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Skin Pharmacology and Physiology

Skin Pharmacol Physiol 2004;17:298–303
DOI: 10.1159/000081115

Received: December 29, 2003
Accepted after revision: July 22, 2004

Ex vivo Cutaneous Absorption Assessment of a Stabilized Ascorbic Acid Formulation Using a Microdialysis System

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Key Words
Skin penetration, ex vivo · Ascorbic acid · Microdialysis

Abstract
Background: Reactive oxygen species generated by ultraviolet light result in photocarcinogenic and photoaging changes in the skin. Antioxidants protect the skin from these insults. ***Objective:*** The aim of this study was to determine the ex vivo ascorbic acid penetration and its degradation in the skin after its topical application from an 8% new formulation. ***Method:*** Ascorbic acid was applied to human skin fragments. Ascorbic acid and its metabolites were collected by microdialysis and assessed by gas chromatography mass spectrometry. ***Results:*** After topical application of the new formulation, the ascorbic acid level achieved was 8.5 times the normal tissue value. This high ascorbic acid dermal concentration remained constant if a topical application was made every 8 h. No degradation of ascorbic acid was detected. ***Conclusion:*** Ascorbic acid penetrates rapidly after its topical application. The persistent reservoir of ascorbic acid provides an important and attractive photoprotection strategy.

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Introduction

Inflammatory insults such as ultraviolet irradiation, pollution and smoking generate reactive oxygen species (ROS) in the skin. ROS cause injury by reacting with biomolecules such as lipids, proteins and nucleic acids as well as by depleting the skin of endogenous enzymic or nonenzymic antioxidants [1, 2]. The body protects itself from ROS by using antioxidants to neutralize them before they cause damage to the skin and its components. Ascorbic acid (Asc) is the most efficient antioxidant in the skin [3]. It is a cofactor for hydroxylation of lysine and proline residues of procollagen and promotes formation of triple helical conformation of mature collagen fibers [4]. Also, Asc was shown to increase the stability of procollagen mRNA [5]. Besides its cofactor activity, Asc is as well required to form competent barrier lipids in the epidermis [6] by stimulating the synthesis of ceramides. It has been shown to stimulate the barrier function of the endothelial cells [7].

Despite these benefits of Asc, some disadvantages need to be overcome. It can be severely depleted after UV irradiation [8], and only a very small portion of Asc ingested penetrates into the skin [9]. Continuous large oral doses of Asc can cause gastrointestinal discomfort and diarrhea. If

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1660–5527/04/0176–0298$21.00/0

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antioxidants could be delivered in high concentration through the stratum corneum barrier into the skin, then the antioxidant protective reservoir could be increased and photoprotection might be enhanced. The use of Asc in cosmetic and pharmaceutical products is however limited due to its low stability. Under aerobic conditions, it is reversibly oxidized to dehydroascorbic acid, which can be irreversibly degraded to oxalic acid, threonic acid and xylose [10].

The aim of this ex vivo work was to determine the skin penetration of Asc from a new topical formulation. The metabolism of Asc in the dermis (target tissue) after the formulation application was also investigated.

Materials and Methods

Products

The internal standard, *p*-hydroxybenzoic acid, and N-(*tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), a silylating agent, were purchased from Sigma Aldrich (St. Quentin Fallavier, France). An 8% Asc serum (Flavo C) was used (pH 3.1). It was composed of water, propylene glycol, *Gingko biloba*, octoxynol 11, polysorbate 20 and imidazolidinyl urea. Four Asc metabolites (dehydroascorbic acid, oxalic acid, threonic acid and xylose) were also purchased from Sigma. Ringer lactate solution (pH = 6, Cl^-: 111 mmol/l; K^+: 5.3 mmol/l; Ca^{2+}: 1.8 mmol/l; Na^+: 130 mmol/l, lactate: 27.6 mmol/l) was obtained from Maco Pharma (Paris, France). The formulation was provided by Auriga International (Belgium).

Microdialysis Experiment

The microdialysis system, used in this investigation, consisted of a CMA/100® syringe pump (Phymep, Paris, France) and a Univentor 820® microfraction collector, which collected samples and stored them at 4°C. The microdialysis probes (CMA/20®), used in this work, had a 20-kD cutoff with a polycarbonate membrane (length 10 mm).

