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Stability, cutaneous delivery and antioxidant potential of a lipoic acid and α -tocopherol co-drug incorporated in microemulsions

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Abstract

The aim of this study was to assess the skin penetration, stability and antioxidant effects of a α -tocopherol-lipoic acid co-drug. To enhance penetration, we evaluated three microemulsions varying in water content and composition of the oil phase (isopropyl myristate with either monicaprylin or oleic acid). The co-drug was incorporated at 1% (w/w). Co-drug hydrolysis in the microemulsion increased with increases in time (up to 48 h) and formulation water content (10–30%, w/w). Microemulsions increased the co-drug delivery into viable layers of porcine ear skin by 2.9–7.8-fold compared to a control formulation (20% monicaprylin in isopropyl myristate) after 24 h. Penetration enhancement was influenced by the oil phase, with the formulation containing monicaprylin displaying the most pronounced effect. Antioxidant activity, assessed in skin bioequivalents using the thiobarbituric acid-reactive substances (TBARS) assay, demonstrated that TBARS levels decreased by 39% after treatment with the co-drug-containing microemulsion compared to the unloaded formulation. In addition to the co-drug, tocopherol ($8.2 \pm 0.6 \mu\text{g}/\text{cm}^2$) was detected in the viable bioequivalent tissues, suggesting that the co-drug was partly hydrolyzed after 12 h. Taken together, these results support the potential of nanodispersed formulations containing a tocopherol-lipoic acid co-drug to improve skin antioxidant activity.

Keywords

skin; α -tocopherol; lipoic acid; transdermal; microemulsion; antioxidant; co-drugs; permeation enhancers; formulations

Introduction

It is well known that continuous exposure to UV radiation results in skin injury. Even though the UV-induced mechanisms leading to these injuries are not fully elucidated, radiation stimulates the generation of reactive oxygen species, which in turn mediates oxidative reactions leading to skin damage, ageing and favoring the development of tumors¹. Cutaneous delivery of antioxidants is a valuable strategy to improve skin protection, especially when combining compounds capable of acting synergistically. α -

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Tocopherol, one of the most studied non-enzymatic antioxidants for topical application², synergizes with lipoic acid to reduce oxidative damage in brain, cardiac ischemia and oxidation of low-density lipoproteins^{3–5}. In a previous study, we demonstrated that topical application of lipoic acid and tocopherol synergistically increased the antioxidant activity in the skin, suggesting the potential of this combination to increase cutaneous protection⁶.

To take full advantage of the synergism among these antioxidants, it is necessary to ensure their co-localization in viable layers of the skin. In this study, we employed the co-drug approach, and synthesized a co-drug of α -tocopherol and lipoic acid (Figure 1) to ensure the co-localization of the compounds. There are two main advantages related to this approach. First, α -tocopherol and lipoic have distinct molecular weight (MW) and lipophilicity: MW of 431 and 206, and calculated logP= 7.8 and 2.3 for α -tocopherol and lipoic acid, respectively^{7–9}. These differences make their delivery and co-localization in viable skin challenging: while lipoic acid's properties are more suitable for cutaneous delivery, α -tocopherol is prone to be retained within the stratum corneum due to its high lipophilicity. Consequently, it is not enough to use a formulation capable of incorporating drugs with variable physicochemical properties; it is necessary to consider the effects of formulation composition and properties on the delivery of each compound. In our previous study for example, co-localization of lipoic acid and α -tocopherol in viable skin layers was achieved only after a careful formulation design, and enhancement of tocopherol delivery was considerably more pronounced compared to lipoic acid⁶. Thus, formulation design for the co-drug (instead of the individual antioxidants) may be simplified. Second, considering that the group related to the antioxidant effect of tocopherol is masked (being bound to lipoic acid) until the co-drug undergoes hydrolysis, part of the co-drug delivered into the skin might function as a depot.

To ensure an efficient cutaneous delivery, the co-drug was incorporated in microemulsions. Our goal was to identify a microemulsion in which the co-drug was stable (i.e. hydrolysis was not pronounced), and at the same time, was capable of delivering sufficient amounts of the co-drug into the skin to increase its antioxidant activity. To reach this goal, we (i) first assessed the influence of the aqueous content and oil phase composition on the stability and cutaneous delivery of the co-drug, and (ii) evaluated whether a selected microemulsion lowered the levels of thiobarbituric acid-reactive substances in skin equivalents as a measure of its ability to increase the tissue antioxidant potential, and thus, skin protection.

Material and Methods

Materials

Propylene glycol (PG), oleic acid, isopropyl myristate, α -tocopherol, lipoic acid, ferrous sulfate, thiobarbituric acid and trichloroacetic acid were obtained from Sigma (St. Louis, MO, USA). Monocaprylin was a kind gift from Abitec Corporation (Janesville, WI, USA). Acetonitrile, ethanol and methanol were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA) and decylglucoside was a kind gift from BASF (USA).

