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Incorporation of quercetin in lipid microparticles: Effect on photo- and chemical-stability

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ABSTRACT

Lipid microparticles loaded with the flavonoid, quercetin were developed in order to enhance its stability in topical formulations. The microparticles were produced using tristearin as the lipid material and phosphatidylcholine as the emulsifier. The obtained lipoparticles were characterized by release studies, scanning electron microscopy and powder X-ray diffractometry. The quercetin loading was 12.1% (w/w). Free or microencapsulated quercetin was introduced in a model cream formulation (oil-in-water emulsion) and irradiated with a solar simulator. The extent of photodegradation was measured by high-performance liquid chromatography. The light-induced decomposition of quercetin in the cream vehicle was markedly decreased by incorporation into the lipid microparticles (the extent of degradation was $23.1 \pm 3.6\%$ for non-encapsulated quercetin compared to $11.9 \pm 2.5\%$ for the quercetin-loaded microparticles) and this photostabilization effect was maintained over time. Moreover, the chemical instability of quercetin, during 3-month storage of the formulations at room temperature and in the dark, was almost completely suppressed by the lipid microparticle system. Therefore incorporation of quercetin in lipoparticles represents an effective strategy to enhance its stability in dermatological products.

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1. Introduction

It is well recognized that the oxidative damage of biological molecules in cells and tissues induced by free radicals plays an important role in many pathological situations such as neurodegenerative disorders, diabetes, autoimmune diseases, cardiovascular diseases, and cancers [1–3]. The oxidative stress is also responsible for skin damage (e.g., skin ageing, tumor promotion, phototoxicity/photosensitivity, cutaneous autoimmune diseases), being mainly the result of increased environmental pollutants and especially exposure to sunlight UV radiation [4–6]. In fact, it has been shown that the UV rays from the sun promote a depletion of the skin antioxidant systems and a marked increase of reactive oxygen species (which include non-radical as well as radical species) and other free radicals [4,6–8]. In order to prevent or reduce the cutaneous adverse effects triggered by the oxidative stress, topical administration of antioxidants has received increased interest [6,8–11]. A further advantage of this strategy is the delivery of antioxidants to the tissue of interest (i.e., the skin)[12].

Flavonoids, a class of polyphenolic compounds widely distributed in plants, are among the most commonly used, naturally occurring antioxidants [3,10–14]. In particular, quercetin is the flavonoid with the highest antioxidant activity [3,6,14,15]. Recently, it has been reported that the topical application of quercetin inhibits oxidative skin damage and the inflammatory processes induced by the solar UV radiation [6,16,17]. An essential requirement to ensure the effectiveness of quercetin as photoprotective agent, is its stability in topical formulations. Although the functional stability (i.e., antioxidant activity) of this flavonoid in dermatological preparations has been investigated [15,17], there are currently no data available concerning the photostability aspect. This is a disadvantage, especially for topical treatments, since the light-induced degradation of quercetin could reduce its protective power against cutaneous photooxidative damage. To explore further the potential therapeutic applications of quercetin, the photochemical behaviour of this flavonoid in topical preparations need to be examined and improved. Accordingly, this investigation focuses on lipid microparticles as biocompatible system for reducing the instability of quercetin during exposure to sunlight.

Lipid microparticles (LMs) are composed of a lipid matrix stabilized by surfactant molecules [18,19]. Their constituents are physiologically compatible, biodegradable and include excipients approved for topical pharmaceutical and cosmetic preparations [18,20]. Additional advantages of LMs are good substantivity (i.e., adhesive properties) for the skin surface and protection of incorporated labile active against degradation [19,21–22].

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The purpose of this study was to produce and characterize LMs loaded with quercetin. The influence of the microencapsulation process on the flavonoid photo- and chemical-stability was examined after incorporation of the LMs in a model formulation (emulsion), suitable for topical application.

