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In Vitro Release Studies on Multiple and Simple Emulsions of α-Tocopherol with *Pistacia* Leaves

Ö.Özer[1], B.Kıvçak[2], B.Mutlu[1], S.Akay[2], H.Sağlam[2], S.Tömek[1]

[1]Department of Pharmaceutical Technology, Faculty of Pharmacy, Ege University, 35100-Bornova-İzmir-Turkey;

[2]Department of Pharmacognosy, Faculty of Pharmacy, Ege University, 35100-Bornova-İzmir-Turkey;

Keywords

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Abstract

α-Tocopherol is the most effective lipid soluble natural antioxidant and free-radical scavenger. The present study aimed to evaluate the performance of the extracts of *Pistacia lentiscus*, *P. lentiscus* var. *chia* and *P. terebinthus* leaves as additives on *in vitro* release of α-tocopherol. The α-tocopherol content of *Pistacia* extracts were determined and the release was investigated from w/o/w multiple emulsion, w/o and o/w simple emulsions through cellulose acetate and cellulose nitrate membranes by using HPLC-UV method. As significant increase in the extent of α-tocopherol release was observed with the addition of *Pistacia* extracts in all emulsions. They could be suggested as suitable additives in α-tocopherol containing formulations.

Introduction

Topical products are important classes of drug delivery systems, and their use in therapy is becoming more widespread. However the stratum corneum has physical barrier functions to most of the compounds. Many strategies have been

suggested to overcome the low permeability of drugs through the skin. The therapeutic efficacy of a topical formulation depends on both the nature of the vehicle and the physicochemical properties of the active substance [1]. It is generally assumed that the nature of the delivery vehicle strongly influences the rate and extent of drug release. The release of the drug may be improved by selecting the appropriate vehicle [2,3].

α -Tocopherol is the most effective natural lipid soluble antioxidant and free-radical scavenger. It is also an anti-aging factor and it can reduce the risk of cancer and delay the progression of precancerous lesions [4]. Vitamin E is a collective term for tocopherols and tocotrienols whereas α-tocopherol is considered as the most active form [5]. A number of studies have examined the photoprotective benefits of topically applied α–tocopherol [6,7].

The genus *Pistacia* belongs to the Anacardiaceae family. *Pistacia terebinthus*, *Pistacia lentiscus* var. *chia* and *Pistacia lentiscus* are evergreen shrubs or small trees native to the Mediterranean countries. They are used as anti-inflammatory, antidiarrheal, antihypertensive, antispasmodic, diuretic, insecticidal and antioxidant in traditional medicine [8-10]. The essential oils and chemical composition of the leaves and twigs of *Pistacia* species [11] and the α-tocopherol content of *Pistacia lentiscus*, *P. lentiscus* var.*chia* and *P. terebinthus* leaves were previously investigated by using GC-MS, TLC-densitometry and colorimetry methods [11-13].

As the synthesis of α-tocopherol was an expensive and difficult procedure one of the aim of this study was to propose *Pistacia* extracts as novel natural source of α-tocopherol by using a well documented HPLC-UV method [14]. In addition, multiple (w/o/w) and simple emulsions (w/o, o/w) containing synthetic α-tocopherol with and without extracts of *Pistacia* leaves were formulated. The *in vitro* release of α-tocopherol through cellulose acetate (CA) and cellulose nitrate (CN) membranes were investigated to evaluate the additive effect of plant extracts and vehicle effects.

Results and Discussion

The α-tocopherol content of *Pistacia* species was determined by using HPLC-UV method. The results were compared with those previously obtained by TLC-densitometry and colorimetry methods [13]. The amount of α- tocopherol in plant extracts was found higher by HPLC-UV method thus the present findings also showed that HPLC separations of α-tocopherol provided fast, sensitive and selective results [15]. This method could be used in quantitative evaluation of α-tocopherol from plants, extracts or formulations. The α-tocopherol content of the *Pistacia* extracts (PE) and the formulations were calculated from the following regression equation of the calibration curve (Figure 1).

$$y = 198{,}13x + 3{,}4285 \quad R^2 = 0.9992$$

Where y is the peak area and x is the α-tocopherol concentration (μg/μl).

