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Comparison of antioxidant activities of tocopherols alone and in pharmaceutical formulations

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Abstract

The objective of the present investigation was to compare the antioxidant effect of different forms of Vitamin E (DL-α-tocopherol, Mixed Tocopherols, Ronoxan MAP® and α-tocopherol acetate) and of topical formulations containing these active pharmaceutical ingredients, using chemiluminescence and the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays. Inhibition of the intensity of chemiluminescence, using the H_2O_2–luminol–horseradish peroxidase (HRP) enzyme system, was measured for 10 min at room temperature in 10 μl samples of each vitamin at different concentrations, and of formulations containing these vitamins. H-donor ability in the DPPH assay, was measured in 10 μl samples at different concentrations of each vitamin, as well as in formulation in ethanol solution; the decrease of absorbency was read at 517 nm. DL-α-Tocopherol, Mixed Tocopherols and Ronoxan MAP® alone or in formulations, markedly inhibited chemiluminescence intensity and decreased absorbency in the DPPH assay in a concentration-dependent manner. α-Tocopherol acetate and formulations containing this vitamin did not show antioxidant activity in either assay. Other components of the formulations did not interfere with the measurements, indicating that the methods employed can be used to evaluate antioxidant activity in topical formulations.

Keywords: α-Tocopherol; Topical formulation; Antioxidant; DPPH; Chemiluminescence

1. Introduction

Human skin is frequently exposed to oxidative injury by a variety of environmental stressors, including solar radiation, ozone, nitrogen oxides, transition metal ions, etc. Oxidative stress and subsequent oxidative damage are likely to be of importance in the pathogenesis of skin cancer and photoaging (Fuchs et al., 2003).

Vitamin E is a very active antioxidant acting as a free radical and singlet state oxygen scavenger, playing a crucial role in the protection of the skin from free-radical-generating factors such as ultraviolet radiation (Duval and Poelman, 1995). Vitamin E is the generic description for all tocopherol and tocotrienol derivatives, which exhibit the same qualitative biological activity as α-tocopherol.

Topical administration of antioxidants is capable of diminishing oxidative injury (Pauling, 1991;

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Werninghaus et al., 1991; Jurkiewicz et al., 1995). Because Vitamin E is the major lipophilic antioxidant of exogenous origin found in tissues, it is an obvious choice for the enhancement of antioxidative protection by topical application (Weber et al., 1997). Yet, Vitamin E can loose its antioxidant potency in the presence of certain solvents (Iwatsuki et al., 1994), which impair the pharmacological activity of vitamin formulations containing such solvents. Quantitative analysis of the antioxidant activity of topical vitamin formulations may furthermore, be rendered difficult by the interference of components of some formulations.

In view of the importance of Vitamin E in the protection of skin from oxidative injury, and of the necessity for correct evaluation of the activity in topical formulations of this vitamin, we have evaluated the antioxidant activity of different tocopherols alone, and following inclusion in formulations, measuring their H-donor capabilities as well as proving their free radical scavenging effects by studying changes of the chemiluminescence intensity of the H_2O_2–luminol–peroxidase system which they may evoke.

2. Materials and methods

2.1. Chemicals

α-Tocopherol acetate was purchased from Galena (Campinas, SP, Brazil); other vitamins (DL-α-tocopherol, Mixed Tocopherols and Ronoxan MAP®) were a kind gift from Roche (São Paulo, SP, Brazil). Luminol, horseradish peroxidase (HRP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); hydrogen peroxide 36% was purchased from Calbiochem (CA, USA). All other chemicals were of reagent grade and were used without further purification.

2.2. Test formulation

All the raw materials for the formulations were purchased from Galena (Campinas, SP). An emulsion stabilized by an anionic hydrophilic colloid (carboxypolymethylene, Carbopol® 940), was developed, based on a commercially available self-emulsifying wax; macadamia nut oil was added as an emollient, and glycerol as a moisturizer. The preservative used was a mixture of phenoxyethanol and parabene. Deionized water was used for the preparation of all formulations (Table 1). Preservative and vitamins were incorporated at room temperature. All formulations were allowed to equilibrate for 24 h prior to use in the study.