2. Materials and methods

2.1. Materials

Quercetin and tristearin were supplied by Fluka (Buchs, Switzerland). Hydrogenated soybean phosphatidylcholine was a gift from Cargill (Hamburg, Germany). The excipients for the cream preparations were obtained from Sigma–Aldrich (Steinheim, Germany) and Henkel (Fino Mornasco, Italy), respectively. Methanol, acetonitrile and water of high-performance liquid chromatography (HPLC)-grade were obtained from Merck (Darmstadt, Germany). All other reagents used were of analytical grade (Sigma).

2.2. High-performance liquid chromatography

The HPLC apparatus comprised a Model LabFlow 3000 pump (LabService Analytica, Bologna, Italy), a Model 7125 injection valve with a 20-μl sample loop (Rheodyne, Cotati, CA, USA) and a Model 975-UV variable wavelength UV–vis detector (Jasco, Tokyo, Japan) set at 370 nm. Data acquisition and processing were performed with a personal computer using Borwin software (JBMS Developpements, Le Fontanil, France). Sample injections were effected with a Model 701 syringe (10 μl; Hamilton, Bonaduz, Switzerland). Separations were performed according to the method of Ishii et al. [23], with minor modifications, using a 5-μm Luna C18 column (150 mm × 4.6 mm i.d.; Phenomenex, Torrance, CA, USA) fitted with a guard column (5-μm particles, 4 mm × 2 mm i.d.) and eluted isocratically, at a flow-rate of 1.0 ml/min, with sodium acetate (pH 3.8; 0.01 M)–acetonitrile (65:35, v/v) containing EDTA (0.5 mM). The mobile phase was filtered through 0.2-μm nylon filters (Alltech Italia, Sedriano, Italy). Chromatography was performed at ambient temperature. The identity of the separated peak was established by co-chromatography with the authentic standard. Quantification was carried out by integration of the peak areas using the external standardization method.

2.3. Lipid microparticle preparation

Unloaded LMs were prepared by adding hot (75 °C) water (50 ml) containing 1.0% (w/v) phosphatidylcholine to melted tristearin (3.6 g) under high-shear mixing (13,500 rpm for 2 min) with an Ultra-Turrax (T25; IKA-Werk, Staufen, Germany) at 75 °C. The obtained emulsion was rapidly cooled under magnetic stirring and the formed LMs were recovered by centrifugation (6000 rpm for 20 min), washed with water and freeze-dried. Quercetin-loaded microparticles were obtained by dispersing quercetin (1.0 g) in the melted lipid phase.

2.4. In vitro release

Quercetin dissolution and release from the microparticles were studied by adding previously sieved (100 μm) quercetin (5 mg) or LMs containing an equivalent amount of the flavonoid to 100 ml of phosphate buffer (50 mM, pH 7.4) containing polysorbate 20 (0.5%, p/p) as solubilizer. The fluid was maintained under stirring (100 rpm) at 37 °C. At fixed time intervals, 1-ml aliquots of the medium were withdrawn and replaced with equal volumes of fresh release medium. The test samples were filtered (0.45 μm membrane filters) and assayed for quercetin by UV spectrophotometry at 370 nm (Lambda 3B, PerkinElmer, Norwalk, USA). Each series of experiments was repeated at least five times.

2.5. Microparticle characterization

Microparticle morphological structure was examined by both scanning electron microscopy (SEM; Cambridge Stereoscan 360, Cambridge Instruments, Bar Hill, UK) and optical microscopy (Nikon Diaphot inverted microscope, Tokyo, Japan). The particle size was determined by computerized image analysis (Micrometrics camera 122 CU and Software Vision 1.0) of at least 150 particles on photomicrographs obtained with the optical microscope.

The powder X-ray diffraction patterns were recorded on a D 5000 powder diffractometer (Siemens, Munich, Germany) using a voltage of 45 kV and a current of 25 mA for the generator, with Cu anode material. The wavelength of the graphite-monochromated radiation was 1.5406 Å. The diffractograms were recorded from 3° (2θ) to 35° (2θ) at an angular speed of 1° (2θ) per minute using 1°–1°–1°–0.15° slits.