Methods

Co-drug synthesis—The ester co-drug was synthesized by covalently coupling the phenolic functionality of α -tocopherol and the carboxylic acid group of lipoic acid. A solution of dicyclohexylcarbodiimide (DCC, 1.1 mmol) in dichloromethane (10 mL) was added in 5 min to a solution of α -tocopherol (1 mmol), lipoic acid (1.25 mmol) and dimethylaminopyridine (0.8 mmol) in dichloromethane (10 mL) under N_2 . After the addition was completed, the cooling bath was removed and the reaction was stirred overnight. The solvent was evaporated and the crude reaction mixture was purified by flash column chromatography on silica gel using hexane:ethyl acetate at 100:0–98:2–96:4 (v/v) to afford the pure product as a yellow viscous oil. The co-drug was characterized by APCI-MS, 1H and ^{13}C -NMR, and IR spectroscopy. The yield was 90%. APCI-MS (m/z): 619.41 (M^++1). Anal calcd for $C_{37}H_{62}O_3S_2$ (618.41): C, 71.79; H, 10.10; O, 7.75; S, 10.36. IR (neat): 2926 cm^{-1} , 2865 cm^{-1} , 1758 cm^{-1} . 1H NMR ($CDCl_3$): δ 3.65–3.55 (1H, q), 3.2 (2H, t), 2.65–2.54 (4H, m), 2.51–2.4 (2H, m), 2.1 (3H, s), 1.95 (3H, s), 1.94–1.89 (2H, m), 1.85–1.71 (5H, m), 1.65–1.45 (6H, m), 1.45–1.32 (10H, m), 1.15–1.31 (4H, m), 1.24 (3H, s), 1.14–1.0 (5H, m), 0.89 (3H, s), 0.85 (3H, s), 0.81 (3H, s); ^{13}C NMR ($CDCl_3$): δ 172.13 (CO, ester), 149.98, 145.02, 126.73, 124.92, 123.14, 117.48, 74.98, 56.44, 40.34, 39.49, 38.61, 37.51, 36.49, 34.76, 33.99, 32.89, 32.33, 29.05, 28.09, 28.01, 24.99, 24.93, 24.91, 24.55, 24.31, 22.83, 22.74, 20.72, 19.86, 19.80, 13.11, 12.27, 11.94.

Pseudo-ternary phase diagram construction and sample preparation

Ternary phase diagrams were constructed using the water titration method at room temperature. Decylglucoside (DG) and Centromix E (CT, also known as Solec, a commercial mixture of soy lecithin, ethoxylated mono-diglycerides and propylene glycol with a HLB value of 12) were chosen as surfactant blend based on the low irritation potential of individual components. Initially, they were mixed and used at 1:2 (w/w) (supplementary material), but due to the low aqueous incorporation, propylene glycol was included in the blend. As oil phase, mixtures of isopropyl myristate with monocaprylin or oleic acid (1:1, w/w) were selected due to their penetration-enhancing properties. An additional reason for employing isopropyl myristate is the previously reported increased stability of pro-drugs in this solvent¹⁰. The oil phase and surfactant blend were mixed at 1:9 to 9:1 ratios (w/w), titrated at room temperature with water under vortexing, and formulations that were fluid, clear, and did not undergo phase separation were assigned to a monophasic region in the phase diagram.

Based on the phase behavior of mixtures and dimensions of the monophasic region in phase diagrams, surfactant-co-surfactant blends composed of DG:CT:propyleneglycol at 1:1:1 (w/w/w) were selected and mixed with isopropyl myristate:monocaprylin (1:1, w/w as oil phase) at a 1:1 ratio for the preparation of three microemulsions: ME10-MC, ME20-MC and ME30-MC (containing 10%, 20 and 30% of water, respectively). A fourth formulation containing oleic acid instead of monocaprylin and 20% of water (ME20-OA) was also selected to study the influence of the oil phase composition on the co-drug skin penetration. The co-drug was added at 1% (w/w).

