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Research paper

Simultaneous absorption of vitamins C and E from topical microemulsions using reconstructed human epidermis as a skin model

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ABSTRACT

Antioxidants provide the mainstay for skin protection against free radical damage. The structure of microemulsions (ME), colloidal thermodynamically stable dispersions of water, oil and surfactant, allows the incorporation of both lipophilic (vitamin E) and hydrophilic (vitamin C) antioxidants in the same system. The objective of this work was to investigate the potential of non-thickened (o/w, w/o and gel-like) and thickened (with colloidal silica) ME as carriers for the two vitamins using reconstructed human epidermis (RHE). The amounts of these vitamins accumulated in and permeated across the RHE were determined, together with factors affecting skin deposition and permeation. Notable differences were observed between formulations. The absorption of vitamins C and E in RHE layers was in general enhanced by ME compared to solutions. The incorporation of vitamins in the outer phase of ME resulted in greater absorption than that when vitamins were in the inner phase. The location of the antioxidants in the ME and affinity for the vehicle appear to be crucial in the case of non-thickened ME. Addition of thickener enhanced the deposition of vitamins E and C in the RHE. By varying the composition of ME, RHE absorption of the two vitamins can be significantly modulated.

1. Introduction

Skin is a tissue subjected to a high degree of oxidative stress from both endogenous and exogenous sources. As excessive levels of free radicals in skin can induce deleterious reactions such as skin ageing, skin disorders and skin diseases, several lines of antioxidant defence against damaging reactive metabolites have evolved, the most important being enzymatic (such as superoxide dismutase, catalase and peroxidase), and non-enzymatic (such as glutathione, α-tocopherol - vitamin E, ascorbate - vitamin C, β-carotene and ubiquinone). A promising strategy for enhancing skin protection from oxidative stress would be to support the endogenous skin antioxidant system. The most intensively studied antioxidants for preventing skin oxidative damage have been vitamin C, vitamin E and β-carotene [1–3].

Vitamin C, unique in its high reactivity with all aggressive oxygen radicals, is a major – and the only essential – antioxidant in the aqueous cell compartment, whereas vitamin E is the most important chain-breaking radical scavenger in the liposoluble compartment, thus constituting the major specific defence line against lipid peroxidation. There is increasing evidence that vitamins E and C, even though present in different compartments of the cell, act synergistically [2,3]. In membranes, vitamin E is oxidised when it quenches peroxyl free radicals. The neighbouring intracellular vitamin C reduces the oxidised vitamin E to regenerate its activity. It has been shown that a topically applied combination of vitamin C with vitamin E gives fourfold protection against UV-induced erythema, compared to twofold protection by either vitamin alone [4–6].

In order to protect skin from antioxidative damage, the antioxidant must first be able to penetrate the effective permeation barrier offered by the skin. An effective way to enhance cutaneous bioavailability of drugs is to choose an appropriate delivery system. Topically applied microemulsions (ME) significantly increase skin absorption of drugs and, in some cases, provide its sustained release [7,8]. ME are clear, thermodynamically stable dispersions of water and oil, stabilized by an interfacial film of surfactant molecules [7]. Moreover, their specific structure allows the incorporation of both lipophilic and hydrophilic drugs in the same system.

Skin is a heterogeneous membrane composed of a variety of cell types, but the upper layer *stratum corneum* is the main barrier for percutaneous absorption. Reconstructed human epidermis (RHE) models have a well-developed *stratum corneum* and demonstrate

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reasonable similarity to the native human tissue in terms of morphology, lipid composition and biochemical markers. EpiSkin®'s model used in our study shows all epidermal layers seen in human skin, but cells of viable parts are organised differently than in native epidermis. All major classes of epidermal lipids are present; the phospholipids content is very close to that of human epidermis, but EpiSkin® model has higher content of di- and triglycerides. The potential of RHE has been evaluated in permeation studies using model lipophilic and hydrophilic drugs [9–11]. However, there are not many studies dealing with the influence of the vehicle on drug permeation through RHE [12–14].

We have demonstrated that ME provide enhanced stability for vitamins C and E [15]. The aim of the present research was to study the influence of formulation on the percutaneous absorption of hydrophilic (vitamin C) and lipophilic (vitamin E) antioxidants following topical application to reconstructed human epidermis. Both antioxidants were simultaneously incorporated in thickened and non-thickened ME.

2. Materials and methods

Isopropyl myristate (IPM) was obtained from Fluka Chemie, Buch, Switzerland and used as the lipophilic phase. Tween 40 – polyoxyethylene [20] sorbitan monopalmitate (Fluka Chemie, Buch, Switzerland) – was used as surfactant and Imwitor 308 – glyceryl caprylate (Condea, Hamburg, Germany) – as cosurfactant. Purified water was used as the hydrophilic phase. α-Tocopherol (vitamin E) and ascorbic acid (vitamin C) were from Fluka, Buch, Switzerland. Colloidal silica (Aerosil 200, Degussa, Dusseldolf, Germany) was used as the thickening agent.