Table 1
Percent composition (w/w), of the emulsion media of the formulations of the different forms of Vitamin E tested

Component	Percentage
Self-emulsifying wax	1.00
Macadamia nut oil	2.00
Glycerol	6.00
Phenoxyethanol and parabene	0.40
Carboxypolymethylene (sol. 3%)	6.00
Triethanolamine	0.15
Deionized water qs	100.0

2.3. Preparation of samples

DL-α-Tocopherol, Mixed Tocopherols and α-tocopherol acetate were solubilized in ethanol and Ronoxan MAP® was solubilized in dimethylsulfoxide (DMSO), to final concentrations of 1, 5, 10, 50, 100 and 200 μg/ml. Formulations containing 2% of each Vitamin E and the Vitamin E-free formulation, were diluted 1:5 with the extraction solution (Tween-20/H_2O 1:5, w/w), mixed, and kept for 20 min prior to the measurement of their antioxidant activity.

2.4. Chemiluminescence assay

Changes of chemiluminescence intensity of the H_2O_2–luminol–HRP system by different concentrations of the vitamins and their formulations, were measured as follows: 10 μl of each sample or 10 μl of ethanol as the control for DL-α-tocopherol, Mixed Tocopherols and α-tocopherol acetate, or 10 μl of DMSO as the control for Ronoxan MAP®, as well as the vitamin-free control formulations, were mixed with 0.1 M phosphate buffer (pH 7.4), and a 2 mg/ml luminol solution in DMSO was added to yield a final concentration of 1.13×10^{-4} M. H_2O_2 was then added to a final 5×10^{-5} M concentration. The reaction was started by adding HRP at a final concentration of 0.2 IU/ml, yielding final 1.0 ml of solution (Krol et al., 1994). Chemiluminescence was measured for

10 min at 25 °C with an Autolumat LB953 apparatus (EG & G Berthold). Experiments were conducted in triplicate for each vitamin sample and formulation.

2.5. Stable free radical scavenging activity

DPPH, a stable nitrogen-centered free radical, was dissolved in ethanol to give a 250 μM solution. 10 μl of each sample or of the respective control (DMSO as the control for Ronoxan MAP®, ethanol as the control for the other vitamins and the vitamin-free control formulation for the formulations), were added to the ethanol solution of DPPH (0.5 ml) mixed with 1.0 ml of 0.1 M acetate buffer (pH 5.5), and the absorbance measured after 10 min. The ethanol solution of DPPH shows maximum absorbency at 517 nm which decreases in the presence of H-donor molecules, and in our experiments was considered to be correlated to the scavenging action of the test compounds and formulations studied. Experiments were done in triplicate for each vitamin sample as such, or in the formulation.

3. Results

3.1. Inhibition of the chemiluminescence of the H_2O_2–luminol–HRP system

Based on the measurements of the areas under the time course curves of chemiluminescence in the presence of, respectively DL-α-tocopherol, Mixed Tocopherols, Ronoxan MAP® or α-tocopherol acetate, we estimated the relative inhibitory activity of each sample at different concentrations. The percent inhibition caused by each sample was calculated as

$$\text{inhibition (\%)} = \frac{100 \times \text{AUC}_1}{\text{AUC}_0}$$

where AUC_0 and AUC_1 represent the areas under the curve observed for the control (ethanol, DMSO and the vitamin-free control formulation) and experimental samples, respectively.

The percent inhibition of chemiluminescence as a function of the concentration of DL-α-tocopherol, Mixed Tocopherols, Ronoxan MAP® and α-tocopherol acetate, respectively is shown in Fig. 1. Fig. 2 shows the time course of the effect of the Vitamin E formulation (A) and the percent inhibition achieved (B).

3.2. Interaction with stable free radical (DPPH)-scavenging action

The change in absorbency produced by reduced DPPH was used to evaluate the antioxidant ability of the compounds tested. Figs. 3 and 4 show the percent decrease of absorbance, caused by different concentrations of DL-α-tocopherol, Mixed Tocopherols, Ronoxan MAP® and α-tocopherol acetate alone or after addition to the formulations, respectively.

