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Influence of cyclodextrin complexation on the photodegradation and antioxidant activity of α-tocopherol

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The influence of complexation with β-cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD) or hydroxypropyl-γ-cyclodextrin (HP-γ-CD) on the antioxidant activity and light-induced decomposition of vitamin E (α-tocopherol) was investigated. The interaction of the vitamin with the cyclodextrins was ascertained by nuclear magnetic resonance spectroscopy. The photodegradation of α-tocopherol was examined in emulsion vehicles and was not significantly influenced by complexation with β-CD (the extent of decomposition was 39.9% for the β-CD complex compared to 47.2% for the free vitamin) whereas HP-β-CD and HP-γ-CD enhanced the light-induced decomposition of α-tocopherol (the loss of the vitamin reached 64.6% for the HP-β-CD complex and 65.8% for the HP-γ-CD complex). On the other hand, accelerated stability studies indicated that the degradation of non-irradiated α-tocopherol was reduced by complexation with HP-β-CD or HP-γ-CD. The radical scavenging activity of α-tocopherol was evaluated *in vitro* using the xanthine/xanthine oxidase enzymatic system. No significant differences were observed between the free form of the vitamin and its complexes with β-CD, HP-β-CD or HP-γ-CD. Therefore, complexation of α-tocopherol with these cyclodextrins does not interfere with the vitamin antioxidant activity.

1. Introduction

The major biologically active vitamin E homologue, α-tocopherol is generally regarded as the most important lipid soluble antioxidant in human tissue (Bieri et al. 1983). It is employed in therapies of imborn metabolic disorders or lipid peroxidation pathologies such as atherosclerosis and ischaemic brain damage (Bieri et al. 1983). Moreover, the topical application of α-tocopherol has been shown to decrease, through a combination of antioxidant and UV absorptive properties, the incidence of skin cancer and immunosuppression induced by sunlight (Kramer and Liebler 1997; Krol et al. 2000). Consequently, α-tocopherol is commonly used in skin care and sunscreen preparations to prevent the harmful effects of the sun UV rays (Idson 1993; Gensler et al. 1996).

However, α-tocopherol is thermolabile, sensitive to oxidative agents and rapidly decomposed by the UV component of the solar radiation (Kramer and Liebler 1997; Kramer-Stickland et al. 1999; Krol et al. 2000). Improved stability is achieved by the prodrug ester, α-tocopheryl acetate but there is little evidence for its conversion to the active free α-tocopherol in the skin (Gensler et al. 1996; Krol et al. 2000). In fact, the esterified form of vitamin E, when applied topically, failed to protect against skin damage caused by solar-simulated UV radiation in mice (Gensler et al. 1996).

An alternative strategy for enhancing the stability of α-tocopherol is based on the complexation of the vitamin with cyclodextrins. Cyclodextrins are cyclic oligosaccharides which can incorporate appropriately sized molecules as guests into their apolar cavity forming non-covalent inclusion complexes (Loftsson and Brewster 1996; Rajewski and Stella 1996). This complexation process can increase the apparent aqueous solubility of the entrapped molecule (Loftsson and Brewster 1996). In addition, the stability to air and light of labile compounds can be improved upon inclusion into the cyclodextrin cavity (Loftsson and Brewster 1996; Scalia et al. 1999; Scalia et al. 2002). Several studies and patents (Koho 1981; Takanori and Yoshitaka 1990; Duchêne et al. 1999; Hisaya and Seiji 2000) deal with the preparation and performance of complexes of α-tocopherol with different cyclodextrins (i.e., β-cyclodextrin, (β-CD) hydroxypropyl-β-cyclodextrin (HP-β-CD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD)). However, none of these reports examined the influence of complexation on the vitamin photochemical behaviour. This is a major drawback since α-tocopherol is widely used in topical preparations (sunscreens, skin care products) which are exposed to solar radiations following application on the skin. In addition, the possible interference of the inclusion process with the reactivity of the phenolic group responsible for the antioxidant properties has not been evaluated before.

In the present study we report on the effect of cyclodextrin complexation on the photodegradation of α-tocopherol. The influence of the complexation on the free radical scavenging activity of α-tocopherol is also presented.