NaCl and orthophosphoric acid were from Prolabo (Fontenay-sous-Bois, France); chicken egg albumin, phosphate buffer saline tablets (pH 7.4) and potassium monobasic phosphate from Sigma Aldrich (Munchen, Germany) and Chremaphor EL from BASF, Ludwigshafen, Germany. Analytical grade acetonitrile and methanol were from Fisher Scientific (Illkirch, France).

Episkin® kits containing 12 cell cultures with 1.07 cm^2 surface area on the nutritive gelatin gel were a gift of Episkin (Lyon, France).

2.1. Preparation of microemulsions (ME)

The components of ME are acceptable for topical use. Non-thickened ME of both types and gel-like ME differ only in quantitative composition (w/w percent) (Table 1).

All ME were prepared in the same way. The surfactant and cosurfactant were mixed in a 1:1 mass ratio with a magnetic stirrer for 5 min at room temperature. IPM and water were then added. Vitamin C (0.4% w/w concentration) and/or vitamin E (1% w/w concentration) were incorporated in ME by stirring with a magnetic stirrer for 30 min. No preservatives were added in order to avoid possible interaction with the vitamins. When colloidal silica was used, it was added (10% w/w) to ME containing vitamins and stirred with a magnetic stirrer for 30 min. The ME were left covered in the dark for at least 24 h. The final concentrations of the vitamins in the formulation were checked by HPLC at the time of dosing. The final vitamin content was always in the range 95–105% of the amount incorporated.

For comparison, the solutions of vitamins were tested (Table 1).

2.2. Rheological measurements

A Rheolab MC 100 Paar Physica, equipped with a cone and plate sensor system MK 22, was used for rheological characterization. Rheological measurements were carried out under destructive conditions at 20 ± 1 and 37 ± 1 °C. Prior to analysis, the samples were exposed to the temperature of the experiment for 30 min. The glass cover was used in order to prevent water evaporation. All measurements were made in triplicate.

2.3. In vitro skin permeation experiments

Upon receipt of the Episkin® kit, cell cultures were removed from the nutritive gel and transferred under aseptic conditions into a sterile 12-well culture dish containing 2 ml of the maintenance medium per well. Maintenance medium was provided by Episkin. The cultures were incubated at 37 °C, 5% CO_2 and saturated humidity until their use.

The RHE were mounted on static Franz diffusional cells (diameter 12 mm, receptor volume 8 mL). The receptor compartment was filled with 8 ml of 0.9% water solution of NaCl to which 3% of albumin was added. Solubilities of vitamins C and E in receptor phase were 652.2 ± 44.7 mg/mL and 40.25 ± 2.3 mg/L, respectively. The receptor solution was stirred continuously at 100 rpm and the water bath kept at 37 ± 1 °C, resulting in RHE surface temperature of 32 °C. Donor compartment contained 1 g of formulation (ME simultaneously containing 0.4% of vitamin C and 1% of vitamin E, 0.4% aqueous solution of vitamin C or 1% oily solution of vitamin E). At time intervals of 1, 2, 3, 4 and 6 h, 1 ml of the receptor medium was removed and replaced by fresh, preheated medium. Extraction of vitamins from receptor fluid was done with 1 ml of methanolic solution of Chremaphor EL (1 mg/ml). Samples were vortexed with glass bills (3 × 2 min with 5 min stops), centrifuged (at 6000 rpm for 15 min) and after filtration (0.45 μm filter) analyzed by HPLC. For recovery determination, a known amount of vitamin C (250, 125 and 50 μg/ml) or vitamin E (20 μg/ml) was added to blank receptor solution. After 6 h of contact, the receptor fluid was submitted to the above described assay of extraction and analysis. Recovery test were done in triplicate. The recovery of vitamin C was always in range 95–105% and that of vitamin E was 72 ± 8%. After 6 h the formulation remaining on the surface of the RHE was collected and analysed for the content of the two vitamins. The RHE samples were cleaned three times with cotton swab soaked in methanol and then dried with fresh cotton swab. Epidermis was separated from collagen, put into eppendorf tube containing 0.75 ml of methanol and cut into small pieces. The tubes were occasionally vortexed. After 45 min, the extract was removed and extraction procedure was repeated with 0.75 ml of

Table 1
Composition of tested formulations (w/w %).

