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Skin absorption and metabolism of a new vitamin E prodrug, δ-tocopherol-glucoside: in vitro evaluation in human skin models

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Abstract

The aim of this study was to investigate the cutaneous penetration and metabolism of the new vitamin E prodrug δ-tocopherol glucoside (δ-TG), as compared to those of common vitamin E acetate, in vitro, both in reconstituted human epidermis and in viable human skin. Better diffusion was observed with α-tocopherol acetate (α-TAc) than with δ-tocopherol glucoside in both skin models, at 0.1% and 0.05% in a myritol solution; however, no metabolism was detected with α-tocopherol acetate. In all conditions tested (two skin models, two concentrations, three test times, and compartmental analysis) the δ-tocopherol glucoside was metabolized into free tocopherol. In the reconstituted human epidermis, after 18 h, over 90% of the δ-tocopherol glucoside was bioconverted. In the viable human skin, the extent of metabolism was about 20%, with 0.12 and 0.10 μg/cm^2 of δ-tocopherol glucoside in the *stratum corneum* and epidermis, respectively. After topical application, the δ-tocopherol glucoside had a considerable reservoir effect, associated with gradual delivery of free tocopherol. The use of this gluco-conjugated vitamin E at a low concentration shows the capability of the skin to metabolize the prodrug in a slow and prolonged manner, making this gluco-conjugated vitamin E an excellent candidate for continuous reinforcement of antioxidants in the skin.

Keywords: δ-Tocopherol glucoside; α-Tocopherol acetate; Percutaneous absorption; Drug metabolism; Topical antioxidant

1. Introduction

Located at the interface between the body and the environment, the skin is the human body's largest organ and its outermost barrier. It is constantly being exposed to environmental sources of reactive oxygen species (ROS) such as ozone, smoke and ultraviolet radiation (UVR). The relationship between ROS and cutaneous oxidative damage induced by UVR has been well documented. In fact, the data suggest that ROS may act as initiators of photoaging [1] or skin cancer [2].

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In vitro and in vivo experiments have shown that topical treatments with the most common lipophilic antioxidant found in the tissues, α-tocopherol (α-Toc) [3], acting primarily through its radical-scavenging properties, have potentially beneficial protective effects against UV induced skin damage, such as epidermal lipid peroxidation [4], sunburn cell formation [5], and thymidine dimer formation [6]. Treatments with α-tocopherol (α-Toc) may contribute to the prevention of photocarcinogenesis. Therefore, such treatments appear to be an effective means of preventing oxidative damage caused by UVR.

As α-Toc is highly unstable to oxidation, the acetate ester of the vitamin is commonly used in cosmetic formulation and clinical trials. A number of studies have examined the photoprotective benefits of this topically applied α-tocopherol acetate (α-TAc). On mice, topical application of α-Toc resulted in a reduction of UV-radiation-induced damage. α-TAc also provided protection, but was less effective than α-Toc [4]. One study, in which mice were treated with thermostable vitamin E esters, showed that UV-induced radical flux in the skin, measured by electron spin resonance, was reduced by α-Toc or α-tocopherol sorbate, but not by α-TAc [7]. In another study, also on mice, it was shown that α-TAc and α-tocopherol succinate not only failed to prevent photo-carcinogenesis, but that they could increase the incidence of squamous cell carcinoma [8]. A similar study on the inhibition of UV-induced carcinogenesis showed that although α-TAc was capable of mitigating DNA damage and p53 expression, it had limited potential in preventing UV-induced proliferation and tumor formation [9]. McVean [6] demonstrated in mice that a lower level of thymidine dimer suppression was produced by α-TAc than by α-Toc. This was most likely the result of less intense UV absorbance of α-TAc [6]. Further studies determined that α-Toc was incorporated into keratinocytes preferentially over α-Tac, and that α-Toc incorporation into keratinocytes is important for maximum DNA protection [10]. A recent in vitro study on mouse keratinocytes showed that α-TAc has the potential to protect the skin from UVB-associated epidermal damage [11].