The isotropicity of the selected formulations was confirmed by polarized light microscopy (Axiotop, Zeiss, Thornwood, NY). Internal phase diameter was determined using a Zetasizer nano series instrument (Malvern, Westborough, MA) at room temperature. To evaluate the internal structure of the systems, electrical conductivity of samples composed of surfactant blend:oil (1:1, w/w) and variable amounts of water (1–42%, w/w) was determined at $25\pm0.5^{\circ}\text{C}$ (Jenway 4520 Conductivity/TDS meter, Techne Inc., Burlington, NJ). Water was added at 5% increments until microemulsions were transformed into turbid systems, and conductivity was measured after each addition. To further evaluate phase transition regions, changes on viscosity as a function of water content were studied. The rheological behavior and viscosity of formulations containing monicaprylin or oleic acid and 10–40% of water were determined using a R/S Plus controlled stress rheometer with cones RC75-1 and RC50-1 (Brookfield Engineering Laboratories, Middleboro, MA), and a Julabo F26MA bath/circulator for temperature control (25°C). All formulations displayed a linear relationship between rate of shear (to 500 s^{-1}) and shear stress consistent with Newtonian behavior. As the viscosity of Newtonian systems is constant at the same temperature, the final viscosity was determined by averaging viscosity values at individual rates of shear.

Drug stability in microemulsions

Because tocopherol and lipoic acid are bound through an ester bond to form the co-drug (which may be hydrolyzed in the presence of water), we assessed the co-drug content after its incorporation in microemulsion, and whether the aqueous content (10–30%) had any influence on the co-drug stability. Microemulsions with an aqueous content between 10 and 30% with and without the co-drug (1%) were kept in sealable glass vials protected from light for 1, 24 or 48 h. Following, they were diluted with acetonitrile to obtain solutions with a theoretical co-drug concentration of 0.3 mg/mL, filtered, and assayed by HPLC.

In vitro skin penetration

Skin penetration of the co-drug was studied using Franz diffusion cells (diffusion area of 1 cm^2 ; Laboratory Glass Apparatus, Inc, Berkeley, CA) with porcine ear skin (obtained from a local slaughterhouse) serving as model tissue. The ears were obtained immediately after the animals were killed. After transportation to the laboratory in coolers, they were rinsed with water, blotted dry with paper tissue, and the skin was excised with scissors and scalpel. Tissue sections were frozen (-80°C) and used for permeation assays within 1 month. No steam-cleaning was performed on the ears, and the stratum corneum was intact after tissue excision. Integrity of the tissue was confirmed by histological analysis of every shipment of ears obtained (Supplementary Figure 1 depicts a skin section to confirm integrity). The ears were considered a waste product of food production, and their use did not require ethical approval by the College's Institutional Animal Care and Use Committee. A receptor phase consisting of PBS containing ethanol (10% v/v) was used and maintained at 37°C under constant stirring (300 rpm). Ethanol was added to aid the co-drug solubility in aqueous medium, which increased from close to the quantification limit of the method to $24.8\text{ }\mu\text{g/mL}$. Microemulsions (100 mg) were placed in the donor compartment of diffusion cells for up to 24 h. This period of time was selected based on a previous report showing no significant alteration in tritiated water flux across the skin during this period of time when ethanol was included in the receptor phase (up to 33%), which indicates that the skin barrier was not

compromised¹¹. Co-drug solutions in isopropyl myristate with 20% monocaprylin were used as control formulations. This concentration was chosen based on the amount of the monoglyceride in the selected microemulsions (20 to 22.5%, w/w). At the end of the experiment, skin samples were wiped to remove excess formulation, and the stratum corneum (SC) was separated from the epidermis (E) and dermis (D) by tape stripping, using 13 pieces of tape. The first piece was discharged and the others were placed in conical tubes containing 4 mL of acetonitrile:dichloromethane (9:1, v/v). The remaining skin (viable epidermis + dermis, ED) was cut in small pieces, placed into conical tubes containing 5 mL of acetonitrile:dichloromethane, homogenized using a hand-held homogenizer (Biospec products, Bartlesville, OK) and bath sonicated for 30 minutes. Samples from SC, ED and aliquots of the receptor phase were assayed by HPLC. The concentrations of drugs in SC and ED were used as indices of cutaneous delivery.

To standardize the extraction of the co-drug from the skin, untreated tissue sections (1 cm²) were subjected to the tape stripping procedure. Following, the tapes containing SC and the ED sections were spiked with the control formulation (400 mg); the extraction solvent was added and extraction was performed as described. Recovery of the co-drug from the SC and ED was 74.8 and 71.8%. Leaving the co-drug in contact with the skin for 24 h before addition of the extraction solvent and homogenization did not significantly ($p > 0.05$) affect the recovery.

Microemulsion effect on skin barrier

Transepidermal water loss was assessed as an index of the ability of microemulsions to induce changes on the cutaneous barrier depending on their composition^{12,13}. We acknowledge that an accurate differentiation of changes on transepidermal water loss caused by molecular disruption of the barrier and possible occlusion by the formulation is a difficult task, and would require a combination of techniques involving quantification of the penetration of formulation components in the skin and changes on the stratum corneum composition and organization. Previous reports have shown that propylene glycol, isopropyl myristate, oleic acid and glycerides of caprylic acid affect the structural organization of biological membranes (including the stratum corneum) as evidenced by NMR, neutron diffraction and attenuated-total-reflectance Fourier transform infrared (ATR-FTIR) spectroscopy^{14–16}. This suggests that their effect (as well as the effect of formulations in which they are used) is not restricted to providing occlusion. Consequently, it seems reasonable to assess changes on transepidermal water loss to compare the ability of formulations to induce changes in the cutaneous barrier.