Composition (w/w %)	o/w ME	o/w ME colloidal silica	w/o ME	w/o ME colloidal silica	gel-like ME	Aqueous solution	Aqueous solution colloidal silica	Isopropyl myristate solution
Tween 40	14.79	13.29	14.79	13.29	14.79	–	–	–
Imwitor 308	14.79	13.29	14.79	13.29	14.79	–	–	–
Isopropyl myristate	24.65	22.15	9.86	53.16	9.86	–	–	99.00
Purified water	44.37	39.87	59.16	8.86	59.16	99.60	89.60	–
Vitamin E	1.00	1.00	1.00	1.00	1.00	–	–	1.00
Vitamin C	0.40	0.40	0.40	0.40	0.40	0.40	0.40	–
Colloidal silica	–	10.00	–	10.00	–	–	10.00	–

Table 2
Recovery of vitamins C and E from epidermis and collagen.

	Vitamin C	Vitamin E
Epidermis	88 ± 5%	107 ± 8%
Collagen	93 ± 10%	105 ± 5%

fresh methanol until no vitamin was detected in extract. For collagen the same procedure as for epidermis was used, but volume of methanol used was 1.5 ml. After filtration the extracts were analysed by HPLC. Specifity and recovery of vitamins' extraction from RHE was proved. RHE that were not in contact with vitamins were submitted to extraction procedure and the retention time of endogenous compounds did not overlap with those of either vitamin. For recovery determination a known amount of each vitamin (10 μl of 5 mg/ml aqueous or oily solution of vitamins C and E, respectively) was added to either epidermis or collagen that was separated prior to experiment. After 6 h of contact, the extractions were done as described previously and recovery was calculated as ratio between the amount of vitamin extracted from tissue and the amount of vitamin added. Recovery of both vitamins from collagen and epidermis is reported in Table 2.

All experiments were performed in six parallels.

2.4. HPLC analysis

HPLC analysis was carried out with an Agilent 1200 series HPLC system. The injection volume was 20 μl for the analysis of standards and for the determination of vitamin content in the formulations and in the extracts of the donor compartment; for all other samples, 100 μl was used. As external standards, methanolic solution of vitamins C (50 μg/mL) or E (50 μg/mL) was used.

For vitamin E analysis, the stationary phase was a 120 × 4 mm ID column packed with 5 μm Nucleosil C18 and the mobile phase methanol-acetonitrile 70:30. The flow rate was 1.5 ml/min, and UV detection at 291 nm. The method was validated for the determination of vitamin E in ME. The limit of quantification (LOQ) for chromatographic determination of vitamin E was determined from the calibration curve to be 0.54 μg/mL. The limit of detection (LOD) was 0.18 μg/mL. Intra- and interday precision according to ICH guidelines for three relevant concentrations (50, 100 and 200 μg/ml) were <0.15% and <0.60%, respectively.

For vitamin C, the stationary phase was a 250 × 4 mm ID column packed with 5 μm Nucleosil C18-NH_2, and the mobile phase methanol acetonitrile 0.02 M phosphate buffer pH 3.5 (20:30:50). The flow rate was 1 ml/min, with UV detection at 243 nm. The method was validated for the determination of vitamin C in ME. The limit of quantification (LOQ) for chromatographic determination of vitamin C was determined from the calibration curve to be 4.0 μg/mL. The limit of detection (LOD) was 1.3 μg/mL. Intra- and interday precision according to ICH guidelines for three relevant concentrations (50, 100 and 200 μg/ml) were <1.0% and <1.4%, respectively.

2.5. Measurement of transepidermal water loss (TEWL)

ME containing no vitamins were applied on the RHE in the same quantity as for permeation tests. After 6 h, the formulations were removed and the surface cleaned with 2 × 300 μl of PBS buffer, pH 7.4, and dried with a cotton swab. The cell cultures were then placed on the nutritive gelatin gel at room temperature for 4 h. The TEWL was measured with an MPA 5 Tewameter®, Courage Khazaka, Germany. The measurements were done in triplicate for epidermis treated with thickened and non-thickened o/w ME, and in duplicate when treated with gel-like MEs and aqueous solution of colloidal silica, and when non-treated.

2.6. Data analysis

Influence of formulation on vitamins' retention in epidermis and collagen as well as on their permeation was evaluated by one way ANOVA. Bonferroni's test was used for post-hoc comparisons. Significance was tested at the 0.05 level of probability.