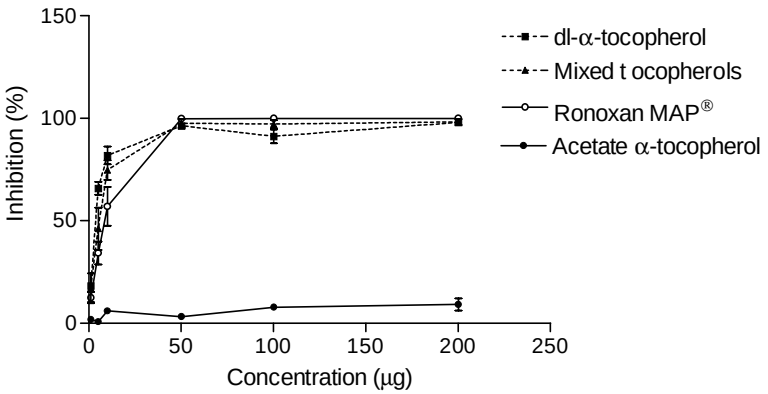

Fig. 1. Percent inhibition by Vitamin E of light emission from the HRP-catalyzed luminescent reactions with luminol. Results are means ± S.D. of three measurements run in parallel.

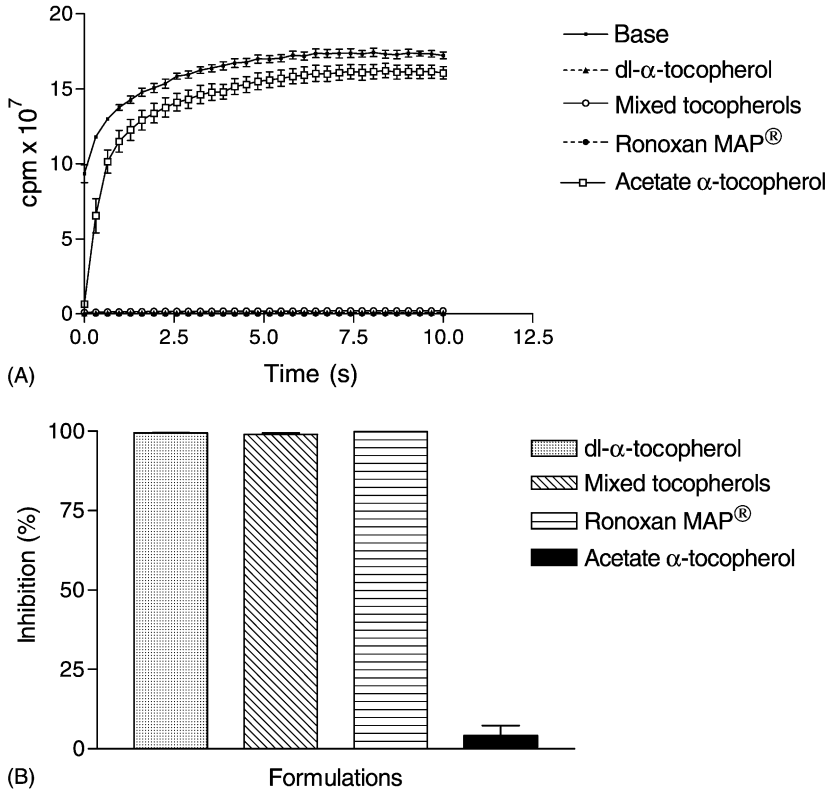

Fig. 2. Chemiluminescence of formulations of tocopherols. (A) Intensity of light emission from HRP-catalyzed luminol reactions vs. time. (B) Percent inhibition of light emission from the HRP-catalyzed luminescent reactions with luminol. Results are means ± S.D. of three measurements run in parallel.