Two experiments were carried out. For these experiments, abdominal skin fragments [10 × 10 cm (surface) × 2.5 cm (±0.5 cm, thickness)] were obtained from a surgery procedure and were used as soon as after their excision. For the first experiment, 3 female skin samples, from cutaneous surgery, were freshly used (37 ± 7 years old). For each sample, 4 microdialysis probes (2 cm distances between each probe) were inserted into the dermis and perfused with Ringer lactate solution at 3 µl/min for 60 min to reach equilibrium. Then, the formulation was applied to the skin surface above 3 microdialysis probes (2 mg/cm^2). The fourth probe was used as control. Microdialysis samples were collected every 30 min for a period of 12 h.

The second microdialysis experiment consisted to apply at 1 and 9 h the formulation to the skin. The samples were then collected every 30 min during 24 h.

Assessment of Dermis Asc and Its Metabolites by Gas Chromatography Mass Spectrometry

L-Asc and its metabolites are derivatized by MTBSTFA [11]. Assessments of silylated products were performed on a Hewlett-Packard 5890 gas chromatograph and a 5971A mass spectrometer. The RTX-5MS gas chromatography column (30 m; 0.25 mm ID; 0.25 µm film thickness) was obtained from Supelco (Paris, France). The injector temperature was 250°C. The detector temperature was 290°C, and the initial column temperature was 180°C. A temperature gradient (15°C·min^{-1}) was applied to the column to reach a final temperature of 300°C. 4 µl of the sample were injected in splitless mode (30 s purge-off time). Ionization was performed by electron impact (at 70 eV). The electron multiplier was set at 2,700 V to increase the sensitivity of the apparatus. Selected ion monitoring was used to assess derivatized Asc. The ions selected were 261, 309, 345, 535, 549 and 575 for silylated oxalic acid, *p*-hydroxybenzoic acid, dehydroascorbic acid, threonic acid, xylose and Asc, respectively.

Results

To confirm the performance of the microdialysis probes, we checked the in vitro and ex vivo recoveries of Asc and its metabolites by passing a solution containing either Asc or its metabolites through the microdialysis probes. The ex vivo recoveries of Asc and its metabolites are comprised between 25 and 32%.

One hour after the beginning of the experiment, the serum was deposed on the skin surface. 2 h after this topical application, dermal Asc concentrations increased (fig. 1). Then, a plateau was obtained during 8 h, which demonstrated that Asc was stable during this period. The Asc level achieved was approximately 8.5 times the normal tissue level. Following the plateau, dermal Asc concentrations decreased and came back to the basal values. Concomitantly to the decreased dermal Asc levels, oxalic acid, threonic acid and xylose concentrations in the dermis increased significantly (fig. 2). The control curve demonstrated that the basal Asc level in the dermis was stable during all the time of the experiment (fig. 1).

A second experiment consisted in applying the serum 8 h after the first application, just before the decrease in Asc levels. The results demonstrated that a plateau was obtained about 2 h after the first application and that this level was stable during all the experiment even after the second application (fig. 3). During the 20 h of the experiment, the concentrations of Asc metabolites remained stable and were not significantly different to the basal values (table 1). During the last 4 h of the experiment, Asc dermal levels decreased and concomitantly its metabolite concentrations increased (fig. 3 and table 1). As a preliminary study [12] demonstrated that Asc is stable in this new formulation, this result proved that Asc is degraded in skin fragments and that it is not possible to use skin fragments for more than 20 h for Asc studies.