A major question when considering the potential of vitamin E compounds as topical skin photoprotectors is the mode of administration. When consumed orally, α-TAc is readily hydrolysed to α-Toc by esterases in the gut. However, the fate of topically applied α-TAc and skin esterase activity are not well described [12]. In a pig skin model, up to 15% [13] of the α-TAc that permeated into the viable skin was shown to have metabolized. Similar results were shown in viable human skin model [14], where 20% of the prodrug was hydrolyzed in basal layers. Another work, also on human skin model [15], showed a bioconversion of a maximum value of 0.5 nmol/cm^2. Mice pretreated with topical α-TAc were found to have an increase in α-Toc levels in the skin prior to UVB irradiation, which provided protection against skin damage [16]. Still another work demonstrated that prior UVB irradiation enhanced the conversion of α-TAc to α-Toc, with an increase in α-Toc levels of up to 30-fold over endogenous α-Toc, apparently by inducing non-specific esterase activity, responsible for hydrolysis of the α-TAc [17]. An in vivo study in rats showed that after a single application of α-TAc, hydrolysis into free tocopherol was not observed, and no UV radiation protection was found. After treatment for 5 days with the acetate, the absolute amount of free vitamin E was only a few times higher than the normal level, but sufficient to afford a UV-protecting activity [18]. A subsequent investigation has shown that although α-TAc is well absorbed in the skin of human volunteers, there is little evidence to support its conversion to α-Toc [19]. Recently, a comparative study with water soluble Vitamin E and α-TAc on mouse skin showed that no bioconversion of α-TAc to α-Toc was observed [20]. It was suggested that photoprotection was efficient [6,10] because α-TAc was able to absorb UVB irradiation and thus act as a suncreen and attenuate photodamage. These works show that the bioconversion of α-TAc into free tocopherol in the skin is not clearly understood. It seems to be mediated by non-specific esterase activity [17] located in the viable epidermis [14]. However, no metabolism of α-TAc was detected [13,14] in the *stratum corneum*, the first skin layer directly exposed to a pro-oxidative environment [21]. Although the *stratum corneum* contains very little free water, metabolic activity has been found in it. Using the tape stripping method [22], enzyme activity has been shown for the serine proteases trypsin and chymotrypsin, which are involved in the degradation of corneodesmosomal linkages in the desquamation process. Also found were phosphatases, phopholi-

pases and β-glucocerebrosidase, which are responsible for the formation of multilamellar lipid layers. The enzyme β-glucocerebrosidase hydrolyzes amphiphilic β-glucocerebrosides into more lipophilic molecules, the ceramides, which constitute more than 40% of the weight of intercellular lipids [23].

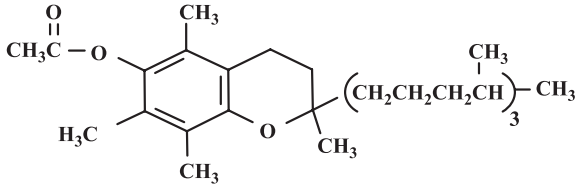
A gluco-vitamin E conjugate, δ-tocopherol glucoside (δ-TG), has been synthesized by making use of β-glucocerebrosidase activity [24]. The glycosidic bond cleavage catalyzed by β-glucocerebrosidase allows the release of the active vitamin E into the *stratum corneum*. As for the acetate form, glucoconjugation improves the stability of this vitamin E prodrug.

In the present study, the skin penetration and metabolism of δ-tocopherol glucoside were assessed in comparison with those of α-tocopherol acetate (Fig. 1), at low concentrations, i.e. 0.1% and 0.05%. These concentrations were chosen for two reasons: first, because it has been shown that too high a concentration of free tocopherol (over 0.1%) could have a pro-oxidant effect [25], and second, because these concentrations are within the range of those recommended for their antioxidant effects [26]. This is particularly true for δ-tocopherol, which has the highest antioxidant action among the natural tocopherols [18,26]. In addition, as a controlled release was expected, the free tocopherol could be reinforced in a closer physiological concentration. These assessments of skin penetration and metabolism were conducted on two human skin models: reconstructed human epidermis (RHE) [27] used as a "screening model", in a preliminary approach, and viable human skin taken from plastic surgery, a model closer to in vivo human skin. The validity of these two models as well as the compartmental distribution of the prodrugs and of the active vitamin E are discussed here.

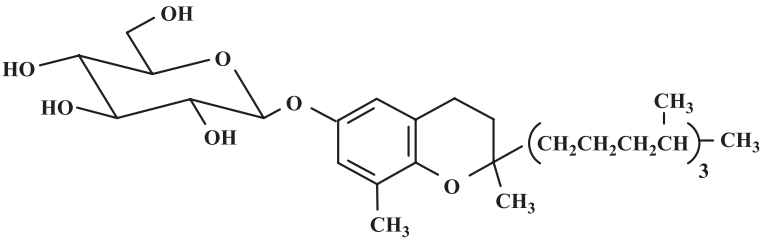
2. Materials and methods

2.1. Chemicals

DL-α-tocopherol acetate (purity>99%) was obtained from Sigma (St. Quentin Fallavier, France). DL-δ-tocopherol glucoside (purity>97%, with less than 0.8% of δ-tocopherol) was supplied by IRPF (Gaillac, France). The glucoconjugate compound was prepared by Schmidt's method [28]. Briefly, after the specific deprotection at carbon 1 of glucopyranose protected in the acetylated form, the reaction with trichloroacetonitrile in the presence of sodium hydride in dichloromethane leads to the imidate α isomer only. The corresponding imidate was then reacted under Lewis acid catalysis with the alcohol unit in the β-config-



α-tocopherol acetate (α-TAc)



δ-tocopherol glucoside (δ-TG)

Fig. 1. The chemical structures of α-tocopherol acetate (α-TAc) and δ-tocopherol glucoside (δ-TG).

uration. Finally, acetyl groups were deprotected using an ion exchange resin (Amberlist A-26) at room temperature.