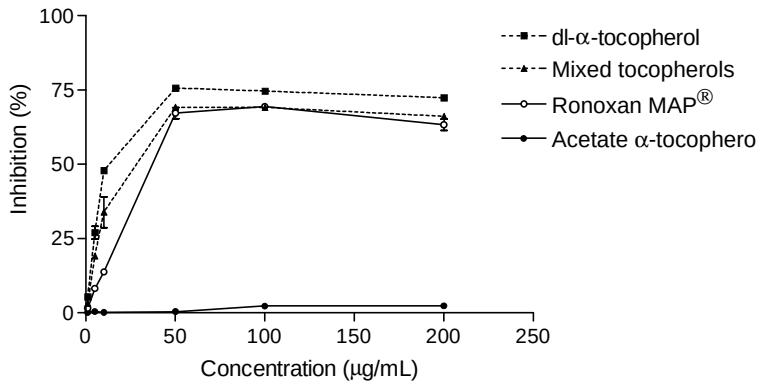

Fig. 3. Inhibition of hydrogen donor activity by Vitamin E samples measured by the DPPH method. Results are means ± S.D. of three measurements run in parallel.

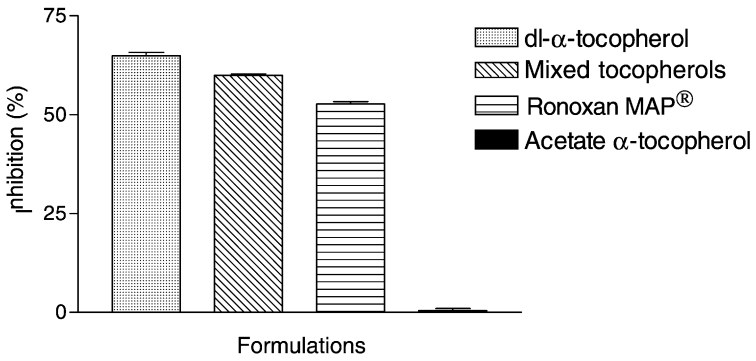

Fig. 4. Inhibition of hydrogen donor activity by formulations of Vitamin E samples, measured by the DPPH method. Results are means±S.D. of three measurements run in parallel.

4. Discussion

The skin, as our major external barrier, is continually exposed to the oxidative injury caused by free radicals. It is considered that topical use of antioxidant vitamins and other antioxidants can protect and possibly correct oxidative skin damage by neutralizing free radicals. Numerous benefits to the skin have been shown to follow topical application of Vitamin E. The primary mechanism of action justifying these effects is the potent antioxidant ability of Vitamin E (Lupo, 2001).

However, Vitamin E may lose or decrease its antioxidant activity depending on the formulation into which it is incorporated. The major objective of this study was to compare the relative activities of different samples of α-tocopherol and of the topical formulations containing these vitamins. To increase effectiveness, two analytical methods were used to evaluate the antioxidant activity of such formulations.

Several recent reviews have reported on the wide applicability of chemiluminescence as a sensitive assay to monitor levels of free radicals and reactive metabolites from cell-free, enzyme, cell, or organ systems as well as for the screening of antioxidant activity (Krol et al., 1990; Yasaei et al., 1996; Kubo and Toriba, 1997; Yildiz and Demiryurck, 1998; Yildiz et al., 1998). Among antioxidant activity assays, chemiluminescence presents advantages due to its high sensitivity and speed (Hirayama et al., 1997). Light emission from luminol can be markedly amplified by the use of the horseradish peroxidase–luminol–hydrogen peroxide system. HRP reacts with hydrogen peroxide to form oxidized HRP, which reacts with the anion of luminol (Dodeigene et al., 2000). Luminol radicals then undergo further reaction resulting in the formation of an endoperoxide which decomposes to yield the electronically excited 3-aminophthalate dianion which emits light when returning to its ground-state (Thorpe and Kricka, 1986).

Addition of antioxidant free radical scavengers to this chemiluminescent mixture leads to a decrease in the intensity of luminol chemiluminescence, permitting the evaluation of their antioxidant activity (Thorpe et al., 1985). Thus the instantaneous reduction of luminol chemiluminescent intensity elicited by the samples tested, can be considered a measure of their antioxidant activity.