3. Results

3.1. Rheological measurements

At 20 °C non-thickened ME of both types are low-viscosity Newtonian fluids with viscosities less than 0.5 Pa s (Fig. 1). Although the difference in the initial viscosities of non-thickened ME and gel-like ME cannot be clearly seen from Fig. 1, a slightly increased viscosity of the latter was proved by absolute viscosity measurement by Hoeppler viscosimeter at 20 °C (37.2 ± 0.1 mPa s vs. 97.6 ± 0.4 mPa s for o/w ME and gel-like ME, respectively). The increased viscosity at higher shear rates and non-Newtonian behaviour of gel-like ME further distinguish gel-like ME from o/w and w/o ME. The addition of colloidal silica significantly increased the viscosity of ME regardless of type, and triggered a transition from ideal Newtonian to pseudoplastic behaviour. The initial viscosities of the thickened o/w and w/o ME were approximately the same. At 37 °C the behaviour of gel-like ME was changed to Newtonian and the viscosity decreased relative to that at 20 °C. In contrast, slightly elevated temperature did not affect the rheological behaviour of thickened ME.

3.2. In vitro skin permeation experiments

The permeation of the two vitamins from six different formulations was determined – in solution (aqueous or isopropyl myristate), in o/w ME, w/o ME, gel-like ME and o/w and w/o ME thickened with 10% of colloidal silica. Three parameters were investigated: the amount of vitamin accumulated in the epidermis, in collagen and in the receptor fluid (Figs. 2–4).

After 6 h, more than 80% of vitamin E and 60–80% of the vitamin C remained on the surface (data not shown). Thus, about 10–15% of the vitamins peneterated into RHE. The amounts of vitamins penetrated into the epidermis and collagen from different formulations are shown in Figs. 2 and 3. The distribution of the vitamins in the RHE generally showed slightly higher amounts in the keratinocytes than in collagen, but the concentrations are within the same range. Only in the case of thickened o/w ME, a significant difference between vitamin C and vitamin E accumulation in epidermis was observed. In the collagen layer, more vitamin E accumulated than vitamin C.

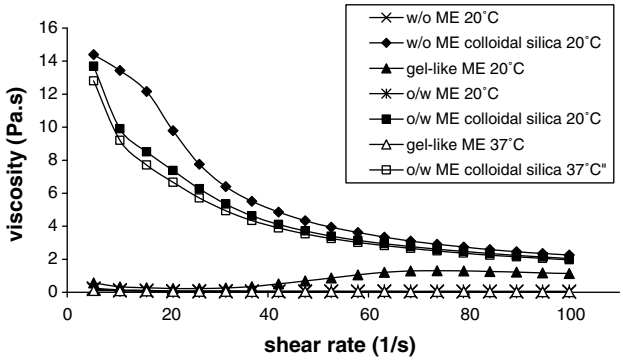

Fig. 1. Viscosity diagrams of thickened and non-thickened ME at 20 and 37 °C.

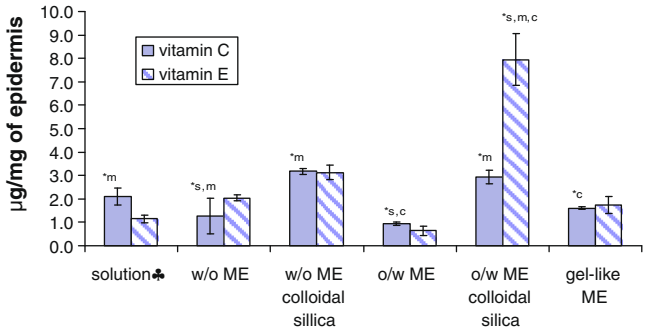

Fig. 2. The amounts of vitamins C and E accumulated in epidermis of RHE from different formulations (♣ aqueous solution of vitamin C and isopropyl myristate solution of vitamin E) after 6 h. The bars indicate the mean value and standard deviation of six experiments for each formulation. Vitamin C: *s – $p < 0.05$ compared to aqueous solution; *m – $p < 0.05$ compared to o/w ME; *c – $p < 0.05$ compared to o/w ME thickened with colloidal silica. Vitamin E: *s – $p < 0.05$ compared to oily solution; *m – $p < 0.05$ compared to w/o ME; *c – $p < 0.05$ compared to w/o MEs thickened with colloidal silica.

3.2.1. Vitamin C

Vitamin C from aqueous solution accumulated in the epidermis and was also found in the collagen layer, but not in the receptor solution. When vitamin C was incorporated in the ME, the tendency to lower accumulation in the epidermis was observed. Moreover, the amount of the vitamin in the collagen increased. The type of ME also influenced its accumulation in the collagen. A difference between o/w ME and gel-like ME was observed, but the amount was the highest for w/o ME. The addition of colloidal silica increased the amount of vitamin C in epidermis, regardless of the type of ME. In the collagen layer, however, the effect was opposite – less vitamin C accumulated, although for o/w ME the difference was not significant.