Tritiated molecules; [3H]-δ-tocopherol glucoside (specific activity 592 GBq/mmol), [3H]-α-tocopherol acetate (specific activity 703 GBq/mmol), [3H]-δ-tocopherol (specific activity 185 GBq/mmol); and [3H]-α-tocopherol (specific activity 1.44 TBq/mmol), with a radiochemical purity of 98% (determined by thin layer chromatography), were provided by Amersham Pharmacia Biotech (Saclay, France). An isotopic dilution was made for the two prodrugs in caprylic/capric triglyceride (trade name: Myritol 318; Cognis, Düsseldorf, Germany). A non-ionic surfactant, ethoxylated oleic alcohol 20 M (trade name: Ameroxol OE 20, Unipex, France) was added in the receptor medium to improve the solubility of vitamin E derivatives. All chemicals were reagent grade. The solvent used in the HPLC assay was HPLC grade.

2.2. Skin models: reconstituted human epidermis and viable human skin

The 4 cm^2 reconstituted human epidermis (RHE) was supplied by SkinEthic Laboratories (Nice, France). The RHE, a three-dimensional multilayered keratinocyte, was obtained by growing the keratinocytes for 17 days at the surface of a defined medium [27] in a CO_2 incubator (5% CO_2, 37 °C). In these conditions, the RHE can be maintained viable for several days. The 1 cm^2 viable human skin, a Natskin® kit, was obtained from Bioprédic (Rennes, France). The fresh skin came from plastic surgery (abdominoplasty). The skin is delivered in a culture insert and is maintained viable with a defined medium in a CO_2 incubator (5% CO_2, 37 °C). The supplier insures viability for 72 h.

2.3. Percutaneous and metabolism studies

2.3.1. Culture insert acting as diffusion cells

The 4 cm^2 RHE consists of an airlifted, living epidermal reconstruct, produced in inserts. Each culture insert containing the RHE was placed in a six-well plate, previously filled with the defined medium. Under these conditions, the insert and the well act as donor and receptor compartments, respectively.

The viable human skin is also in culture inserts and was placed in a multi-well plate, filled with the defined medium. The insert and the well act as donor and receptor compartments, respectively. As these skin tissues do not result from tissue reconstruction, adhesive washers were provided in the NatSkin kit to define the limits of the application area and to act as a sealant.

2.3.2. Comparison of α-TAc and δ-TG diffusion on RHE

The preliminary series of experiments was carried out on RHE using a 0.1% α-TAc and δ-TG myritol solution, in an infinite dose. 100 μl per cm^2 was gently spread onto the 4 cm^2 epidermis surface. In the defined medium, acting as the receptor medium, 6% of ethoxylated oleic alcohol 20 M, was added in order to obtain a good solubility of the two prodrugs and to maintain sink conditions during an 18-h permeation study. RHE distribution was assessed through analysis of the surface, of one tape stripping and of the epidermis. Three samples were analyzed per experiment and each experiment was performed in triplicate.

2.3.3. Kinetics of the metabolism of δ-TG on RHE

As these first assays (Section 2.3.2) showed hydrolysis with δ-TG only, a second experiment was carried out with the gluco-conjugated compound on the RHE at 2, 6 and 18 h, in order to determine the kinetics of the prodrug metabolism. This experiment was conducted with a finite dose, 10 μl per cm^2 of a 0.05% δ-TG myritol solution, which was deposited onto the RHE. Two RHE samples were analyzed per test time and each experiment was performed in duplicate.

2.3.4. Comparison of α-TAc and δ-TG diffusion on viable human skin

Finally, 10 μl of the 0.05% myritol solution of the two prodrugs was tested on the human skin. As before, the metabolism was analyzed at 18 h for α-TAc and δ-TG, and a kinetic study was carried out for the δ-TG at 2, 6 and 18 h. The distribution in the skin of the prodrug and its metabolite was assessed, using a compartmental approach. The *stratum corneum* was removed by 10 tape strippings, and the viable epidermis and the dermis were separated

in warm (55 °C) water. Three skin explants were analyzed per test time and each experiment was performed in duplicate.