The amount of quercetin entrapped in the lipidic microparticles was determined by dissolving the microparticles (25 mg) in ethanol under sonication (15 min). The obtained sample was diluted to volume (10 ml), filtered (0.45 μm membrane filters) and assayed by HPLC, as outlined above. The encapsulation efficiency was calculated as the percentage ratio between the quantity of quercetin entrapped in the microparticles and the amount of flavonoid added to the melted lipid phase. Data were calculated from the average of at least three determinations.

2.6. Photodegradation studies

Photodecomposition experiments were performed in cream preparations (oil-in-water emulsions) containing non-encapsulated quercetin (0.5%, w/w) in conjunction with blank LMs or an equivalent amount of the flavonoid loaded in LMs. The emulsion excipients were: cetearyl alcohol (1.0%), squalene (3.8%), macadamia oil (4.8%) and Polawax® (8%; emulsifying wax based on cetearyl alcohol and ethoxylated fatty acid esters of sorbitan) for the internal phase and glycerin (5.0%), EDTA (0.1%), Phenonip® (0.4%; phenoxyethanol and parabens), ethanol (1.8%) and deionized water (71.0%) for the external phase. The creams were prepared according to the common procedure used in compounding practice [24]. Quercetin (solubilized in ethanol), blank and loaded microparticles (3.6–4.0 g per 100 g of cream, dispersed in water) were added in the cooling phase of the emulsion preparation at about 40 °C. Portions (ca. 40 mg) of the test creams were evenly spread by means of a syringe onto the bottom of a beaker (surface area, 9.6 cm^2) and then irradiated for 2 h with a solar simulator (Suntest CPS+, Atlas, Linsengericht, Germany) equipped with a Xenon lamp, an optical filter to cut off wavelengths shorter than 290 nm and an IR-block filter to avoid thermal effects. The solar simulator emission was maintained at 500 W/m^2. The applied UV energy was equivalent to 10 minimal erythemal dose (MED), which is representative of the solar emission during half-day close to the equator [25]. After the exposure interval, the beaker was removed, its content quantitatively transferred into a 20-ml calibrated flask with ethanol (3× 6 ml) and subjected to sonication (15 min). The resulting sample was adjusted to volume (20 ml), filtered (0.45-μm membrane filters) and analysed by HPLC. The degree of photodegradation was measured by comparing the peak areas of quercetin from the irradiated samples, with those obtained by analysis of an equivalent amount of the non-exposed formulations. The results were the average of at least eight experiments.

2.7. Long-term stability studies

Stability studies were performed on the same formulations utilized for the photodecomposition experiments. The emulsions were stored into stoppered containers for 3 months, at room temperature and in the dark. At appropriate time intervals, aliquots (40–50 mg) were withdrawn from the emulsions and transferred into calibrated flasks (20 ml). The samples were extracted with ethanol under sonication, diluted to volume, filtered (0.45-μm membrane filters) and analysed by HPLC for the assay of the remaining quercetin content. All measurements were performed in quintuplicate.

2.8. Assay validation

Cream test samples were prepared by adding quercetin at levels of 0.2% and 0.5% (w/w) to the formulation components listed in Section 2.6. The percentage recoveries were calculated by comparing the peak areas of quercetin extracted from the test samples with those obtained by direct injections of equivalent concentrations of the analyte dissolved in ethanol.

The chromatographic precision was evaluated by repeated analyses ($n = 6$) of the same sample solution from a cream containing 0.5% (w/w) quercetin. The method precision was calculated by extraction and HPLC assay of independent samples ($n = 6$) from the same cream formulation.

Calibration curves of peak area versus concentration were generated with placebo extracts spiked with known amounts of quercetin in the concentration range 0.002–0.02 mg/ml.

2.9. Statistical analysis

Statistical analyses were performed using Student's unpaired *t*-test, analysis of variance (ANOVA) and Tukey's post-test. Significance was taken as $P < 0.05$. All computations were carried out using the statistical software GraphPad Instat (Graphpad Software, San Diego, CA).