The amount of vitamin C accumulated in the receptor solution was significantly lower with thickened than with non-thickened ME (Fig. 4), the exception being with w/o ME. In the latter case, vitamin C was found in the receptor solution only after 6 h (40 μg/cm^2), whereas in the two other types, detectable amounts were found after 1 h already (Fig. 4). Thickened o/w ME delivered more vitamin C into the receptor solution than thickened w/o ME and its steady-state flux was also higher (Fig. 4, Table 3). In contrast, the steady-state flux of thickened systems was significantly lower from that of non-thickened.

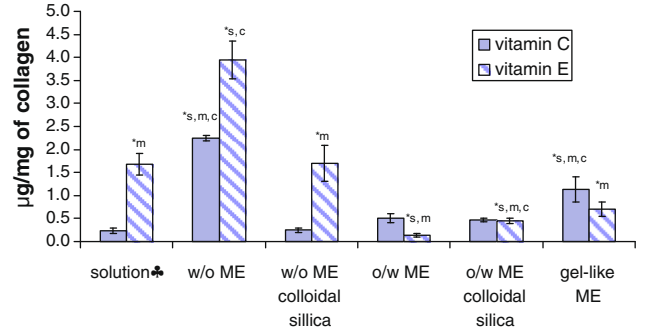

Fig. 3. Vitamins C and E absorbed into collagen layer of RHE from different formulations (♣ aqueous solution of vitamin C and isopropyl myristate solution of vitamin E) after 6 h. Vitamin C: *s – $p < 0.05$ compared to aqueous solution; *m – $p < 0.05$ compared to o/w ME, *c – $p < 0.05$ compared to o/w ME thickened with colloidal silica. Vitamin E: *s – $p < 0.05$ compared to oily solution; *m – $p < 0.05$ compared to w/o ME; *c – $p < 0.05$ compared to w/o ME thickened with colloidal silica.

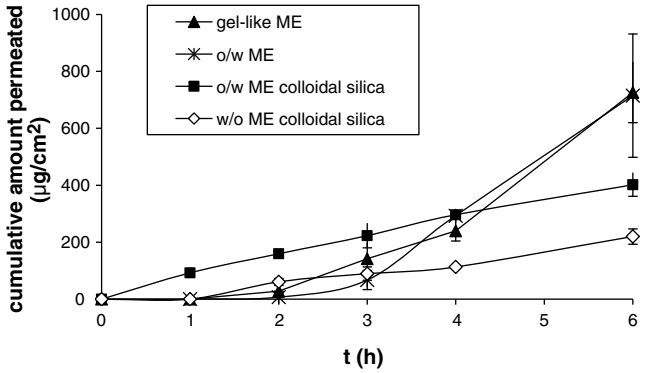

Fig. 4. Permeation profiles of vitamin C from different formulations through RHE.

Table 3
Steady state flux (J) for vitamin C from different formulations.

Formulation	J (μg/hcm^2)
o/w ME	185[b]
Gel-like ME	176[b]
w/o ME	–
o/w ME colloidal silica	62[a]
w/o ME colloidal silica	40[a]

[a] $p < 0.05$ compared to o/w ME.
[b] $p < 0.05$ compared to o/w MEs thickened with colloidal silica.

In order to obtain more detailed information on the influence of colloidal silica on the skin absorption of vitamin C, three additional formulations were tested: vitamin C aqueous solution thickened with 10% colloidal silica and o/w ME containing vitamins C and E, each thickened with 5 and 15% of colloidal silica. Results concerning vitamin C are shown in Table 4a. The addition of colloidal silica in the aqueous solution facilitated the passage of vitamin C into the receptor solution, although the quantity was low. There was no significant difference between the vitamin C permeation parameters and accumulation in the collagen for the ME o/w thickened with 5 and 10% of colloidal silica (Table 4a), whereas the addition of 15% of silica resulted (compared to 10% colloidal silica) in lower concentrations of vitamin in the RHE and in the receptor solution, where the vitamin was found only after 6 h.

3.2.2. Vitamin E

Vitamin E passed into the receptor solution only when incorporated in ME containing colloidal silica (334 and 24 μg/cm^2 for thickened w/o and o/w ME respectively). Even in this case, the vitamin E was detected in the receptor solution only after 6 h. The

Table 4a
Compartmental analysis (epidermis, collagen, receptor fluid) of vitamin C concentration from different formulations.