2.4. Skin extraction

Lipophilic antioxidants were extracted from the surface, the tape strippings, the RHE and the viable human skin. The vitamin and oily residues were washed off from the skin surface using a cotton swab soaked with ethanol/water (3:1), and then wiped with a dry swab. For the infinite dose study only, excess oil was removed with a micropipette prior to this washing procedure.

The cotton swabs (and excess oil in the infinite dose study) were transferred to a 10 ml glass tube containing 1 ml of ethanol/water (3:1) and were shaken vigorously for 10 min. 100 μl of the solution was injected into the HPLC.

The tape strippings were transferred to a 15 ml glass tube containing 5 ml ethylic ether and 1 ml ethanol/DMSO (1/1). This was shaken vigorously for 30 min at room temperature. The contents of the 15 ml tubes, excluding the disks, were transferred to a 10 ml glass tube. Then ethylic ether and ethanol were evaporated under nitrogen gas flow. The rest of the solvent (DMSO) was transferred into an Eppendorf tube and centrifuged for 1 min at maximum speed (4000 rpm). A 100 μl of the clear supernatant was injected into the HPLC.

The skin (epidermis and dermis) and the RHE were cut into small pieces and transferred into a 15 ml glass tube containing 5 ml ethyl acetate. Each vial was shaken vigorously for 10 min. The solvent was transferred into another glass tube and evaporated under nitrogen. The residue was dissolved in 300 μl ethanol and injected into the HPLC.

2.5. Radio-HPLC analysis of samples from skin extracts

The HPLC system consisted of a Hewlett Packard Model 1100 diode array detector set up in line with a flow scintillation analyzer (Radiomatic 500TR, Packard), equipped with a 1 ml TR-LSC cell (Packard), using Ultima Flo AP (Packard) at an effluent to scintillation fluid ratio of 1:1. The products were eluted using a prepacked C18 column (Nucleosil, 5 μm, 4.6×250 mm, Macherey Nagel) and an isocratic mobile phase consisting of 100% methanol (flow rate 0.4 ml/min). For the liquid scintillation counter the energy window selected was 0–10 KeV. The integration of the data was carried out with a Flo-One integration package (Packard). Calibration curves over the range of 0.1–2.4 kBq were linear. The detection limit was 30 Bq.

2.6. Calculations

The fraction of the vitamin E prodrug metabolized into free tocopherol was calculated as a percentage from the data in dpm (disintegration per minute). As these calculations were related to the specific activity per mol, the difference in molar mass was not taken into account. Therefore, the percentage was calculated according to the following equation:

$$\text{Fraction metabolized (\%)} = \left[Q_{\text{ft}}/\left(Q_{\text{p}} + Q_{\text{ft}}\right)\right] \times 100 \quad (1)$$

where Q_{ft} and Q_{p} are the quantities of dpm counts for the free tocopherol and the prodrug, respectively.

The total amount which permeated into the skin tissue in μg/cm^2 is expressed in prodrug mass. For these calculations, the amount of free tocopherol was corrected by the prodrug mass, by taking into account the difference in molecular mass. Thus the total amount is the sum of the prodrug mass (not metabolized) added to the free tocopherol corrected by the prodrug mass. The calculations of the skin distribution of the prodrugs and the metabolites were carried out according to the COLIPA guidelines for percutaneous penetration [29].

3. Results

3.1. Comparison of α-TAc and δ-TG diffusion on RHE

Table 1 shows the amount of the vitamin E prodrugs in μg/cm^2 at the surface, in one tape stripping and in the epidermis. Tape stripping was performed in order to obtain a compartmental distribution, and as previous trials have shown that more than two tape strippings cause a wrenching of the thin and fragile epidermis from its polycarbonate

Table 1
Prodrug absorption and metabolism in RHE after 18 h [mean (S.E.)] (n=9)

Analyte	Surface in μg/cm^2	One tape stripping in μg/cm^2	Epidermis in μg/cm^2
α-TAc	79.2 (23.3)	1.19 (0.97)	1.26 (1.19)
α-Toc	Not detected	Not detected	Not detected
% of metabolite	–	–	–
δ-TG	92.9 (14.5)	0.30 (0.13)	0.35 (0.25)
δ-Toc	2.58 (2.23)	0.07 (0.05)	0.23 (0.15)
% of metabolite	2.8	17.0	31.6

membrane, only one tape stripping was performed here on the RHE.