We had three goals with this experiment: (i) to evaluate whether variations in composition affected the formulation's ability to disrupt the cutaneous barrier function, (ii) to evaluate whether the structural organization of microemulsions was relevant to this effect (or whether solutions of the enhancers monocaprylin or oleic acid in isopropyl myristate would have a similar effect), and (iii) to evaluate when changes on skin permeability could be detected. Based on the co-drug penetration profile, ME20-MC and ME20-OA displayed an enhancement effect at 6 h. Thus, we hypothesized that changes on the skin permeability

might be detected around this period of time, and searched for changes on transepidermal water loss at time points preceding (4 h) and following (8 h) the 6-h period.

Transepidermal water loss was measured before and after treatment with unloaded (not containing the co-drug) ME20-MC, ME20-OA or control vehicles (isopropyl myristate containing 20% of either monocaprilin or oleic acid) using a closed chamber evaporimeter (Vapometer, Delfin Technologies Ltd., Kuopio, Finland) equipped with an adaptor to fit the diffusion cell (Hanson Research, Chatsworth, CA, diffusion area of 1.7 cm²) opening¹³.

Skin sections were mounted in diffusion cells and treated with 250 mg of the formulations for 5 minutes, 4 and 8 h. After each period, skin samples were carefully wiped with tissue paper to remove the formulations, placed in diffusion cells and left undisturbed for 15 minutes before measuring the transepidermal water loss¹⁴. We did not rinse the skin since preliminary results from our laboratory and other reports have demonstrated influence of rinsing on transepidermal water loss^{17,18}. We acknowledge that tissue wiping may alter the barrier integrity, and the measurement at 5 minutes was performed to take into consideration any impact of the wiping. Results were expressed as *transepidermal water loss*, calculated as the difference between transepidermal water loss values after treatment (4 or 8 h) and 5 minutes.

Antioxidant activity

The antioxidant activity of co-drug-treated skin was evaluated using the thiobarbituric acid-reactive substances assay (TBARS assay)^{6,19}. Because the co-drug was designed to release the antioxidants after hydrolysis in the skin, the antioxidant activity was evaluated in viable bioengineered skin equivalents (EpiDerm, MatTek, Ashland, MA). The skin equivalent model was chosen because its metabolizing capacity appears to be representative of human skin²⁰. The level of TBARS after ferrous sulfate-induced damage in microemulsion-treated tissue was quantified and normalized to the levels in untreated tissue. By measuring TBARS levels, we have an indirect estimate of reactive oxygen species generated in the tissue¹⁹. Skin equivalents were treated for 3 h with ME20-MC (50 mg); following, the formulation was carefully removed (with the help of sterilized cotton tips), and tissues were left in the incubator at 37° C and 5% CO₂ for additional 9 h. We could not rinse the tissues as contact with water could induce co-drug hydrolysis, leading to an overestimation of α -tocopherol content. We opted to remove the formulation after 3 h of exposure since previous studies from our group showed significant changes on tissue viability after exposure to microemulsions (compared to PBS) for longer periods of time²¹.

Two sets of tissues were used. Both were treated with unloaded microemulsion and the microemulsion containing the co-drug. One set was used for quantification of the co-drug and free tocopherol by HPLC; the other set was subjected to the antioxidant assay in which the homogenates were combined with ferrous sulfate to initiate the lipid peroxidation reaction for 30 minutes at 37° C with mixing. Following, 40% trichloroacetic acid was added, and tubes were centrifuged at 15,000 rpm for 5 minutes. The supernatant was incubated with thiobarbituric acid solution (1%) at 90° C for 20 minutes, and samples were analyzed by spectrophotometry at 532 nm using a standard curve generated from malondialdehyde (5–500 μ M)⁶.

Quantification of the co-drug and tocopherol

High performance liquid chromatography was selected for quantification of the co-drug and tocopherol using a Shimadzu HPLC equipment (Prominence series, LC-20AB dual pump, SIL-20A autosampler, SPD-M20A photodiode array detector and Class-VP software). Separation was performed using a C8 column (87nm, XBridge™, 4.6 mm x 150 mm, 3.5µm) equipped with a C8 precolumn, and isocratic mobile phase consisting of acetonitrile:methanol:dichloromethane (60:35:5, v/v) at 0.75 mL/min. The quantification limit of the method was 500 ng/mL, and linearity up to 340 µg/mL was observed.