Formulation	Concentration in epidermis (μg/mg)	Concentration in collagen (μg/mg)	Concentration in receptor fluid (μg/cm^2)
Aqueous solution	2.11 ± 0.35[a,b]	0.23 ± 0.05	0.0
Aqueous solution 10% colloidal silica	2.98 ± 0.61[b,c]	0.23 ± 0.02	68.9 ± 5.3[b]
o/w ME	0.93 ± 0.06[a]	0.51 ± 0.10[a,c]	504 ± 141[a,c]
o/w ME 5% colloidal silica	1.36 ± 0.35[a]	0.37 ± 0.19	438 ± 118[a,c]
o/w ME 10% colloidal silica	2.93 ± 0.30[b,c]	0.47 ± 0.04[a,c]	402 ± 32[a,c]
o/w ME 15% colloidal silica	1.63 ± 0.03[a]	0.23 ± 0.07	46.7 ± 3.3[b]

[a] $p < 0.05$ compared to aqueous solution 10% colloidal silica.
[b] $p < 0.05$ compared to ME o/w 5% colloidal silica.
[c] $p < 0.05$ compared to ME o/w 15% colloidal silica.

Table 4b
Compartmental analysis (epidermis, collagen and receptor fluid) of vitamin E concentration from different formulations.

Formulation	Concentration in epidermis (μg/mg)	Concentration in collagen (μg/mg)	Concentration in receptor fluid (μg/cm^2)
o/w ME	0.65 ± 0.20	0.14 ± 0.04	0
o/w ME 5% colloidal silica	3.15 ± 2.10	0.14 ± 0.05	1610 ± 16
o/w ME 10% colloidal silica	7.94 ± 1.10[a,b]	0.44 ± 0.06[a,b]	88 ± 6
o/w MEs 15% colloidal silica	3.12 ± 0.58	0.28 ± 0.12	0

[a] $p < 0.05$ compared to MEs o/w 5% colloidal silica.
[b] $p < 0.05$ compared to MEs o/w 15% colloidal silica.

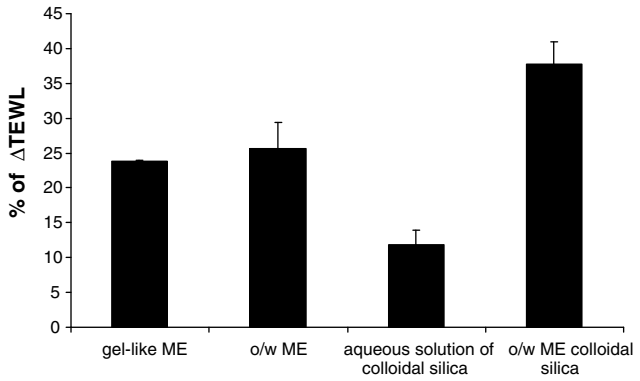

Fig. 5. The change (in %. compared to non-treated RHE) of TEWL 4 h after the removal of the formulation at room temperature. RHE were treated with formulations for 6 h.

addition of colloidal silica also enhanced the epidermal content of vitamin E when compared to other formulations (Fig. 2). The accumulation of vitamin E in the collagen reflects the influence of the vehicle, the accumulation being highest for w/o ME, followed by isopropyl myristate solution and thickened w/o ME, and lowest for ME that contained water as the continuous phase, even if the thickener was used (Fig. 3).

The results of an additional experiment with o/w ME thickened with 5 and 15% of colloidal silica showed that, for vitamin E, the addition of 5% colloidal silica enhanced its passage into the receptor solution, but did not significantly modify its retention in the RHE (Table 4b). The addition of 15% colloidal silica was the least favourable of all the concentrations of thickener tested, but still superior to the ability of non-thickened ME of the same type to deliver the vitamin to the RHE.

3.3. Transepidermal water loss

The measurement of TEWL is a well established method for assessing the integrity of the skin barrier. When skin is damaged, its barrier function is impaired resulting in greater water loss [16]. Transepidermal water loss was measured for four formulations – thickened and non-thickened o/w ME, gel-like ME, and an aqueous solution of colloidal silica and non-treated keratinocytes. The results are shown as the percentage of the difference at room temperature between the TEWL of treated and non-treated RHE 4 h after removal of the formulation (Fig. 5). There is no significant difference between the two non-thickened ME, but the addition of thickener significantly raised the TEWL, which was almost 40% higher than in the case of non-treated RHE. The aqueous solution of colloidal silica also enhanced TEWL, by more than 10%, when compared to non-treated RHE, but significantly less than for RHE treated with ME.

4. Discussion

4.1. Rheological measurements

Non-thickened ME used in our experiments are stable, transparent liquids that consist of fine droplets of inner phase dispersed in continuous medium [17–19]. The application of non-thickened ME to the skin is possible but preparations with increased viscosity are more convenient to apply. A possible answer to the viscosity problem is to use systems with lamellar structure and consequently increased viscosity [18], as in the case of gel-like ME. The term gel-like ME was chosen only to distinguish this system from liquid o/w ME. The gel-like ME still consists of droplets of oil in water, but the higher content of water allows strong hydration of the hydrophilic chains of surfactant. Hydrated chains are connected by hydrogen bonds, resulting in strong interactions between droplets [17], which is expressed by slightly increased dynamic viscosity and can also be seen from Fig. 1 as increased viscosity at higher shear rates. On the basis of the rheological behaviour at room temperature, gel-like ME was shown to be appropriate for dermal use. When the gel-like ME was exposed to 37 °C in order to simulate the experimental conditions of permeation studies its rheological behaviour changed to that of a low-viscosity Newtonian fluid, indicating the destruction of the gel-like structure. Changes in microstructure due to change of temperature are common phenomena in ME [8].