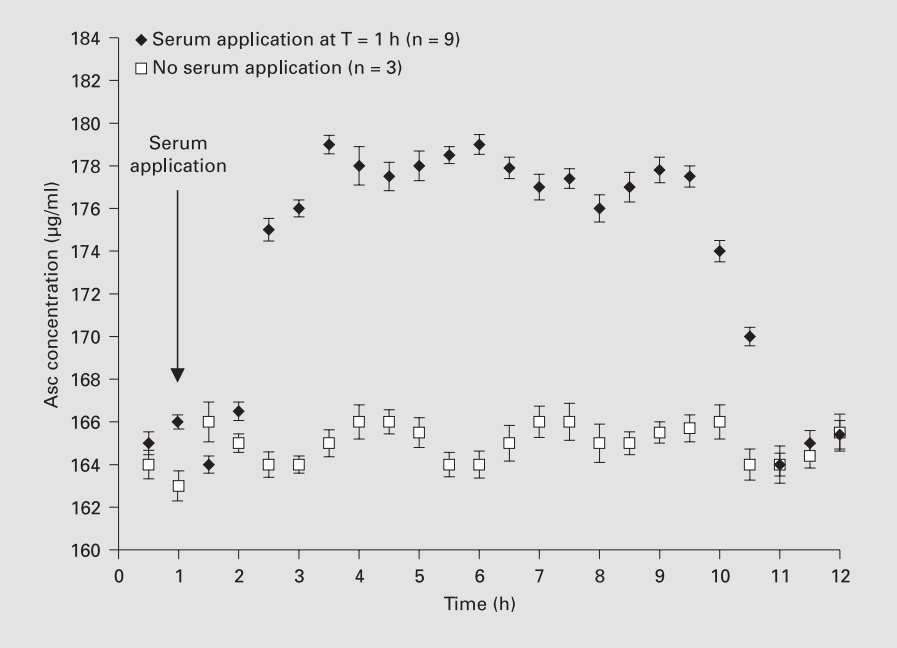


Fig. 1. Cutaneous absorption of Asc after the serum deposition at T = 1 h compared to basal concentration of Asc in microdialysates. Each value represents the mean ± SEM (n > 3).

Discussion

In the skin, Asc is a part of the antioxidative network of the stratum corneum and therefore, along with lipophilic antioxidants, proteins and other biomolecules, a target to the oxidative stressors of the environment. A decrease in the concentration of Asc was measured in mouse dermis [2] or in human skin equivalent [13] following UV irradiation. Therefore, supplementation of the skin with antioxidants, thereby strengthening its antioxidant capacity, is a further approach in limiting UV-induced skin damage. However, according to Dreher et al. [14], no significant protective effect of Asc was obtained when it was applied after UV exposure. Thus, that means that Asc prevents oxidative damage only when it is present in relevant concentrations at the site of action beginning and during oxidative stress. Maximum skin levels of Asc from ingestion are regulated by active transport mechanisms that preclude increasing levels by further ingestion. For this reason, it is important to develop new stable topical formulations to increase Asc dermis concentrations before ROS cause damage to the skin. Pinnell et al. [15] reported the critical importance of formulation pH for percutaneous absorption of Asc. According to these authors, topical application of an acidic pH formulation (less than 3.5) resulted in increasing Asc skin concentrations. However, the required duration of therapy is relatively long (weeks to months) and skin irritation could occur with acidic pH formulations. Therefore, the current formulation is less acidic than those used by Pinnell et al. [15]. The pH of our formulation was 3.1.

The presented data demonstrate that topical application of Asc enhances its steady-state dermal levels during 8 h (fig. 1). After this period, Asc dermal concentrations decrease to the basal level. Although Asc is a very hydrophilic compound with a partition coefficient between octanol and water of 0.02 ± 0.002, the skin permeation of Asc has been confirmed by several scientists. In 1937, Kasahara and Kiwashima [16] reported that Asc can be absorbed through human skin, as shown by the increase in vitamin C in mother's milk following the application of a 30% solution of Asc to mammalian skin. Lee and Tojo [17] reported the skin permeation rate of unstable compounds such as Asc. In 1998, these authors [18] proved that Asc was found to permeate rapidly through the skin, in spite of its low lipophilicity. The diffusivity determined from the lag time was approximately 1,000 times higher in the stripped skin, compared with whole skin. There was a dramatic increase in the permeation rate in stripped skin indicating that the major barrier to the skin permeation of Asc is the stratum corneum. Therefore, Asc derivatives such as magnesium ascorbyl phosphate or ascorbyl-6-palmitate were synthesized. In addition, these derivatives offer the advantage of being more stable than Asc