3. Results and discussion

3.1. Microparticle preparation and characterization

Lipid microparticles loaded with quercetin were developed through a hot emulsion technique, which circumvents the use of organic solvents [18,22]. Tristearin was selected as lipidic material since it is a commonly used excipient in LMs [18] and hydrogenated phosphatidylcholine as the surfactant because of its physiological compatibility. To evaluate the retention capacity of the LMs, in vitro release studies were performed using a medium (phosphate buffer containing polysorbate 20 as solubilizer) in which quercetin was sufficiently soluble to ensure sink conditions [17], whereas the lipoparticles remained intact. As illustrated in Fig. 1, the quercetin release from the LMs was significantly lower (ANOVA and Tukey's post-test) than its dissolution and did not exhibit burst-effect phenomena, which indicated that the flavonoid was entrapped in the lipid particle core. SEM analysis on the obtained LMs revealed that the particles were mainly irregular with uneven surfaces (Fig. 2). The particle size was between 10 and 45 μm, the majority (75.2%) of the population being in the 15–35 μm range, suitable for topical applications [26]. Additional information on the solid state of the LMs was obtained by powder X-ray diffractometry. As illustrated in Fig. 3, the physical mixture of quercetin with blank microparticles (Fig. 3a) displayed the diffraction peaks of the flavonoid (10.6°, 12.2°, 26.4°, 27.2°) and those due to tristearin (5.1°, 21.3°). The characteristic signals of quercetin were also detected in the diffractogram of the loaded particles (Fig. 3b), suggesting that the flavonoid is, at least partially, in the crystalline state in the LMs.

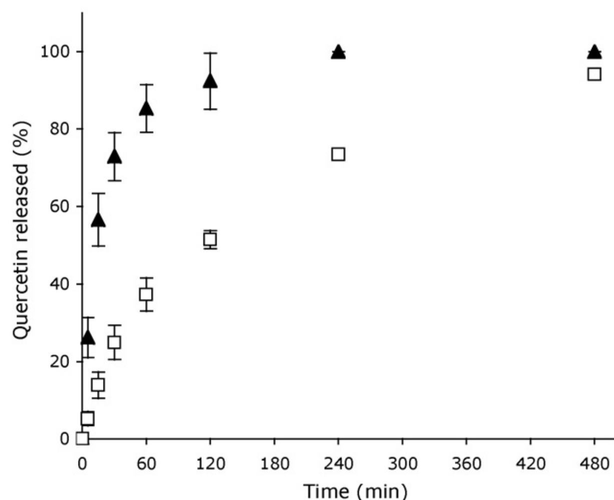

Fig. 1. Quercetin dissolution (▲) and release from lipid particles (□). Values are means ± S.D. ($n = 5$).

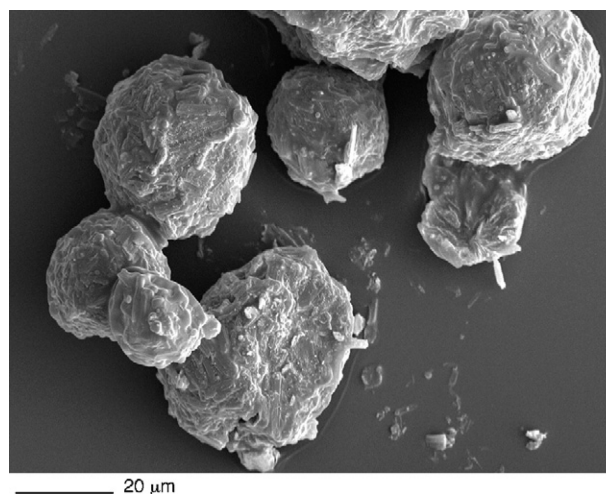

Fig. 2. Scanning electron microscopy (SEM) photomicrograph of lipid microparticles loaded with quercetin.