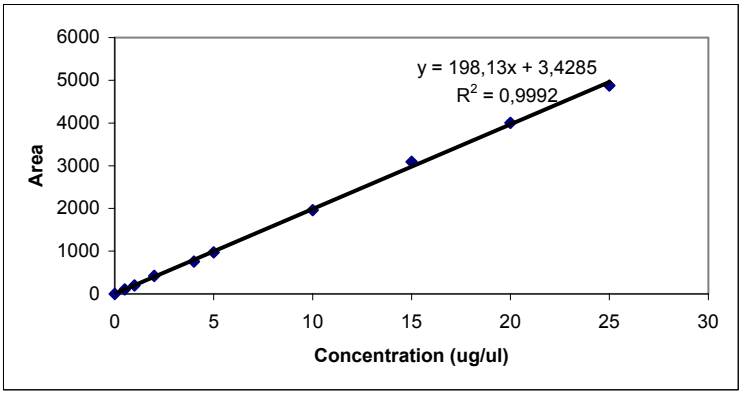

Fig. 1. The calibration curve for α-tocopherol by the HPLC-UV method

The major industrial source of α-tocopherol is a residue obtained from the distillation of soja bean oil and the content of α-tocopherol in soja bean is only 0.0051-0.011% [16]. In this study the amount of α-tocopherol was calculated as 0.02373, 0.02368 and 0.01073 % in the extracts of the leaves of *P. lentiscus*, *P. lentiscus* var. *chia* and *P. terebinthus,* respectively. The results indicated that PE extracts could be considered as a new and cheaper source of α-tocopherol.

In vitro evaluations

Main characteristics of the emulsions were given in Table 1. The conductometric and microscopic analysis have shown the emulsion type and the presence of both primary and the multiple characters.

Tab. 1. Characteristics of the w/o/w multiple, w/o and o/w simple emulsions

Code of formulation	Microscopic aspect	Macroscopic aspect	Conductivity	Stability	
				25° C	40° C
F1	Multiple globules 8-12µm	Homogeneous, very compact, white	19 µS	>3m	3m
F2	Multiple globules 8-12µm	Homogeneous, very compact, white-yellowish	19 µS	>3m	3m
F3	Simple globules 2-3 µm	Homogeneous, compact, white	0.02 µS	>3m	3m
F4	Simple globules 2-3 µm	Homogeneous, compact, white-yellowish	0.02 µS	>3m	3m
F5	Simple globules <2µm	Homogeneous, very compact, white	25 µS	>3m	3m
F6	Simple globules <2µm	Homogeneous, very compact, white-yellowish	25 µS	>3m	3m

The thermal stability tests were performed at 25±1^{0}C and 40±1^{0}C and centrifuge assay showed that the addition of the PE did not comprimise the structure of the skin creams and all the formulations were stable at these conditions.

The *in vitro* release studies which measure drug/vehicle interactions are considered to be useful in pre-formulation step to choose the best vehicle in further experiments. In the development of topical formulations the release of the drug from artificial membranes is crucial for predicting an appropriate vehicle [17]. Therefore, for an 8h period α-tocopherol diffusion from multiple (w/o/w) and simple emulsions (w/o, o/w) through CA and CN membranes was examined by using HPLC-UV method.

The highest release rate was observed in o/w emulsion with and without PE with the values of 0,211-0.119 mcg/cm^2. In respect of release rates, statistically significant vehicle effects were found ($p<0,05$) (Table 2).

Tab. 2. Release amount and release rate of α-tocopherol from w/o/w, w/o and o/w emulsions with and without PE ($p<0.05$)

Vehicles	Released amount (μg/cm^2)		Release rate (μg/cm^2/h)	
	CA	CN	CA	CN
F1	0,029± 0,001	0,072±0,021	0,003±0,001	0,009±0,005
F2	0,0560±0,001	0,085±0,016	0,007±0,002	0,011±0,003
F3	0,104±0,001	0,180±0,008	0,013±0,002	0,022±0,012
F4	0,190±0,011	0,251±0,025	0,023±0,001	0,031±0,003
F5	0,119±0,010	0,211±0,036	0,014±0,011	0,026±0,002
F6	0,211±0,002	0,260±0,046	0,026±0,003	0,032±0,003

According to the release results, it could be concluded that the best vehicle for α-tocopherol was o/w emulsion. It was shown that o/w emulsion increased the release rate by up to 2-3 times when compared with w/o and w/o/w emulsions, respectively.

The n-octanol/buffer partition coefficient (P_o) is commonly used in the pharmaceutical industry to reflect the lipophilicity of a potential drug compound. Octanol/buffer partition coefficient of α-tocopherol is reported as 12.2 [18].

Therefore, depending on the highly lipophilic character of α-tocopherol, it could better escape from the o/w emulsion and produce a stronger affinity to the lipophilic stratum corneum resulting in permeating into the skin easily. In the case of o/w emulsion, the free concentration in the external phase was greater than that in the internal oil phase, increasing the rate of release from this emulsion.