After 18 h, in an infinite dose, more than 80% of α-TAc remained at the skin surface. An equivalent distribution was found in the tape stripping and in the epidermis, 1.19 (±0.97) μg/cm^2 and 1.26 (±1.19) μg/cm^2. Accordingly, about 1% of the applied dose has permeated into the RHE. More than 90% of the total amount of δ-TG at 18 h is located at the surface, but 2.6 μg/cm^2 of free tocopherol was also detected in this compartment. The distribution in the RHE showed 0.30 (±0.13) μg/cm^2 and 0.35 (±0.25) μg/cm^2 of δ-TG in the tape stripping and in the epidermis, respectively, associated with 0.07 (±0.05) μg/cm^2 and 0.23 (±0.15) μg/cm^2 of δ-Toc, the free vitamin E. The fraction of metabolized prodrug in this experiment was about 3% of the total δ-TG applied. If bioconversion occurred for α-TAc, it was under the detection limit.

3.2. Kinetics of the metabolism of δ-TG on RHE

As only the δ-TG was metabolized into free tocopherol, a study of the kinetics of the metabolism was carried out in the RHE. But only 0.3% of the prodrug had penetrated in the above conditions. Accordingly, a finite dose (10 μl/cm^2) was then applied on the RHE. Table 2 shows the RHE surface, the tape stripping and the epidermis amounts of δ-TG and δ-Toc, also expressed in the percentage of metabolite at the three test times.

Soon after 2 h, 1.56 μg/cm^2 of the δ-Toc was found at the surface of the RHE, representing one-third of the total amount of the prodrug deposited. The δ-Toc quantities were lower in the tape stripping and in the epidermis, 0.39 and 0.19 μg/cm^2, respectively, than in the surface (1.56 μg/cm^2). However, these amounts represented about 90% of the metabolism of δ-TG. At 6 h, a similar distribution was observed in each compartment, but was accompanied with an increase in the extent of the metabolism. In fact, at the surface this percentage had increased from 37.6% to 70%. In the tape stripping and in the epidermis the percentage reached 96%. Moreover, a greater amount of free tocopherol was found in the epidermis, clear evidence of diffusion. At 18 h, the content of δ-Toc was similar to that of 6 h, in spite of a greater metabolite percentage of roughly 86%. In the tape stripping and in the epidermis, a very low concentration of δ-TG remained, representing a biotransformation into δ-Toc of 98%. But the largest amount was located at the RHE surface. The high content of δ-Toc at the RHE surface is the result of a important metabolism in the SC, and a solubilization into the myritol solution, remaining at the RHE surface, where δ-Toc solubility is probably better than in the tissue. Due to the increase in the metabolism of δ-TG, the δ-Toc content in the tissue increased with time. Actually, in the epidermis, the quantities were 0.19, 0.52 and 0.89 μg/cm^2 at 2, 6 and 18 h, respectively.

Fig. 2 shows the overall kinetics of the prodrug conversion into free tocopherol in the RHE, including surface, tape stripping and epidermis contents, also expressed by the extent of the metabolite. Soon after 2 h, 2.14 μg/cm^2 was found, representing about 45% of the metabolite. The amount of free tocopherol then increased to reach 76% and 90% at 6 and 18 h, respectively. These results showed that at

Table 2
Compartmental analysis (surface, tape stripping and epidermis) of δ-tocopherol-glucoside (δ-TG) conversion into δ-tocopherol (δ-Toc) in RHE at 2, 6 and 18 h [mean (S.E.)] (n=4 for each test time)

Time (h)	Analyte	Surface in μg/cm^2	One tape stripping in μg/cm^2	Epidermis in μg/cm^2
2	δ-TG	2.59 (0.44)	0.05 (0.02)	0.02 (0.01)
	δ-Toc	1.56 (0.26)	0.39 (0.19)	0.19 (0.09)
	% of metabolite	37.6	89.1	90.2
6	δ-TG	1.09 (0.21)	0.02 (0.02)	0.02 (0.01)
	δ-Toc	2.60 (0.49)	0.55 (0.79)	0.52 (0.32)
	% of metabolite	70.4	96.5	96.1
18	δ-TG	0.41 (0.17)	0.05 (0.08)	0.02 (0.01)
	δ-Toc	2.60 (1.04)	1.13 (0.91)	0.89 (0.49)
	% of metabolite	86.2	96.0	97.8

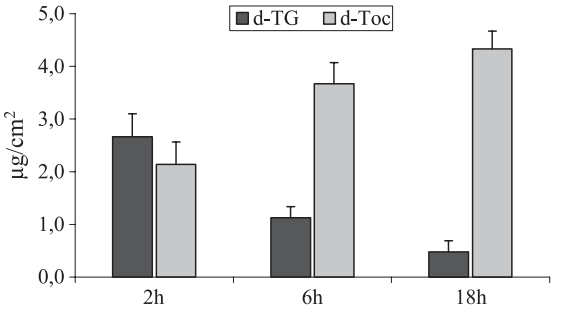


Fig. 2. Total δ-tocopherol glucoside (δ-TG) and δ-tocopherol (δ-Toc) found in RHE at 2, 6 and 18 h [mean (S.E.)] (n=4 for each test time). The extent of metabolism was 45%, 76% and 90% at 2, 6 and 18 h, respectively.

this concentration and in finite dose, the RHE was able to metabolize almost all the δ-TG prodrug into free tocopherol.