The quantity of quercetin incorporated into the LMs was 12.1 ± 0.3% (w/w), corresponding to an encapsulation efficiency of 62.0%. This relatively low entrapment efficacy can be traced to lim-

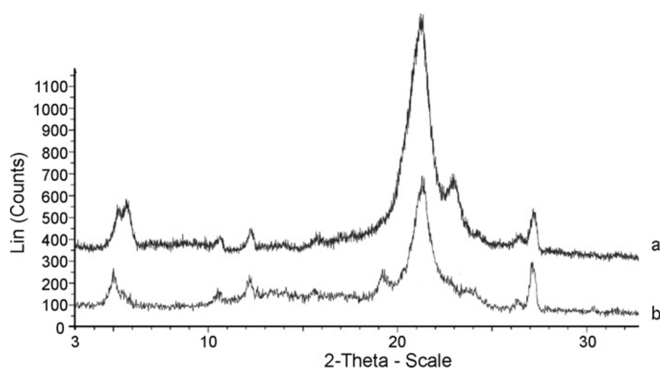

Fig. 3. Powder X-ray diffraction patterns of quercetin-blank lipoparticles physical mixture (a) and lipoparticles loaded with quercetin (b).

ited affinity of the moderately apolar quercetin (log P = 2.0) for the lipid matrix.

3.2. Photodegradation studies

Previous investigations on the light-induced decomposition of quercetin were performed in solutions, suspensions or in the solid state [27–29]. Although these studies provided valuable information on the photochemistry aspects, their relevance to the real conditions encountered in the use of topical preparations is limited. In order to overcome this drawback, in the present investigation the photochemical behaviour of free and microencapsulated quercetin was examined using a cream (oil-in-water emulsion) as a vehicle. This system was selected as a model formulation since it represents the most common type of topical preparation [30] and hence reproduces the actual applicative conditions of dermatological products. Creams containing non-encapsulated quercetin in combination with empty LMs or the flavonoid-loaded lipoparticles were exposed to the solar simulator and the extent of degradation was measured by HPLC (representative chromatograms are shown in Fig. 4). No interference was observed from the cream excipients. The accuracy of the method was examined by recovery experiments. The average recoveries of quercetin from the cream matrices were satisfactory, with values higher than 96.2%. The precision of the method was shown by relative standard deviation (R.S.D.) values of 1.4% and 3.3% for the chromatographic and the method precision, respectively. Calibration curves (n = 6) were linear over the range 0.002–0.02 mg/ml, with correlation coefficients greater than 0.996. The intercept with the y-axis were not significantly different from zero (P > 0.05). The minimum quantifiable amount (i.e., 0.002 mg/ml) corresponded to a quercetin concentration in the final formulation of 0.1% (w/w).

In the chromatogram of the irradiated preparation (Fig. 4b), no peaks originated from the decomposition of quercetin were detected (UV detection, 370 nm). This is due to the photoproducts (dihydroxybenzoic acids) absorbing at shorter wavelengths (<300 nm) [27,31] and exhibiting markedly different chromatographic retention [32] as compared to the parent flavonoid compound. In the cream containing plain quercetin in conjunction with unloaded LMs, 23.1% of the flavonoid content was lost following irradiation (Table 1). This result compared favourably with the data obtained for the light-induced decomposition (17–20%) of quercetin in solutions, although different irradiation conditions were employed [27,29]. At variance with this, quercetin has been reported to be sufficiently stable under UV-B (290–320 nm)

Table 1
Photodegradation values for free and microparticle-entrapped quercetin in cream formulations, immediately after preparation and after 3-month storage.

Sample	% quercetin loss[a]		P[b]
	t = 0	t = 90 days	
Free quercetin	23.1 ± 3.6	20.9 ± 5.2	
Quercetin-loaded LMs	11.9 ± 2.5	11.3 ± 2.4	< 0.01

[a] Each value is the mean ± S.D. of eight determinations.
[b] P values (unpaired t-test) vs. free quercetin.

radiation [28]. However, this condition does not simulate natural exposure to sunlight which comprises UV-B and also UV-A (320–400 nm) wavelengths, the latter being the predominant component. A marked reduction (48.5%) in the extent of quercetin photodegradation to 11.9% (Table 1) was attained in the emulsion containing the microencapsulated flavonoid, which demonstrated the protective effect of the lipid particle carrier. In order to evaluate whether the enhancement of quercetin photostability achieved by the LMs system varied with time, additional photolysis experiments were performed after 3-month storage of the cream samples at room temperature and in the dark. The percentage loss of the flavonoid upon irradiation of the examined formulations was 20.9% for the non-encapsulated quercetin and 11.3% for the microparticle-entrapped flavonoid (Table 1). Therefore, the photostabilization properties of the LMs in the cream preparation were retained after the above time interval.