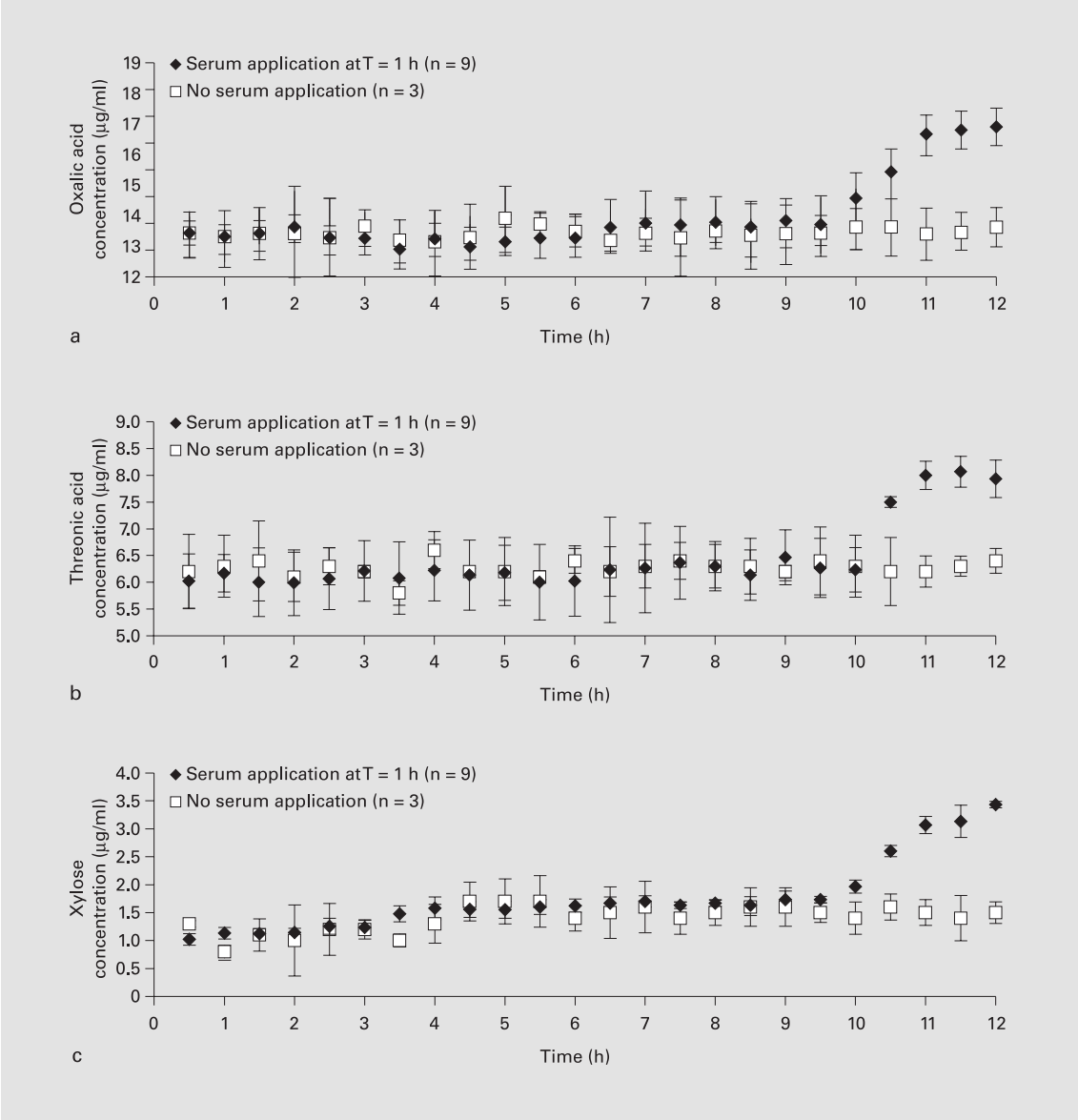


Fig. 2. Asc metabolite concentrations in microdialysates collected from dermal tissue with and without serum deposition: oxalic acid (**a**), threonic acid (**b**) and xylose (**c**). Each value represents the mean ± SEM (n > 3).

[19, 20]. However, according to Pinnell et al. [15], topical application of these Asc derivatives did not increase Asc skin levels. In addition, ascorbyl-6-palmitate is not Asc [21]: Meves et al. [22] showed that ascorbyl-6-palmitate contributes to the generation of oxidized lipid metabolites that are toxic to epidermal cells. These data suggest that, despite its antioxidant properties, ascorbyl-6-palmitate may intensify skin damage following physiological doses of ultraviolet radiation.

This study demonstrates that the concentrations of Asc metabolites (oxalic acid, threonic acid and xylose) increased when Asc dermal levels decreased (fig. 1, 2). A second topical application of Asc was done before its degradation (fig. 3). The data demonstrated that Asc remained stable and that Asc metabolites were not formed (table 1). These results showed the necessity to apply the new Asc formulation every 8 h to obtain an optimal effect.