2. Investigations, results and discussion

HP-β-CD, HP-γ-CD and β-CD were selected for this study since these cyclodextrins are most commonly used

Table 1: ^{1}H NMR chemical shift changes ($\Delta\delta$, ppm) for α-tocopherol in the presence of cyclodextrins

Protons	$\Delta\delta$ [a]		
	β-CD	HP-β-CD	HP-γ-CD
5-CH_3	0.018	0.018	0.031
7-CH_3	0.017	0.016	0.028
8-CH_3	0.016	0.015	0.027
6-OH	0.029	0.028	0.058

[a] $\Delta\delta = \delta_{\text{with cyclodextrin}} - \delta_{\alpha\text{-tocopherol alone}}$

in the preparation of the inclusion complexes with α-tocopherol (Koho 1981; Takanori and Yoshitaka 1990; Duchêne et al. 1999; Hisaya and Seiji 2000). The solid complexes were prepared in a molar ratio of 1 : 1 and 1 : 2 (α-tocopherol : cyclodextrin) using different methods (i.e., kneading, co-evaporation and freeze-drying). The highest α-tocopherol recovery values (>89.2%), as measured by HPLC, were obtained by the kneading procedure which was selected for further experiments. The interaction between cyclodextrins and α-tocopherol was verified by ^{1}H NMR spectral studies which provide the most conclusive evidence of complex formation (Hedges 1998). The major changes in the ^{1}H NMR chemical shift values of α-tocopherol in the presence of HP-β-CD, HP-γ-CD or β-CD are listed in Table 1 (the chemical shift variations of the other proton signals were <0.01 ppm). The largest shifts were observed for the methyl and the hydroxyl protons of the aromatic ring substituents suggesting a participation of this portion of the α-tocopherol molecule in the complexation with the cyclodextrins. Moreover, the changes in the ^{1}H signals measured in the presence of HP-β-CD and β-CD were of lower magnitude than those induced by HP-γ-CD (Table 1) indicating a stronger interaction of α-tocopherol with the latter cyclodextrin.

In order to study the effect of HP-β-CD, HP-γ-CD and β-CD on the photochemical behaviour of α-tocopherol, the photolysis experiments were performed on a cream (oil-in-water emulsion) as a medium. This vehicle was selected as a model formulation since it represents the most common type of skin care preparation (Wittern 1991) and hence simulates the actual conditions in the finished product. Free or complexed (1 : 1, guest : host) α-tocopherol was incorporated into the cream and irradiated for 1 h with the solar simulator. In a previous study (Scalia et al. 1999), we reported that the complexation efficiency can be reduced in cream formulations by the excipient competitive displacement of the guest molecule from the cyclodextrin cavity. In order to minimize this effect, an excess (5%, w/w) of cyclodextrin was included in the creams containing the complexes. During the light-stability measurements, the applied UV-B energy was equivalent to 10 Minimal Erythemal Doses (MED) which is considered representative of daily sunlight irradiance (Scalia et al. 2002). The percentage loss of α-tocopherol following irradiation with the solar simulator was measured by HPLC (representative chromatograms are shown in Fig. 1). No photoproducts were detected. Employing a C_{18} column with a high eluotropic strength mobile phase (i.e. 30% methanol in ethyl acetate), Krol et al. (2001) have identified photo-dimers and -trimers of α-tocopherol. However, under the HPLC elution conditions (70% methanol in acetonitrile) selected in the present study, these products are strongly retained on the reversed-phase packing and hence the corresponding peaks are not observed in the chromatograms obtained (Fig. 1). In the preparation containing free α-tocopherol, the degree of photodegradation was 47.2% which decreased to 39.9% in the cream containing the α-tocopherol/β-CD complex, this difference being not statistically significant (Table 2). At variance with the data obtained for β-CD, the irradiation-induced decomposition of α-tocopherol was significantly increased (Table 2) by complexation with HP-β-CD (percentage loss, 64.6%) or HP-γ-CD (percentage loss, 65.8%). These data demonstrate that complexation with HP-β-CD or HP-γ-CD enhances the photo-induced degradation of the vitamin. Similar results were obtained for the complexes with a 1 : 2 (α-tocopherol : cyclodextrin) molar ratio.

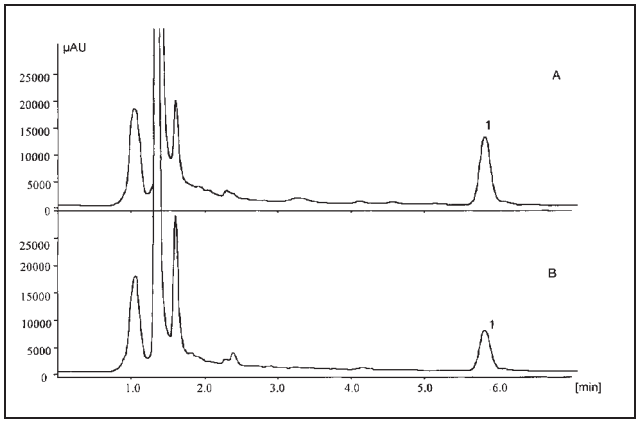

Fig. 1: HPLC of sample creams containing: (A) free α-tocopherol or (B) α-tocopherol/HP-β-CD complex after 1 h irradiation with the solar simulator. 1 = α-tocopherol. Operating conditions as described in Experimental.