3.3. Long-term stability studies

To examine the effect of the microencapsulation process on the chemical stability of quercetin, an aging study was performed on the same formulations submitted to the foregoing photodegradation experiments. The emulsions were analysed for quercetin over 3 months at room temperature and in the dark and the generated results are illustrated in Fig. 5. The curve for the cream containing the non-encapsulated flavonoid showed the quercetin level falling below 86% of the initial concentration within 3 months (Fig. 5). This is in accordance with previous stability studies evaluating the antilipoperoxidative activity of quercetin-containing creams [15]. On the other hand, no decrease of the quercetin content was detected, after the same time interval, in the emulsion prepared with the flavonoid-loaded lipoparticles (Fig. 5). The differences between the two formulations were statistically significant (ANOVA and Tukey's post-test) and demonstrated that incorporation of quercetin in LMs enhanced the chemical stabil-

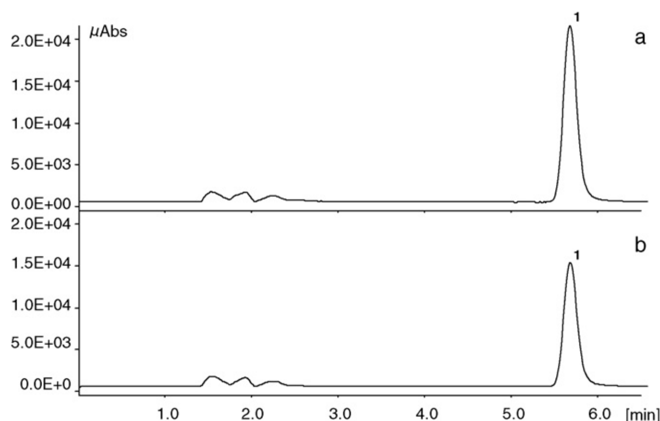

Fig. 4. HPLC traces of a cream preparation containing quercetin with blank lipoparticles, before (a) and after (b) 2 h irradiation with the solar simulator. Other operating conditions as described in Section 2. Peak 1 = quercetin.

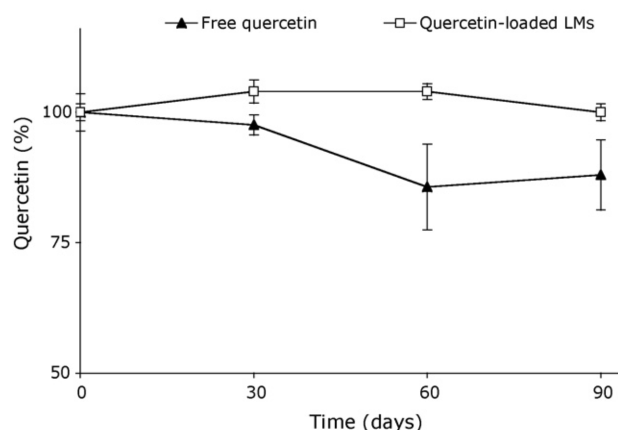

Fig. 5. Quercetin content in its formulations at different storing times. Operating conditions as described in Section 2. Values are means ± S.D. (n = 5).

ity of the flavonoid in the cream vehicle. In addition, the standard deviations of the data reported in Fig. 5, indicated that the variability associated with the assay of quercetin, during storage of the tested formulations, was higher for the cream containing the non-encapsulated flavonoid. This suggested that the microparticle carrier ensured a more uniform composition for the quercetin-based formulation.

4. Conclusions

In previous investigations on the stability of quercetin in topical formulations, the photochemical behaviour of the flavonoid was not examined. This represents a limitation for the potential applicability of quercetin in the protection of the skin against oxidative damage induced by exposure to the solar radiation [6]. From the results reported in this study, it can be deduced that incorporation of quercetin in LMs improves the photo- and chemical-stability of the flavonoid. Moreover, the biocompatibility of the lipoparticle carrier system represents an additional advantage for the development of quercetin-based products for skin care.

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