In contrast, release from w/o/w emulsion was significantly slower than other emulsions as w/o/w multiple emulsion contains larger emulsion droplets and the polymeric lipophilic surfactant Abil EM®90 in its oil phase. It was shown that the *in vitro* release of active substance after encapsulation in the inner phase of multiple emulsions was slower than pure solutions containing the same active substances. When active molecules were introduced into multiple emulsions, a greater power of sustained release was seen than in the case of simple emulsions as it was also found in this study [19-21]. For delivering actives through the skin or in other sites multiple emulsions have great promise because of the potential of these systems to protect the drug in the internal phase of the emulsion and the possibility to control the release of actives [22]. From a usage point of view, multiple emulsions are much more pleasant to use than w/o emulsions because of their less greasy feel. It was observed that the highest amount of α-tocopherol was remained at the membran surface. As it was concluded also by other authors, α-tocopherol had a resorvoir effect, associated with gradual delivery [6].

The release of α-tocopherol from w/o/w and w/o emulsions was slower. This result indicated a better encapsulation of α-tocopherol in the oily phase of these emulsions. The solubility of the active substance in the vehicle was an important factor for predicting the release. Increased solubility of the active substance in the vehicle helps to improve the product homogeneity, but at the same time it diminishes the release of the active if there is a great propensity for it to remain in the excipient.

The relase increased 1.8 and 1.3 fold by the addition of PE, from cellulose acetate and cellulose nitrate membranes, respectively (Table 2). The PE increased

the α-tocopherol content in all formulations. As a result, release profile was improved. The essential oils and chemical composition of leaves and twigs of the *Pistacia* species have been investigated previously [11]. On phytochemical screning [23,24], all extracts gave positive tests for α-tocopherol and the terpenes which are major constituents of essential oils. Naturally occuring terpenes that are non-toxic and non-irritan towards the skin are great interest as skin penetration enhancers [25]. So, the increase in release rate in the presence of *Pistacia* extracts might also be attributed to the terpen components in the essential oils of the *Pistacia* species.

The membranes used should not react with the drug product or the receptor medium in any way, should be permeable to the drug substance and should not be rate limiting in the drug release process. Despite, the comparison of the diffusion across the membranes revealed the rank order cellulose nitrate>cellulose acetate for all formulations, no significant difference were observed. These observations confirm that the characteristic of the membrane didn't contribute to release rate of formulations, provided that sufficient porosity of the membrane was maintained.

The release of active ingredient from heterogeneous vehicles results from exchanges occuring between the continuous and discontinuous phases. However results indicate that the release of α-tocopherol from different emulsions depended upon the emulsion type and the plant extracts were able to increase the release rate depending on their terpen and α-tocopherol components.

In conclusion, α-tocopherol content of of *Pistacia lentiscus*, *P. lentiscus* var. *chia* and *P. terebinthus* leaves were quantitatively determined by HPLC-UV method for the first time in this study. The obtained results showed that the types of vehicle had remarkable effect on *in vitro* release. The release of α-tocopherol in multiple and simple emulsions was found to be increased by the addition of the *Pistacia leaf* extracts. In addition, o/w emulsion base could be suggested as a suitable vehicle for the topical delivery of α-tocopherol.

Experimental

Materials

α-Tocopherol was obtained from Sigma. The oil used was liquid paraffin (Birpa, Turkey). The lipophilic surfactants were Abil EM®90, a cetyl dimethicone copolyol (Goldschmid, France) and Span®80, a sorbitan oleat (ICI France) and the hydrophilic surfactants were Tween®80, a polyoxyethylen sorbitan oleat, synperonic PE/ F®127, an ethoxylated propylene oxide copolymer (ICI, France). The viscosifying agent was carbopol 974P®, a synthetic polimer (BF Goodrich, Polyplastics, France) and triethanolamine was used for the neutralization of the polymer (Sigma). Hydrated magnesium sulfate was purchased from Merck as a marker to discriminate the emulsion type and to increase stability. All other chemicals used for analysis were analytical reagent grade.

Cellulose acetate membrane (por size: 0.2μm; Sartorius AG, Germany) and cellulose nitrate membrane (por size:0.45μm; Sartorius AG, Germany) were rinsed with distilled water and soaked in the receptor fluid. The methanol (Lab-Scan) was HPLC grade.

Preparation of plant extracts

P.lentiscus and *P. lentiscus* var.*chia* leaf samples were collected from West Anatolia Cesme/Izmir, in February 2001. *P. terebinthus* leaf samples were collected from West Anatolia in Buca/Izmir, in May 2001 and identified by B. KIVÇAK. The voucher specimens (No 1269,1268, 1267) were deposited in the herbarium of the Faculty of Pharmacy, Ege University, Izmir.