3.3. α-TAc and δ-TG diffusion, and kinetics of metabolism of δ-TG on viable human skin

The diffusion and metabolism of the two 0.05% prodrugs in myritol were carried out on viable human skin. Distribution was determined at 18 h using a compartmental approach, evaluating the surface, the *stratum corneum* (10 tape strippings were performed), the epidermis and the dermis. These results are shown in Table 3. As already seen on the RHE (Table 1), the diffusion of α-TAc was higher than that of δ-TG in human skin. After 18 h, a very small amount of α-TAc remained at the skin surface although more than 3.4 µg/cm^2 of δ-TG remained. The highest amount of α-TAc was located in the *stratum corneum* (3 µg/cm^2), 0.8 µg/cm^2 was found in the epidermis and in the dermis. 0.8, 0.35 and 0.16 µg/cm^2 of δ-TG were found in the *stratum corneum*, the epidermis and the dermis, respectively. In spite of the accumulation of α-TAc in the skin, no metabolism of α-TAc into α-Toc was detected in any skin compartment.

Table 3
Compartmental analysis (surface, *stratum corneum* [10 tape strippings], epidermis and dermis) of the two prodrugs (δ-tocopherol-glucoside and α-tocopherol acetate), their conversion into free tocopherol and percentage of metabolism after an 18-h incubation in viable human skin [mean (S.E.)] (n=6)

Analyte	Surface in µg/cm^2	*Stratum corneum* in µg/cm^2	Epidermis in µg/cm^2	Dermis in µg/cm^2
α-TAc	0.17 (0.04)	2.98 (0.18)	0.80 (0.27)	0.79 (0.45)
α-Toc	Not detected	Not detected	Not detected	Not detected
% of metabolite	–	–	–	–
α-TG	3.44 (0.45)	0.78 (0.27)	0.35 (0.24)	0.16 (0.09)
α-Toc	0.04 (0.04)	0.12 (0.07)	0.10 (0.04)	0.02 (0.02)
% of metabolite	1.1	15.3	29.2	13.1

Table 4
Total δ-tocopherol glucoside (δ-TG) and δ-tocopherol (δ-Toc) found in viable human skin (*stratum corneum*+epidermis+dermis) and percentage of metabolism at 2, 6 and 18 h [mean (S.E.)] (n=6 for each test time)

Analyte in µg/cm^2	2 h	6 h	18 h
δ-TG	0.99 (0.21)	1.67 (0.69)	1.28 (0.33)
δ-Toc	0.05 (0.02)	0.20 (0.05)	0.24 (0.06)
% of metabolite	5.4	12.3	18.8

The extent of δ-TG metabolism was found to be lower in the viable human skin than in the RHE; however, it did occur in the viable human skin (Table 3). A low amount of δ-Toc was found at the skin surface (0.04 µg/cm^2). The highest quantity of free tocopherol was located in the *stratum corneum* and in the epidermis, 0.12 and 0.10 µg/cm^2, respectively, representing about 15% and 29% of the metabolite. A small amount (0.02 µg/cm^2) was also found in the dermis, probably the result of skin diffusion of δ-Toc.

Accordingly, only the kinetics of the δ-TG metabolism were evaluated in human skin at 2, 6 and 18 h. These results appear in Table 4 and Fig. 3. Table 4 shows the total amount of δ-TG and its metabolite, δ-Toc, in the viable human skin at the three test times. At

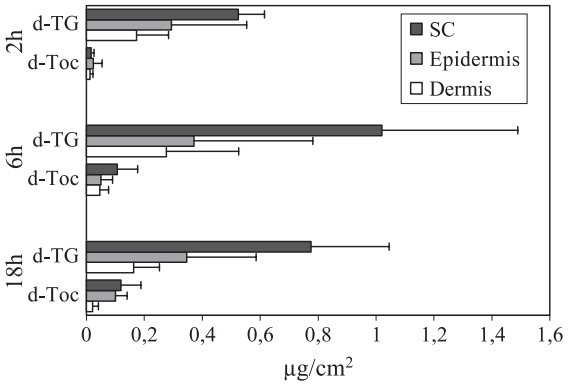


Fig. 3. Compartmental analysis (*stratum corneum* [10 tape strippings], epidermis and dermis). Total δ-tocopherol (δ-TG) and δ-tocopherol (δ-Toc) found in viable human skin at 2, 6 and 18 h [mean (S.E.)] (n=6 for each test time).