The more usual way to solve the viscosity problem is to add a suitable thickening agent that can modify rheological behaviour without significant influence on other features of ME, such as stability, transparency and spontaneous formation [20,21]. It has been shown that the addition of 10% colloidal silica does not affect the stability and microstructure of our ME [15] and, as can be seen from Fig. 1, elevated temperature (37 °C) did not significantly affect the viscosity of the system.

4.2. In vitro skin permeation

Antioxidants such as vitamin E and C are known to play a significant role in preventing oxidative stress in skin. However, to provide satisfactory protection they must first cross the *stratum corneum* which acts as permeation barrier. RHE 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 [22]. In spite of these limitations, RHE is an useful model in the study of percutaneous absorption of drugs.

Even though human skin models have been shown to be more permeable than human skin *ex vivo*, they can correctly predict the permeability rank order of compounds with different physicochemical properties [11,12,23].

As it is generally recognised that penetration of drugs into the skin may be improved by selecting the appropriate vehicle [24–26], we evaluated the influence of different formulations on the percutaneous absorption of a lipophilic and hydrophilic antioxidant. The absorption of vitamins from thickened and non-thickened ME was compared to that from solution.

When considering the penetration of a drug from different formulations into the skin, several factors have to be considered: vehicle-drug interactions, influence of the carrier on the RHE, and influence of RHE and receptor solution on the formulation. During the experiment, ME remained clear and transparent. There was no visual indication that water from the receptor compartment influenced the ME, which is not surprising, as a relatively large amount of formulation was used as a donor. On the contrary, our results clearly show that the vehicle plays an important role in the skin absorption of the two vitamins under investigation.

4.2.1. Vitamin C

Vitamin C from aqueous solution accumulated in epidermis but only a small quantity was found in collagen, proving that the keratinocytes provide an efficient barrier for this hydrophilic vitamin. The incorporation of vitamin C in the ME resulted in permeation of this vitamin across the RHE, which can be attributed to the large amount of surfactants in the formulation that facilitated the passage of vitamin, mostly by compromising the barrier function of RHE, as observed as an increased TEWL. As no difference was seen between the two ME tested for TEWL, it can be concluded that impairment of the barrier function of RHE probably depends on the quantity of surfactants. However, despite all ME containing the same amount of surfactant, significant differences in skin deposition and permeation of vitamin C were observed. In fact, two groups of ME can be distinguished – one that contains water as the continuous phase (o/w and gel-like ME) and other that contains oil as the continuous phase (w/o ME). The absence of differences between o/w and gel-like ME can be explained by the destruction of the gel structure of the latter when exposed to 37 °C, which was proved by rheological measurements. On the basis of solubility of vitamin C in the ME (21.79 mg/g in w/o ME, 85.54 mg/g in o/w ME and 170.4 mg/g in gel-like ME) the more complex partition of vitamin among all the constituents of the vehicle can be suggested, limiting its transfer through the RHE.

The addition of colloidal silica to the ME enhanced the deposition of vitamin C in epidermis, but not in the collagen layer. TEWL measurements showed that the RHE barrier was influenced after the application of formulations containing colloidal silica. The use of colloidal silica as a thickening agent enhances the release rate of a hydrophilic drug from w/o ME [21]. It was assumed that colloidal silica modifies the physicochemical properties of the external phase, thus making the drug diffusion faster. The total amount of vitamin passed in the receptor solution was however smaller in the case of thickened ME, even though the epidermis was more permeable, probably due to the increased viscosity of the vehicle. The addition of thickener significantly increased the viscosity of the formulation, which slowed down permeation of vitamin. Pseudo zero order kinetics was observed in all the permeation profiles. This was expected because of the surplus vitamin C on the donor side. The concentration gradient between receptor and donor phase therefore remained constant, resulting in constant flux [27]. The steady-state flux was determined from the linear part of the permeation curve (Table 3) for all samples except for w/o ME where the duration of experiment was too short for its determination. As expected, it was higher in the case of non-thickened ME. No significant difference was observed between gel-like and o/w ME. There is also a difference between the two thickened systems; when vitamin C is in the inner phase, its steady-state flux decreases because the external oil phase presents a barrier to the diffusion of drug molecule.