Statistical analyses

The results are reported as means $\pm$ SD. Data were statistically analyzed using the ANOVA test followed by Tukey post-hoc test (GraphPad Prism software). Values were considered significantly different when $p < 0.05$. To evaluate the relationship between time, water and co-drug content in stability studies, a contour plot was created from a dataset that included individual values recorded at 1, 24 and 48 h. The dataset was interpolated on a XYZ plane using Origin Pro 9.0 (Origin Lab, Northampton, MA).

Results

Microemulsion formation and phase diagrams

Isotropic, single phase and fluid formulations consistent with microemulsions were assigned to the monophasic dark-shaded region in the pseudo-ternary diagrams in Figure 2. The percent area of this region in relation to all phases displayed was influenced by the ratio between surfactant/co-surfactant and type of oil phase. The dark area corresponded to 25% when surfactant mixtures containing decylglucoside and CT (DG:CT, 1:2 w/w) were used (Supplementary Figure 2), with a maximum of approximately 35% of water being incorporated, leading us to consider propylene glycol addition into the surfactant blend to increase water incorporation²². The area increased to 52% when propylene glycol was included in the surfactant blend at the same amount as the other components (DG:CT:PG 1:1:1), but reduced to 37% when its ratio doubled (Figure 2, panels A and B). These results suggest the existence of optimum ratios of propylene glycol in the blend. To maximize the area of microemulsion formation, a ratio of 1:1:1 (DG:CT:PG) was chosen.

Replacing monacapylin with oleic acid decreased the isotropic and fluid dark-shaded area from 52 to 34% (Figure 2C), which might be attributed to a decrease in the polarity of the oil phase: while oleic acid was attributed an approximate HLB value of 1, the HLB value of monacapylin is 8^{23,24}. This is not surprising as other studies reported similar findings when systems containing monoglycerides, triglycerides or other less polar oils were compared^{23,25}. The water solubilization capacity of surfactants seems greater when lower molecular weight and more polar components are used²⁵.

Microemulsion characterization and selection

To gain a better understanding of the influence of water content on the internal structure, electrical conductivity was measured. DG:CT:PG at 1:1:1 (w/w/w) were mixed with the oil phase (isopropyl myristate with monacapylin or oleic acid, 1:1 w/w) at a 1:1 (w/w) ratio,

and water was added in small increments. The gray lines in phase diagrams (Figure 2B and C) represent the dilution lines used to study conductivity changes. As can be observed in Figure 2D, conductivity of monocaprylin-containing systems increased modestly until 10% of water was added, suggesting w/o structure. Water addition between 12 and 30% led to a sharp rise in conductivity values, suggesting an increase in aqueous droplets' interlinking process and emergence of bicontinuous structures^{26,27}. Further addition led to a less steep rise in conductivity until system destabilization occurred with 41% of water. This limit for water incorporation is consistent with the diagram depicted in panel B (Figure 2). The percolation threshold was further confirmed in the plot of $d\text{conductivity}/d\text{water}$ as a function of water content, which exhibited maxima at 21% water (Figure 2F). In systems containing oleic acid in the oil phase, conductivity increased more sharply with water over 15% (Figure 2E), but stability loss occurred with much less water (26%) compared to the system containing monocaprylin, and the percolation threshold could not be confirmed.

To further evaluate phase transition regions, we assessed changes on viscosity as a function of aqueous content. Clustering of droplets at the percolation threshold generally leads to an increase in viscosity, while transformation into water-continuous systems is often accompanied by a decrease in viscosity^{28,29}. A slight increase in viscosity was observed in systems containing monocaprylin as water increased to 20% (which coincides with the maxima in the $d\text{conductivity}/d\text{water}$ plot, Figure 2F). Viscosity decreased as water increased further (to 40%). Taken together, results from conductivity and viscosity assays suggest formation of bicontinuous and o/w systems with water at 20 and 40%, respectively. On the other hand, formulations containing oleic acid with 10 and 20% of water presented similar values of viscosity (Figure 2E), and further water addition was not possible due to system destabilization. Thus, no conclusion could be drawn regarding regions of structure transformation in oleic acid-containing formulations.

Based on these studies, three microemulsions containing monocaprylin were selected: ME10-MC, ME20-MC and ME30-MC containing 10, 20 or 30% of water, respectively. Based on conductivity and viscosity plots, ME10-MC is most likely w/o, whereas the structures of ME20-MC and ME30-MC are most likely consistent with bicontinuous systems. Since these are not theoretically organized as dispersed droplets (even though they undergo continuous fluctuations with water- or oil- rich domains)^{30,31}, only the internal phase diameter of ME10-MC was determined, and found to be 58.0 ± 3.8 nm. One oleic acid-containing microemulsion with 20% of water (ME20-OA) was also selected for comparison of the effect of oil phase composition on co-drug penetration. Its internal structure could be consistent with both w/o and bicontinuous systems. Thus, internal phase diameter was not determined. All formulations were isotropic under the polarized light microscope.