DL-α-Tocopherol, Mixed Tocopherols (containing *R,R,R*-α-, *R,R,R*-β-, *R,R,R*-γ- and *R,R,R*-δ-tocopherol, D-tocopherols and tocopherol excipient) and Ronoxan MAP® (containing ascorbyl palmitate, Dry Mixed Tocopherols 30% and maltodextrin) inhibited chemiluminescence intensity in a concentration-related manner over an antioxidant range from 1.0 to 50 μg/ml. Above 50 μg/ml, these samples reached a plateau corresponding to a 99% inhibition of chemiluminescent intensity.

α-Tocopherol acetate did not inhibit the chemiluminescence reaction, even when employed at concentrations above 50 μg/ml. This result may be due to the acetylated condition of the hydroxyl group of α-tocopherol, which is responsible for its antioxidant activity. It has been shown that to be an effective antioxidant in skin, α-tocopherol acetate must first be converted to free α-tocopherol (Fuchs, 1998).

In order to evaluate the antioxidant activity of the formulations following addition of these vitamins, they were diluted 1:5 in the extraction solution and 10 μl were added to the reaction mixture, yielding a final concentration of 40 μg of the active principles per ml. Proper homogenization with an adequate extraction solution is a necessary measure, since in other kind of solutions, the formulations showed a low degree of inhibition of the reaction, suggesting that Vitamin E had not been correctly solubilized in the mixture (data not shown). For this reason a 1:5 solution of Tween-20 in water was chosen to give better solubilization of the active principles.

In order to verify whether some formulation components could interfere with the measurement of antioxidant activity, several dilutions of the formulations were made. The formulation without vitamin was diluted in the extraction solution 2.5, 5, 10, 7.5 and 15 times, respectively and 10 μl of each diluted sample was added to the mixture for the chemiluminescence reaction. The different dilutions caused no effect on the observed chemiluminescence intensity (data not shown), demonstrating that the formulation components did not interfere with the antioxidant activity measurements.

The use of formulations with the vitamins led to a 99% inhibition of the chemiluminescence intensity relative to that of the base without antioxidant. There was no significant difference in the antioxidant activity of DL-α-tocopherol, Mixed Tocopherols and Ronoxan MAP®, showing these materials to be equally potent when added to the topical formulation. The formulation containing α-tocopherol acetate in contrast did not cause inhibition of chemiluminescent intensity (see above).

The DPPH assay, originally developed by Blois (1958) is widely used for the measurement of free radical scavenging capacity in phytotechnology, food technology, and pharmacology/toxicology. DPPH is a free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule; DPPH scavenging can be measured in vitro or in vivo by EPH spectroscopy (Fuchs et al., 2003). It can accommodate a large number of samples within a short period, and is sensitive enough to detect low concentrations of the active principles (Yokozawa et al., 1998).

The vitamin samples tested in the present study for their H-donor ability, measured by the stable free radical DPPH assay, showed a lower antioxidant activity when compared to their capability to inhibit the chemiluminescence reaction. At the concentration of 50 μg/ml, DL-α-tocopherol, Mixed Tocopherols and Ronoxan MAP® decreased absorbency at 517 nm by about 70%. Higher concentrations caused no further decrease. DL-α-Tocopherol showed the highest H-donor capability, while α-tocoferol acetate again showed no antioxidant activity. The reaction of tocopherols with DPPH is very fast. Tocopherol compounds behave as effective scavengers of free radicals: the factors responsible for the high rate of reaction with DPPH are likely to be the same as those responsible for tocopherol's effectiveness as an antioxidant (Rao et al., 1982). This effect, to be explained by its hydrogen donor capacity, most probably accounts in large part for these compound's protective effect against UVB cytotoxicity to human skin fibroblasts and against intracellular-flavin-induced photo-sensitization (Duval and Poelman, 1995).

Tocopherol formulations showed the same activity profile as their respective raw materials, confirming data obtained by the chemiluminescence assays. As in the case of the inhibition of chemiluminescence intensity, a good correlation between the DPPH scavenging activity of the vitamins as such, and of the formulations containing these active principles, was found. These results show that both methods are suitable for antioxidant activity evaluation, since the formulation components did not interfere with the antioxidant measurements by either one of them.

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