The permeation experiment with an aqueous solution of colloidal silica revealed the impact of the selected thickener on the skin absorption of vitamin C. It confirmed that the increased concentration of vitamin C in the keratinocyte layer is primarily due to the presence of colloidal silica. The behaviour of vitamin C, which is a weak acid with pK_a 4.2, is also affected by the pH of the formulation. The addition of colloidal silica to o/w ME changed the pH from 4.98 to 4.34 which could have changed the degree of ionisation in the favour of the non-ionised form that can more easily penetrate into the epidermis. The effect of silica also appears to be concentration dependent, as 5% concentration increased the concentration of vitamin C in the RHE, as compared to non-thickened o/w ME, but its concentration in the keratinocyte layer was lower than in the case of ME thickened with 10% silica. However, increasing the concentration of the thickener above 10% did not increase the efficacy of the formulation, probably due to the high viscosity of the formulation thickened with 15% of silica.

4.2.2. Vitamin E

In the *in vitro* skin permeation studies across RHE, generally no vitamin E was found in the receptor solution, the exception being ME containing colloidal silica. Either vitamin E did not permeate the skin or the amount was below the limit of detection of HPLC. Moreover, the extraction procedure did not allow 100% extraction of vitamin E from the receptor solution. Probably, the interaction of vitamin E with albumin was not completely eliminated by the single extraction with methanol.

It is known that, *in vivo*, dermis is more likely to be a barrier for lipophilic substances than the keratinocyte layers [28]. Generally, transport experiments with lipophilic substances through RHE might suggest a higher transport through the skin than it actually is, because of the lack of dermis [11]. The Episkin model is an exception, as it has a dermal substitute in the form of a collagen layer. Our results show that vitamin E could pass into the collagen layer, but remained blocked in this layer for the majority of formulations tested (solution and non-thickened ME). The poor solubility of vitamin E in water does not favour its partitioning from collagen to receptor fluid. We can conclude that, in the binding of vitamin E to the collagen, the latter behaves as an additional receptor compartment that results in reduced drug permeation through the model. These results are in accordance with the penetration studies of vitamin E from a lipophilic vehicle into rabbit skin [29]. A significant penetration of vitamin E from isopropyl myristate solution into RHE was observed. This is not surprising, since isopropyl esters of fatty acids enhance the skin permeation of drugs [30]. The incorporation of vitamin E in the outer phase of ME further increased its deposition in the collagen. This could be due to the impairment of the barrier function of keratinocytes by the surfactants. Surprisingly, the concentration of vitamin E in the collagen did not reach that in the solution, using either o/w ME or gel-like ME. In both cases, vitamin E was trapped in droplets of the inner phase, so it first had to be released from the internal to the external phase. Vitamin E is insoluble in water, so it cannot partition freely from the inner oily phase of ME into the aqueous continuous phase that is in contact with RHE. The vitamin E concentration in the collagen was slightly greater for gel-like ME than for o/w ME. Lipophilic vitamin E can be solubilised in the oily phase of the formulation and probably also in the surfactant film and micelles. As gel-like ME contains a smaller percentage of oil in the formulation (10%) than o/w ME (25%), the affinity of vitamin E for the former is less and its diffusion from the vehicle is thus facilitated [25]. The addition of colloidal silica to the formulation resulted in permeation of vitamin E across RHE, which is facilitated by at least two factors. The first is influence on the barrier function of RHE, and the second the changed partition coefficient (P_K) of vitamin E. The log P_K for isopropyl myristate/water is 4.82 as opposed to that for isopropyl myristate + colloidal silica/water which is 2.46 (unpublished data), indicating the higher affinity for the hydrophilic phase of vitamin E incorporated into oily solution containing colloidal silica.

5. Conclusion

The present study shows that drug-vehicle interactions, as well as properties of RHE, change on the incorporation of drugs into different formulations and that the penetration characteristics and permeation profile of a drug can consequently be altered. The percutaneous absorption of vitamins C and E from ME was, in general, enhanced relative to that from aqueous solutions. The incorporation of vitamin C or E in the outer phase of ME resulted in greater absorption than that when vitamins were in the inner

phase, meaning that, depending on the hydrophilicity of the drug, the same formulation can act as a permeation enhancer or retarder. However, according to our results vitamin C was less affected by its location in the vehicle. Consequently, w/o ME appears to be better vehicle for simultaneous delivery of vitamins C and E than o/w ME into RHE. Colloidal silica enhanced the deposition of vitamins E and C in the RHE, not only by perturbation of its barrier function but also by changing the pH of the vehicle and hence the affinity of vitamin C for vehicle. Moreover, the addition of colloidal silica decreased the steady-state flux of vitamin C, which is probably related to the increased viscosity of the thickened formulation.

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