An accelerated stability study was also performed on the formulations submitted to the photolysis experiments. After 5 months in the dark at 45 °C, the cream containing free α-tocopherol retained 73.9 ± 2.4% (n = 3) of the original vitamin content. Under the same storage conditions, the preparation containing the α-tocopherol/β-CD complex produced no significant effect on the chemical stability of the vitamin (remaining vitamin content, 73.5 ± 2.8%, n = 3). However, in the creams containing the α-tocopherol/HP-β-CD complex or the α-tocopherol/HP-γ-CD complex a statistically significant ($P < 0.01$) reduction in the vitamin loss to 84.4 ± 0.7% (n = 3) and 87.4 ± 1.5% (n = 3) of the initial concentration, respectively, was recorded. Therefore, despite of its photo-destabilization activity, complexation with HP-β-CD or HP-γ-CD enhances the chemical stability of α-tocopherol, in accordance with the data reported in the literature (Koho 1981; Takanori and Yoshitaka 1990; Duchêne et al. 1999; Hisaya and Seiji 2000). These results can be ascribed to the different mechanisms involved in the photo- and chemical degradation of α-tocopherol (Kramer and Liebler 1997; Krol et al. 2001).

In addition, the stronger effects produced in the stability tests by HP-γ-CD as compared to HP-β-CD can be ex-

Table 2: Comparative photodegradation data for free and complexed α-tocopherol, in cream preparations, after 1 h irradiation with the solar simulator

Sample	% α-Tocopherol loss [a]	P[b]
Free α-tocopherol	47.2 ± 8.6	
α-Tocopherol/β-CD	39.9 ± 5.5	>0.05
α-Tocopherol/HP-β-CD	64.6 ± 8.7	<0.002
α-Tocopherol/HP-γ-CD	65.8 ± 5.0	<0.001

[a] Each value is the mean ± S.D. of eight determinations.
[b] P values (unpaired t-test) vs. free α-tocopherol. Significance was taken as $P < 0.05$

plained in terms of a higher degree of interaction of α-tocopherol with HP-γ-CD, as indicated by the NMR data (Table 1).

For the *in vitro* evaluation of the scavenging activity of α-tocopherol and of its complexes with β-CD, HP-β-CD or HP-γ-CD, the xanthine oxidase enzymatic system was used to generate superoxide radicals ($O_2^{\cdot-}$) from xanthine. The amount of radicals produced was evaluated by spectrophotometric analysis in the presence of cytochrome c. In aqueous solutions, $O_2^{\cdot-}$ reduces cytochrome c yielding an increase in absorbance with a well-defined maximum at 550 nm (Halliwell and Gutteridge 2000). Any radical scavenger present in the medium will remove $O_2^{\cdot-}$ thereby decreasing the amount of reduced cytochrome c and hence inhibiting the absorption enhancement at 550 nm. In the solution containing free α-tocopherol, the absorbance at 550 nm showed a marked decrease (57.3%) compared to the sample containing the xanthine/xanthine oxidase system only (Fig. 2). Under the same experimental conditions, the complexes (1 : 1 molar ratio) of α-tocopherol with β-CD, HP-β-CD or HP-γ-CD produced a reduction of the absorption peak at 550 nm which was not significantly different (unpaired t-test, $P > 0.05$) from that observed for free α-tocopherol. As an example, the absorption spectrum of cytochrome c in the presence of the xanthine/xanthine oxidase system and the α-tocopherol/HP-β-CD complex is included in Fig. 2. The scavenging activity was not influenced by the cyclodextrins which exhibited absorption curves superimposable to the control (data not shown). In order to compare the scavenging properties of α-tocopherol and its cyclodextrin complexes with that of a well-known superoxide scavenger, the antioxidant assay was performed also on samples containing superoxide dismutase (SOD) instead of α-tocopherol. Through these comparative analyses it was possible to express the scavenging capacity of the different systems in terms of I.U. of SOD, thus allowing more accurate quantitative evaluations. The results obtained (see Table 3) indicated that under the experimental conditions adopted in this study the scavenging activity of α-tocopherol was equivalent to 1641.3 I.U. of SOD. Moreover, statistical analysis of the data (Table 3) showed that the differences between free α-tocopherol and its inclusion complexes with β-CD, HP-β-CD or HP-γ-CD were not significant ($P > 0.05$). This indicates that complexation does not affect the antioxidant properties of α-tocopherol. In this case too, the 1 : 2 (α-tocopherol:cyclodextrin) complexes exhibited the same behaviour as the complexes with a 1 : 1 stoichiometry.