2 h, about 1 μg/cm^2 of δ-TG and 0.05 μg/cm^2 of δ-Toc were found in the skin, representing a metabolism of 5%. The amount of δ-Toc increased to 0.2 and 0.24 μg/cm^2, representing a metabolism of 12% and 19%, at 6 and 18 h, respectively. Fig. 3 shows the compartmental distribution of the δ-TG and its metabolite, δ-Toc, in viable human skin at 2, 6 and 18 h. The amount of δ-TG which penetrated into the skin was about 60% in the *stratum corneum*, 30% in the epidermis and 10% in the dermis. Concerning the free tocopherol, the distribution was quite different, with higher concentrations in the *stratum corneum* and epidermis than in the dermis, especially at 18 h.

4. Discussion

4.1. Skin models

Reconstructed human epidermis is known to have a lower barrier function than human skin. The TEWL is higher and the rate of penetration of molecules such as caffeine is 10 times higher in the RHE than in human skin [30]. Moreover, due to its thinness, only one tape stripping was done on the RHE model, strongly limiting the compartmental approach. This SC thinness also limits the experimental approach using an infinite dose, mainly for oily solutions. In spite of these limitations, RHE is an interesting model in the study of the metabolism of topical prodrugs in the skin [31,32]. Accordingly, as diffusion is higher, the bioavailability of a prodrug is better, and if metabolism occurs, it can be very effective. Our results with δ-TG have confirmed this, where 90% of the metabolite was evaluated at 18 h (Table 2 and Fig. 2). However, as shown in our work, in spite of good penetration of α-TAc [13,14,19], and better than that of δ-TG, the prodrug bioconversion was not detected (Tables 1 and 3). In a skin penetration and metabolism study, viable human skin model is a good complement to the "low barrier" RHE model. It is recognized as a predictive model for percutaneous absorption [33]. Moreover, as it has a normal *stratum corneum*, similar to that of native human skin, a compartmental approach can be easily carried out. The viability of the skin allows to determine if metabolism occurs, and, where a prodrug is concerned, to follow the controlled release of bioactive molecules. The work described herein confirms better diffusion of α-TAc than of δ-TG on viable human skin, as already seen on the RHE. In spite of this good diffusion, no bioconversion of α-TAc was detected in viable human skin, similar to results previously seen on the RHE. This could be due to the concentration used in this study (0.05%), because using similar skin models, RHE and viable human skin, Nabi et al. [15] detected bioconversion into free tocopherol after topical application of an emulsion containing 1% of α-TAc. Conversely, for δ-TG, metabolism occurred after topical application and increased with time. Due to better barrier function, it is of a lesser extent than that observed in the RHE. It is obvious that in vitro bioconversion differs from in vivo metabolism. Recently, Sintov [34] found, in an in vitro–in vivo comparison, a similar drug accumulation in pig skin, but with a significantly higher drug metabolism in vivo than in vitro. A preliminary in vivo study of δ-TG skin metabolism on humans [35] measured a threefold higher content in free tocopherol in the first three tape strippings than in the control area, after treatment for 14 days. These preliminary in vivo data confirm our in vitro results, but are not sufficient to establish an in vivo–in vitro correlation especially using a viable human skin model.

4.2. α-tocopherol acetate (α-TAc) diffusion and metabolism

The diffusion of α-TAc in both skin models confirmed the good availability of the vitamin E prodrug [13,14,19]. However, despite a better diffusion than that of δ-TG, no metabolism was detected in any of the conditions tested (two skin models, two concentrations, and compartmental analysis) using radio-HPLC detection. That the skin is a metabolic organ capable of hydrolyzing esters is recognized, as confirmed by the metabolism of retinyl palmitate [36] or salicylate esters [34] and α-TAc [15,17], but researchers have suggested that skin esterases have a relatively low rate of hydrolysis [18]. Nabi [15] detected hydrolysis into δ-Toc reaching a maximum of 0.5 nmol/cm^2 (0.21 μg/cm^2) at 14 h, following an application of 4 mg/cm^2 of an emulsion containing 1% of α-TAc. De Vries [18] found that the percentage of α-TAc hydrolyzed in free vitamin E in the epidermis was about 5% after treatment for 5 days.