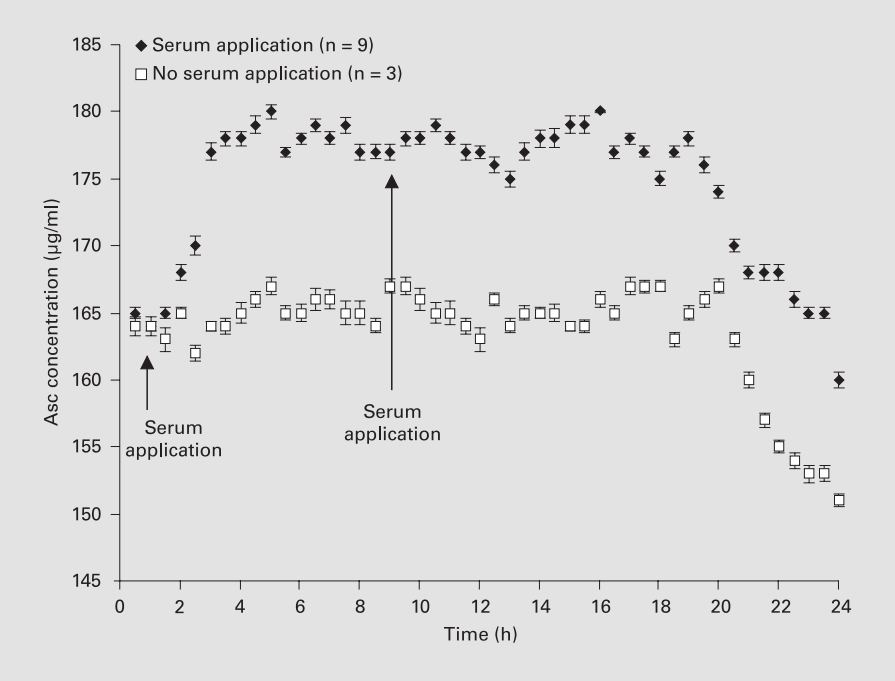


Fig. 3. Asc acid concentrations in microdialysates collected from the dermis with and without serum application. Each value represents the mean ± SEM (n > 3).

Table 1. Asc metabolite concentrations in microdialysis samples (n = 9) collected from the dermis after the cream application at T = 1 h and T = 9 h

Time	Dehydro-ascorbic acid, µg/ml	Xylose µg/ml	Threonic acid µg/ml	Oxalic acid µg/ml
0 h	8.0 ± 0.6	1.0 ± 0.1	6.0 ± 0.5	13.4 ± 0.4
12 h	7.9 ± 0.5	1.1 ± 0.2	6.1 ± 0.4	13.8 ± 0.8
20 h	8.2 ± 0.8	0.9 ± 0.1	6.8 ± 0.3	13.7 ± 0.5
24 h	8.1 ± 0.5	3.5 ± 0.2*	8.1 ± 0.1*	16.2 ± 0.5*

* $p < 0.05$, Mann-Whitney test.

Dermal levels of Asc applied topically had been previously determined in animal [15] skin by using biopsies. Using a formulation with the same pH (pH = 4.5) than our new formulation, tissue levels of Asc did not increase [15]. Therefore, our new formulation seemed to improve Asc skin penetration. In addition, microdialysis offers two major advantages compared to biopsies: microdialysis is a slightly invasive technique, and, in addition, proteins are not collected by this method. Consequently, no extraction is necessary before the sample analysis. Therefore, the sample preparation time is considerably reduced.

The ex vivo results showed an 8% increase in Asc dermal levels following topical application of the new formulation. An in vivo topical application of a 5% Asc formulation demonstrated an improvement of the photodamaged skin and induced modifications of skin relief and ultrastructure, suggesting a positive influence of topical vitamin C on parameters characteristic of sun-induced skin aging [23]. However, the dermal concentration of Asc following the topical application had not been determined by Humbert et al. [23]. Therefore, it is difficult to know if the modest increase in Asc level found in our study is enough for effect. Thus, it would be interesting to determine the in vivo effect of our new formulation. In addition, Ault et al. [24] demonstrated that the blood circulation has a large impact on the drug skin penetration. As Asc is hydrophilic, the blood flow impact is likely to be large. In vivo experiments are necessary to determine the skin effect of this new serum and also the blood circulation impact on the Asc dermal levels.

Conclusion

This work demonstrated that this new 8% Asc formulation permeated rapidly through the skin. After being delivered into the dermis, Asc is stabilized and remains in the tissue for several hours. The results demonstrated that microdialysis was a powerful tool to determine Asc skin penetration and its degradation after its topical application. The next step will consist in determining the in vivo penetration of Asc after the application of this new formulation and also to determine the in vivo effect of this new serum.

Acknowledgement

This study was supported by AURIGA International Ltd. and Alfred Marchal - PhD, Waterloo, Belgium.

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