Drug stability in microemulsions

Considering that tocopherol and lipoic acid are bound through an ester bond to form the co-drug, its hydrolysis in water-containing microemulsions is possible. To evaluate whether the co-drug is hydrolyzed after its incorporation in the microemulsion and the influence of water content (10–30%) on co-drug stability, we performed a short-term stability study. The

interplay between aqueous content, time and co-drug remaining in the formulation is represented in the contour plot depicted in Figure 3, with the axis representing the variables water and time, and the contour, specific levels of the co-drug. Larger co-drug content (darker regions) can be observed with reduced water fraction and time.

One hour after formulation preparation, co-drug content varied from 80 to 93%, with the content decreasing as water increased. A drop between 4 to 7% on drug content was observed at 24 h post-preparation, and an additional decrease between 0.6 (for ME10-MC) and 2% (for the others) occurred at 48 h, suggesting that degradation was more pronounced within the first 24 h independently on the aqueous fraction. No significant ($p > 0.05$) changes on co-drug content were observed in surfactant-oil phase mixtures or in the control formulation (data not shown), indicating that degradation most likely results from contact with water in the formulation. Because the co-drug content was the lowest in ME30-MC and the same components ratio did not produce a stable formulation using oleic acid in the oil phase (see phase diagrams), only ME10-MC, ME20-MC and ME20-OA were further investigated as cutaneous delivery systems. For all subsequent experiments, microemulsions were used right after preparation.

Skin Penetration

Co-drug penetration into the stratum corneum (SC) and viable layers (viable epidermis and dermis, ED) is shown in Figure 4. Penetration increased with time, and more co-drug was detected in the stratum corneum than in viable skin layers.

All microemulsions were more efficacious than the control formulation (20% monicaprylin in isopropyl myristate) to deliver the co-drug into the skin. At the last time point (24 h) co-drug delivery to the SC and ED increased 2- and 2.9-fold ($p < 0.05$), respectively, using ME10-MC compared to the control formulation. ME20-MC was more effective, delivering 3.5- and 7.8-fold more co-drug in SC and ED respectively, which suggests that increasing water content enhanced penetration. When monicaprylin was replaced by oleic acid (ME20-OA), a less pronounced enhancement of penetration in both SC (2.5-fold) and ED (5-fold) was observed. This result agrees with previous studies on the importance of the choice of oil phase for penetration^{21,32}. Co-drug in the receptor phase was below the limit of quantification of the method (0.5 $\mu\text{g/mL}$). Considering that its solubility in the receptor phase was approximately 50-fold higher than the quantification limit, and that at 24 h, the maximum amount of drug delivered into viable skin layers was $17.7 \pm 1.8 \mu\text{g/cm}^2$ (which would correspond to approximately 6 $\mu\text{g/mL}$ of drug in the receptor phase if 100% of this amount would permeate into the later), it is reasonable to suggest that transdermal delivery was not restricted by co-drug solubility in the receptor phase.

Comparing the penetration rates in whole skin (sum of SC and ED), the maximum rate occurs at or before 6 h for all formulations including the control, suggesting that penetration is relatively fast and that neither water content nor the type of oil phase modified the time necessary to reach the maximum rate (Figure 5A). The highest maximum rate was observed for ME20-MC, which is consistent with the largest amount of co-drug delivered at all time points by this formulation.

Microemulsion effect on skin barrier

To verify whether differences in penetration could be attributed to an oil phase-dependent effect of the microemulsion on the skin, we assessed differences in transepidermal water loss after 4 and 8 h of treatment induced by ME20-MC and ME20-OA. As can be observed in Figure 5B, no difference was observed in the *transepidermal water loss* comparing formulations containing oleic acid or monicaprylin at 4 or 8 h.

In the same experiment, we also evaluated whether the structural organization of microemulsions was relevant for tissue permeability or whether a solution of the enhancers monicaprylin and oleic acid in isopropyl myristate would have a similar effect. No significant difference was observed at 4 h, but at 8 h, the *transepidermal water loss* resulting from microemulsions treatment was significantly ($p < 0.05$) higher. Considering that microemulsions increased co-drug penetration at 6 h, the fact that no significant changes on transepidermal water loss were observed at 4 h comparing microemulsions and control solution suggests that changes on cutaneous barrier may be subtler (and not detectable by the technique) before 6 h. Additionally, other mechanisms might contribute to the penetration-enhancing effect of microemulsions; drug solubility and retention in the microemulsions and solutions might be different, which in turn affect delivery³³.