Our work performed on two human skin models does not show this bioconversion, in spite of a duration of 18 h. Therefore, the time factor cannot explain this absence of metabolism. The vehicle used, myritol, could be inappropriate. Bashong [14] has shown that in myritol, α-TAc stayed at the skin surface or diffused into the *stratum corneum* only. We found a considerable amount of α-TAc (Table 3) in the epidermis, where the metabolism of the prodrug occurs, suggesting that myritol does not act as a limiting factor for the diffusion of α-TAc into the skin. Finally, the concentration used in our work (0.1% and 0.05%) is probably too low to observe metabolism. Actually, many authors who find metabolism have deposited α-TAc in concentrations of 0.5% to 5% [13–17]. However, these concentrations are higher than those recommended for this cosmetic ingredient [26]. Morevoer, the hydrolysis occurred only in the deeper epidermis [14,17], and not in the *stratum corneum*, which is one of the areas most exposed to environmental oxidative stress. This implies that this first skin layer should be reinforced in active antioxidants, as confirmed by the works showing the photoprotective efficacy of α-Toc [3,4,7,10].

4.3. δ-tocopherol glucoside (δ-TG) diffusion and metabolism

The skin diffusion of δ-TG in a myritol solution was lower than that of α-TAc. In infinite dose conditions (Table 1), only 0.3% of the applied dose permeated into the RHE. On human skin, which has a thicker *stratum corneum*, and in finite conditions (Table 3), only 30% permeated into the skin. However, bioconversion into free tocopherol was detected in all conditions tested (two concentrations, two skin models, three test times, and compartmental analysis). The β-glucocerebrosidase is located at the *stratum granulosum–corneum* junction and also in the *stratum corneum*. This enzyme hydrolyzes β-glucocerebrosides into ceramides. Accordingly, and as δ-TG is an optimized substrate for this enzyme [22], even though a low concentration of prodrug was present in the *stratum corneum*, bioconversion did occur (Table 1). This has been shown in this work through the kinetic study on RHE (Table 2), where 90% of the prodrug was metabolized in both tape stripping and epidermis at 2 h. This percentage increased with time to reach about 98% at 18 h. As the quality of the barrier function of the RHE is low, the prodrug diffuses more easily through the *stratum corneum* and becomes available for bioconversion. The proportion of the metabolite also increased at the RHE surface, to reach more than 85% at 18 h, certainly due to a better solubility of δ-Toc in this vehicle.

The kinetics of metabolism were observed to be similar in both the RHE (Table 2) and the viable human skin (Table 4). However, as the viable human skin differs from the RHE, having thicker layers and a more efficient barrier function, the extent of metabolism was lower, but increased with time to reach about 20% at 18 h. The compartmental approach gives more details on the dynamics of this metabolism (Fig. 3). The highest amount of the prodrug was located in the *stratum corneum*, which acts as a reservoir. The prodrug slowly diffused in the skin, but soon after 2 h, metabolism occurred, and the δ-Toc was found in each skin compartment. At 6 h, the highest amount of free tocopherol was in the *stratum corneum*. It increased at 18 h (Table 3) to reach an equivalent amount, 0.12 and 0.1 μg/cm^2, in the *stratum corneum* and epidermis, respectively. Due to the location of the β-glucocerebrosidase, these data suggest that the prodrug is metabolised in the *stratum corneum*, and that the free tocopherol diffuses into the other skin compartments such as the epidermis and dermis. Finally, we have shown that topical treatment of δ-TG is an efficient prodrug in the reinforcement of antioxidants in the skin, through a gradual delivery of free tocopherol in each skin compartment, from the *stratum corneum* to the dermis. These results are confirmed by an preliminary in vivo δ-TG skin metabolism study [35]. Finally, Nakayama [20] has shown that a water soluble provitamin E converted into free tocopherol was efficient in preventing UV-induced skin damage. This suggests that reinforcement in δ-Toc [10], using the gluco-vitamin E conjugate, should also protect the skin against UV-induced damage [10,18]. Pharmacological studies on skin models are ongoing to investigate this further.

5. Conclusion

Our study has provided new data on the percutaneous absorption and metabolism of two vitamin E

prodrugs, by using two human skin models, reconstituted human epidermis and viable human skin. The RHE, due to its low barrier function, can be used as a screening model. Confirmation on the viable human skin model gives more reliable data, due to its more efficient barrier function, useful for clinical studies. α-TAc was found not to metabolize on the two models used, probably due to the low concentration. In contrast, the new gluco-conjugated vitamin E was found to have a lower diffusion rate but was metabolized in all conditions tested. With topical application, δ-TG had a considerable reservoir effect, associated with a gradual delivery of free tocopherol, first in the *stratum corneum*, and then in the other skin compartments. The use of gluco-conjugated vitamin E at a low concentration shows the capability of the skin to metabolize a prodrug in a slow and prolonged manner, inducing continuous reinforcement of antioxidants in the skin.

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