studies, IPAA was shown to be an effective vitamin C derivative to improve skin conditions. In addition, according to Maia Campos et al. [16], the derivative had a good antioxidant activity against lipid peroxidation, when compared to hydrophilic magnesium ascorbyl phosphate. Jurkovic [39] studied the antioxidant activity of ascorbyl palmitate against UV-induced free radicals formation when applied on porcine skin and concluded that this substance decreases the level of free radicals and therefore has antioxidant activity. However, the compound is described as an unstable substance in topical formulations [33]. IPAA, in contrast, showed a good stability in the present study, which is very important in terms of efficacy.

Efficacy studies showed that formulations containing IPAA had pronounced moisturizing effects on stratum corneum and on viable epidermis. In addition, these formulations also improved skin microrelief especially in relation to the skin smoothness and roughness.

5. Conclusions

Results of chemical stability studies of tetra-isopalmitoyl ascorbic acid (IPAA) suggest a shelf-life of 6–12 months, a medium stability, for formulations containing the ascorbic acid derivative studied.

Pre-clinical studies on hairless mice skin showed that formulations containing IPAA had a pronounced effect on the stratum corneum water content and in the reduction of skin roughness. Viable epidermis thickness observed and the ratio U_v/U_e (viscoelastic-to-elastic ratio) can be related to hydration of viable epidermis that was also increased. Consequently, the hydration effect observed is not limited to the upper cell layers but is also present in the deeper layers.

In the clinical studies on human volunteers, formulations containing IPAA showed significant improvement effects on skin hydration and microrelief, especially in relation to skin smoothness parameter.

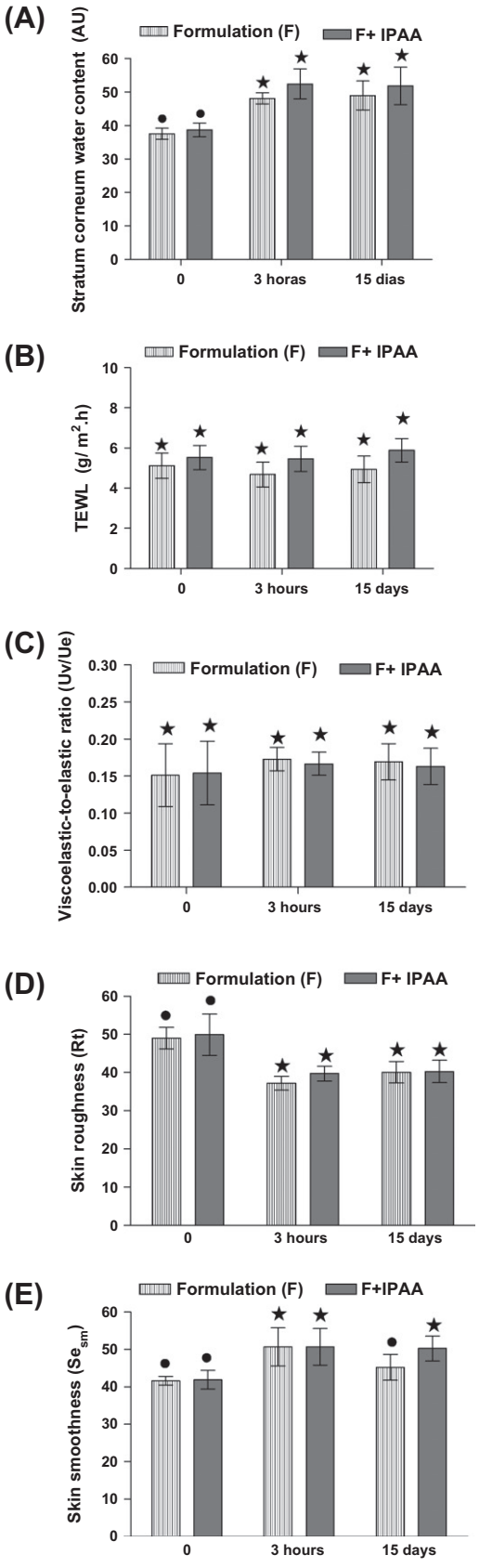

Fig. 5. Application of formulations and control on the forearm of human volunteers. Results were analyzed 3 h and 15 days after. (A) Stratum corneum water content. (B) TEWL. (C) Viscoelastic-to-elastic ratio. (D) Skin roughness expressed as R_t. (E) Skin smoothness expressed as SE_{sm}. Different symbols signal statistically significant differences ($p < 0.05$). Median and confidence interval of 95%.

The improvement in skin conditions shown by the efficacy studies confirms through objective analyses how effective are cosmetic formulations containing this liposoluble derivative of ascorbic acid when the objective is hydration, protection and anti-ageing effects, reducing the disadvantages of ascorbic acid use, such as its poor stability.

In addition, the results contribute to the understanding of cosmetic stability and efficacy in formulations containing tetra-isopalmitoyl ascorbic acid, because there are no studies evaluating the stability and effects of IPAA when vehiculated in topical formulations, mainly using objective measurements, which are an important tool in clinical efficacy studies of these products.

Finally, due to its good stability and efficacy, tetra-isopalmitoyl ascorbic acid may be easily used in cosmetic products, when compared with vitamin C free form, to improve skin conditions.

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