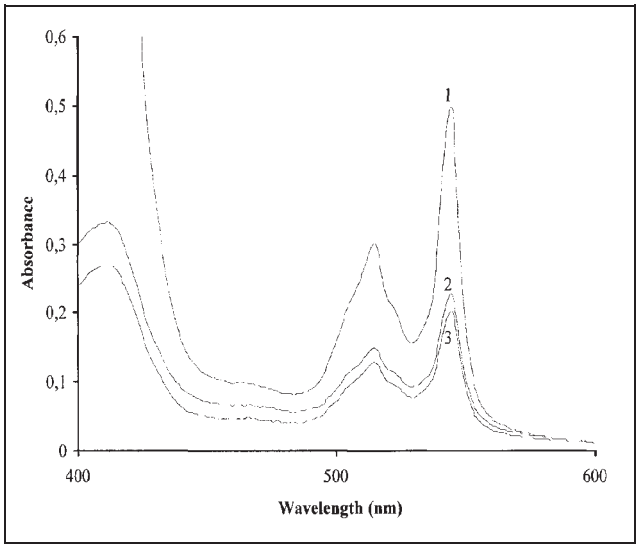

Fig. 2: Absorption curves of cytochrome c in phosphate buffer containing: (1) xanthine/xanthine oxidase; (2) xanthine/xanthine oxidase and free α-tocopherol; (3) xanthine/xanthine oxidase and α-tocopherol/HP-β-CD complex. Operating conditions as described in Experimental.

Table 3: Comparative data for the scavenging activity (expressed as I.U. of SOD) of free and complexed α-tocopherol

Sample	SOD I.U.[a]	P[b]
Free α-tocopherol	1641.3 ± 319.0	
α-Tocopherol/β-CD	1735.2 ± 263.9	>0.05
α-Tocopherol/HP-β-CD	1732.2 ± 190.9	>0.05
α-Tocopherol/HP-γ-CD	1545.5 ± 309.4	>0.05

[a] Each value is the mean ± S.D. of five determinations.
[b] P values (unpaired t-test) vs. free α-tocopherol. Significance was taken as $P < 0.05$

In conclusion, although complexation of α-tocopherol with cyclodextrins is an effective strategy to improve the physico-chemical characteristics of this vitamin, attention should be payed to the possible effects of cyclodextrins on the photochemical behaviour and antioxidant activity of α-tocopherol. The results reported in the present study indicate that the radical scavenging function of α-tocopherol is not significantly altered by inclusion into the cyclodextrin cavity. On the other hand, the photodegradation of α-tocopherol is increased by complexation with HP-β-CD and HP-γ-CD, whereas β-CD does not influence the vitamin photolability. Hence, appropriate evaluation of the photochemical behaviour of systems containing α-tocopherol is necessary to provide better knowledge of their efficacy.

3. Experimental

3.1. Materials

The cyclodextrins used in this study included: β-CD, HP-β-CD (average molar substitution, 0.6) and HP-γ-CD (average molar substitution, 0.6). They were purchased from Aldrich Chimica (Milan, Italy). α-Tocopherol was supplied by Sigma (Milan, Italy). Methanol and acetonitrile were of HPLC-grade from Merck (Darmstadt, Germany). Xanthine, cytochrome c from beef heart, xanthine oxidase (ammonium sulfate suspension, grade III from buttermilk) and superoxide dismutase from bovine erythrocytes (3750 units/mg protein) were obtained from Sigma. All other chemicals were of analytical-reagent grade (Sigma).

3.2. Highperformance 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 set at 290 nm (Jasco, Tokyo, Japan). Data acquisition and processing were accomplished with a personal computer using Borwin software (JBMS Developpements, Le Fontanil, France). Sample injections were effected with a Model 80365 syringe (10 µl; Hamilton, Bonaduz, Switzerland). Separations were performed on a 5-µm Luna C_{18} column (150 × 4.6 mm i.d.; Phenomenex, Torrance, CA) fitted with a guard column (5-µm particles, 4 × 2 mm i.d.) and eluted isocratically, at a flow-rate of 1.0 ml/min, with methanol/acetonitrile (70 : 30, v/v). The identity of the α-tocopherol peak was assigned by co-chromatography with the authentic standard. Quantification was carried out by integration of the peak areas using the external standardization method.