Antioxidant activity

Having determined that ME20-MC delivered the largest amount of co-drug into the skin, we next determined whether treatment of skin equivalents with this formulation increased the tissue antioxidant activity. As control, a blank (not containing co-drug) microemulsion was used. As can be observed in Figure 6, $39.3 \pm 4.0\%$ reduction on TBARS levels was observed when tissues were treated with the co-drug-containing microemulsion, suggesting the protective potential of the co-drug administration. We found 13.4 ± 1.6 and $8.2 \pm 0.6 \mu\text{g}/\text{cm}^2$ of co-drug and α -tocopherol, respectively in viable skin equivalents after the total incubation period. The amount of tocopherol in 50 mg of formulation (amount applied to the tissue) 3 hours after its preparation was 20.3 μg .

Discussion

Co-drugs are considered more advantageous compared to pro-drugs since all compounds chemically or enzymatically released are active³⁴. This is especially interesting for drugs displaying a synergistic effect. Examples of co-drugs developed for delivery to the skin include 6- β -naltrexol-hydroxybupropion for drug addiction, vitamin E-vitamin C for enhancement of tissue antioxidant potential, and azelaic acid-salicylic acid for acne^{35–37}. However, the resulting molecular weight and solubility properties of the co-drug might make its delivery across biological barriers more challenging compared to the parent compounds. The co-drug studied here is lipophilic and has a molecular weight superior to the optimum range associated with efficient skin penetration³⁸. To improve co-drug delivery into viable skin layers was the main reason why we used microemulsions.

An additional reason relates to previous observations that microemulsions might provide drug protection against degradation^{39–41}. However, co-drug content in microemulsions

decreased with increases in water fraction and time, indicating that the formulations studied could not provide a similar level of protection against hydrolysis as the aqueous-free vehicle (the control formulation). Previous reports suggest that formulation-mediated protection seems to be more pronounced for drugs incorporated within the surfactant interface^{39–41}. Even though no studies to determine drug location in the structure were undertaken, previous results from our group have demonstrated that lipophilic drugs seem to prefer the oil phase of microemulsions containing monicaprylin⁴². Since co-drug content was the lowest in the formulation with 30% of water, and the same components ratio did not produce stable microemulsions if oleic acid was used, only formulations containing 10 and 20% of water were further investigated for cutaneous delivery.

Even though co-drug hydrolysis increased with addition of water in formulations, the skin penetration also increased. This can be observed in Figure 4, in which the skin penetration of the co-drug in a control formulation without water (isopropyl myristate solution containing the penetration enhancer monicaprylin) and in microemulsions was compared. These results agree with previous reports demonstrating enhancement of skin penetration of lipophilic compounds due to increases in formulation aqueous content, possibly due to changes on drug solubility (and thermodynamic activity) and formulation-mediated skin hydration^{30,43} (relevant since water is considered an enhancer)⁴⁴. Maintaining co- and pro-drugs stability has been challenging in other studies as well^{35,45}. Ideally, the optimized formulation should provide a suitable balance between co-drug stability and other properties (such as solubility and penetration enhancement). A mixture of phosphate-buffered saline and methanol was selected to serve as donor formulation for retinyl ascorbate co-drugs because it provided a suitable balance between co-drug solubility and low decomposition³⁵. A solution of pH 5.0 was chosen as donor vehicle for a naltrexone pro-drug because it ensured that only a small portion (<10%) of naltrexone coming from pro-drug would be formed in the donor solution during the studied penetration period⁴⁵.

In this study, ME20-MC was selected as the optimized formulation for antioxidant studies because it provided a good balance between co-drug stability and penetration enhancement, maintaining over 80% of the co-drug intact in the formulation during 24 h while delivering 3.5 and 7.8-fold more co-drug (in SC and viable skin, respectively) compared to the control formulation. These results support the relevance of using microemulsions instead of an aqueous-free solution of monicaprylin in isopropyl myristate for co-drug penetration enhancement. One option to optimize co-drug delivery while avoiding stability issues related to presence of water in formulations would be to use a physical method to more efficiently overcome the stratum corneum. As an example, the control solution (in which the co-drug is stable) could be applied after skin treatment with microneedles. The microneedle platform was successfully employed to deliver other drugs and pro-drugs^{45,46}, and may be a useful approach for the co-drug developed here as long as the application area is limited. Extemporaneous formulations and formulations in which water is kept in a separate compartment and mixed before use are other options to maintain co-drug stability during longer periods of time⁴⁷. Examples of a similar design are the multiple chamber nutrition bags (produced by Baxter, USA, for example). For microemulsions, this approach would be feasible since the formulation requires minor energy input (in the form of gentle mixing, for example) to form once oil phase, water and surfactant are placed in contact⁴⁸.

The superiority of the microemulsion compared to the control solution may be related to the presence of other components (in addition to isopropyl myristate and monicaprylin) that can influence the skin barrier. Decylglucoside was not able to induce barrier disruption in our previous study, but phosphatidylcholine and propylene glycol were previously used as penetration enhancers^{21,44}. Additionally, Hathout et al. have previously demonstrated that formulating components in a microemulsion increased their relative uptake into the stratum corneum compared to the pure components, and the more efficient uptake from the microemulsion might imply the possibility of mutual enhancement⁴⁹.

Skin penetration was also affected by the choice of oil phase. It has been suggested that oleic acid is more effective for penetration of hydrophilic compounds, and its penetration-enhancing effect more pronounced in more hydrophilic microemulsions^{30,50}. Since no significant difference on barrier disruption between oleic acid- and monicaprylin-containing systems was detected, the differences in penetration enhancement might be related to the oil phase influence on drug release. Oleic acid is more lipophilic²⁴, and given the fact that the co-drug is also lipophilic, a more pronounced retention in the oleic acid-containing microemulsion is possible.

The reduction in TBARS and presence of tocopherol in viable bioengineered tissues suggest co-drug hydrolysis in the tissue with a consequent increase in the antioxidant potential. An alternative explanation for reduction of TBARS relates to the antioxidant effect of the co-drug *per se*, since the portion corresponding to lipoic acid is exposed in the molecule. However, we have previously observed a much less pronounced ($19.2 \pm 4.9\%$) lipoic acid-induced reduction in TBARS levels in cutaneous tissue after longer exposure periods⁶. Since co-drug hydrolysis was observed in the formulation, one could argue that free tocopherol penetrated in the tissue. However, it is unlikely that almost 50% of the tocopherol present (as result of hydrolysis) in the formulation was transported into skin equivalents within 3 h, especially if we bear in mind previous results showing that 4.2% of the applied dose of tocopherol was delivered into porcine skin by a cationic microemulsion within 12 h⁶. Thus, it is reasonable to suggest that at least part of the co-drug was hydrolyzed in the skin and increased the antioxidant activity of the tissue. Considering the existence of intact co-drug (with protected tocopherol) in the tissue that can form a depot and undergo hydrolysis at longer time points, the co-drug approach might potentially provide longer tissue protection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Representation of the co-drug structure.

Figure 2.

Phase behavior of mixtures containing various surfactant blends, aqueous and oil phases. As oil phases, isopropyl myristate (IPM) was mixed with either monocaprylin or oleic acid (1:1, w/w). Phase diagrams were constructed using DG:DT:propylene glycol (PG) as surfactant blend at 1:1:2 (A) or 1:1:1 (B, w/w/w) with IPM and monocaprylin as oil phase or DG:DT:PG with IPM and oleic acid as oil phase (C). The dark shaded area in phase diagrams represents formation of fluid, transparent, single-phase formulations classified as microemulsions, whereas the grey line represent the dilution line used to study changes on formulation electrical conductivity. Changes on conductivity and viscosity of mixtures of surfactant and oil phase containing monocaprylin (D) or oleic acid (E) at 1:1 (w/w) as a function of aqueous content are also depicted. The percolation threshold of samples containing monocaprylin was confirmed in the plot of dconductivity/dwater as a function of water content (F).

Figure 3. Variations in the content of the co-drug in microemulsions as a function of time and formulation aqueous content. Each point represents average of 3 replicates $\pm$ standard deviation.

Figure 4.

Penetration of the co-drug from control vehicle (isopropyl myristate containing 20% of monocaprylin), ME10-MC, ME20-MC and ME20-OA in the stratum corneum (SC) or viable skin layers (ED) as a function of time. Each point represents average of 3–5 replicates $\pm$ standard deviation.

Figure 5.

Influence of microemulsion composition on skin penetration rates and tissue permeability.

(A): Penetration rates of the co-drug as a function of time; (B): transepidermal water loss induced by treatment with control vehicles, ME20-MC and ME20-OA. Values were calculated as difference in transepidermal water loss after treatment (4 or 8 h) and 5 minutes of formulation application. Each point represents average of 3-7 replicates $\pm$ standard deviation. * $p < 0.05$ compared to skin treated with control vehicles (IPM+MC or IPM+OA).

Figure 6. Influence of microemulsion treatment on TBARS levels in bioengineered cutaneous tissues. TBARS levels were calculated by normalizing levels in microemulsion-treated skin to values in untreated skin. * $p < 0.01$ compared to skin treated with the unloaded (not containing co-drug) formulation.