3.3. Preparation of the complexes

The inclusion complexes were prepared in molar ratios of 1 : 1 and 1 : 2 (α-tocopherol:cyclodextrin) by three different methods, namely kneading, freeze-drying and co-evaporation. In the kneading method the calculated amount of α-tocopherol (107.7 mg) and β-CD, HP-β-CD or HP-γ-CD was weighed, wetted in a mortar with a small volume (0.5 ml) of a methanol/water solution (1 : 1, v/v) and the slurry was mixed thoroughly for 30 min. After evaporation of the solvent, the obtained sample was dried under reduced pressure at room temperature for 2 days and stored in a desiccator. The co-evaporation procedure was as follows: the cyclodextrin was dis-

solved in purified water (6 ml) and added to a solution of α-tocopherol (107.7 mg) in methanol (6 ml). The obtained mixture was maintained under stirring for 24 h at room temperature and shielded from light. The solvent was then evaporated under vacuum at 40 °C by rotary evaporation and the residue was kept in a desiccator until used. For the preparation of the complexes by freeze-drying, α-tocopherol was added to an aqueous solution containing the cyclodextrin. The opalescent mixture was stirred for 24 h at room temperature and shielded from light. The water was removed by sublimation and the residue stored in a desiccator. The content of α-tocopherol in each complex was determined by HPLC after proper dilution.

3.4. NMR spectroscopy

^{1}H NMR spectra were recorded on a Bruker AC spectrometer (300 MHz). Samples were solubilized at a concentration of ca. 0.01 M in DMSO-d_6. Chemical shifts are reported in ppm (δ) relative to TMS. Typical parameters for the ^{1}H spectra were 0.4 Hz/pt resolution, 16 scans, 18 s relaxation delay, 90° pulse.

3.5. Photodegradation studies

Photochemical experiments were carried out in cream preparations (oil-in-water emulsion) containing α-tocopherol (0.1%, w/w) free or complexed with cyclodextrins. The cream excipients were: sorbitan monostearate, polyoxyethylene sorbitan monostearate, butylated hydroxyanisole, sodium benzoate, isopropyl isostearate (Henkel, Fino Mornasco, Italy), cetearyl isononanoate (Henkel), cetearyl alcohol (Henkel), D-sorbitol, dehydroacetic acid, EDTA and water. A portion of the test sample (200 mg) containing uncomplexed or complexed α-tocopherol, was spread by means of a syringe onto the bottom of a beaker and then irradiated for 1 h at a temperature of 37 °C 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 250 W/m^2. After the exposure interval (1 h), the beaker was removed and its content quantitatively transferred into a 10-ml calibrated flask with methanol. The resulting sample was diluted to volume, filtered (0.45-μm membrane filter) and a portion (10 μl) of the clear solution was analysed by HPLC. All samples were protected from light both before and after irradiation. The degree of photodegradation was measured by comparing the peak areas of α-tocopherol from the irradiated samples, with those obtained by analysis of an equivalent amount of the unirradiated preparation.

3.6. Accelerated stability studies

The chemical decomposition of α-tocopherol was evaluated in the same formulations used for the photodegradation studies after 5-months sample storage at 45 °C, in the dark. Portions (200 mg) of the test creams were analyzed by HPLC, as outlined above, for the remaining vitamin content.

3.7. In vitro antioxidant activity

The *in vitro* determination of α-tocopherol scavenging activity was carried out according to a previuosly reported technique (Halliwell and Gutteridge 2000) with minor modifications. In brief, to 0.53 ml of phosphate buffer (10 mM, pH 7.4) were added 0.2 ml of 20 mM free or complexed α-tocopherol in propylene glycol, 0.05 ml of 1 mM xanthine in phosphate buffer (10 mM, pH 7.4) and 0.2 ml of 0.1 mM cytocrome c in phosphate buffer (10 mM, pH 7.4). In some experiments, superoxide dismutase (1500 I.U.) in phosphate buffer (10 mM, pH 7.4) was included in the medium in place of α-tocopherol. The assay was started by the addition of 0.02 ml of xanthine oxidase diluted with purified water (1 : 1, v/v). Absorbance curves from 400 to 600 nm were monitored after 5 min at 25 °C in 2-ml cuvettes (path-length, 1 cm) using a Kontron Uvikon 860 spectrophotometer (Kontron Instruments Everett, MA, USA).

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