A sample (100 mg) of accurately weighed, air-dried and powdered leaves was extracted with n-hexane (2x600ml first 5 h and then for 8 h) under stirring. The combined organic phases were filtered and distilled in vacuo to yield the extract, which was stored at –20 °C until analysis [26].

HPLC analysis

Standard and sample solutions: The standard solutions were prepared by dissolving α-tocopherol in methanol to yield the concentrations of 2.5, 5, 10, 20, 25, 50, 75, 100, 125 g/100 ml. 20 µl of the standard solutions were injected on the HPLC column. Then the calibration curve was drawn. Sample solutions were prepared by dissolving the n-hexane extracts of the leaves in methanol (10 mg/2.5 ml). 10 µl of each aliquot was injected on the HPLC column. Each analysis was carried out in triplicate**.**

The HPLC system consisted of a QuatPump (Hewlett Packard Series 1100), an injector fitted with a 20- µl loop, and a UV detector (HP 1100) set at 292 nm. A Hichrom 5 C18 column (25 cm x 4.6 mm i.d.) was eluted with methanol at a flow rate of 2 ml/min. The column temperature was adjusted to 40^{o}C.The injection volume was 20µl. The retention time of α-tocopherol was found to be 5.06.

Preparation of Formulations

A two-step process was used to prepare the multiple emulsions (F1) [27]. The formula was 24% paraffin oil, 4% Abil EM©90, 0.8% Synperonic PE/F©127, 0.7% $MgSO_4.7H_2O$, 70.5% distilled water, by weight.

The simple emulsions (w/o-F3 and o/w-F5) were formulated by adding aqueous phase to the oil phase containing α-tocopherol. In the case of o/w emulsion, aqueous phase contained 3.25% Tween©80, 0.375% carbomer, 0.3% triethanolamine and oil phase consisted of 1.75% Span©80 while w/o emulsion (F5) was containing 5% Span©80 in oil phase [27].

The concentration of α-tocopherol was 0.02 % in all of the formulations . This concentration was chosen because of being within the range of those recommended for the antioxidant effect. 10 mg of each extract of *Pistacia* leaves was also added to the oil phase of formulations in order to investigate their effect on the release characteristics and stability. The code of the formulations containing the mixtures of extracts of *Pistacia* leaves (PE) were F2, F4 and F6, for w/o/w multiple emulsion, w/o and o/w simple emulsions, respectively.

Tab. 3. The codes and the compositions of the formulations

Formulation	F1 w/o/w	F2 w/o/w+PE	F3 w/o	F4 w/o+PE	F5 o/w	F6 o/w+PE
Mineral oil (Paraffinum liquidum)	24 g	24 g				
Abil EM®90	4 g	4 g				
Synperonic PE/F®127	0,8 g	0,8 g				
Span 80			5 g	5 g	1,75 g	1,75 g
$MgSO_4$.7H2O	0,7 g	0,7 g				
Tween 80			3.25 g	3.25 g	3.25 g	3.25 g
Carbomer			0.375 g	0.375 g	0.375 g	0.375 g
Triethanolamin			0.3 g	0.3 g	0.3 g	0.3 g
Pistacia extracts		10 mg		10 mg		10 mg
α-tocopherol	0.02	0.02	0.02	0.02	0.02	0.02
Water distilled	q.s.p.	q.s.p.	q.s.p.	q.s.p.	q.s.p.	q.s.p.

Characteristics of the formulations

The conductivity of the emulsions was measured with a conductimeter (Jenway 4071, U.K.) in order to discriminate the emulsion type. An optical immersion microscope at 1000 x magnification after diluting the emulsions was used for the microscopic observations.

Stability was tested at 25±1^{0}C and 40±1^{0}C at equal time intervals. Centrifugation at 4000 rpm for 15 minutes was performed on freshly prepared and 24-hour old systems to examine any phase separation.

In vitro release studies

α-Tocopherol release was determined with dialysis tubes through CA and CN membranes. The effective diffusion area was 1.54 cm^2. The membranes were allowed in distilled water for half an hour. The receptor phase used was ethanol. Typically, in release studies the receptor phase is usually an isotonic solution or another water-based medium. In this study the use of such mediums was impossible due to the extremely low solubility of α-tocopherol. Therefore, organic solvents were used to maintain the skin condition. Preliminary experiments showed no interactions of the receptor phase with either the membrane or the formulations. The receptor phase was kept at $37^0C\pm$ 0.5 in a well-closed container system and it was continuously stirred with a small magnetic bar to ensure homogenity at 100 rpm. The donor compartment was filled with 1g vehicles and occluded by parafilm. After application of emulsions on membranes, samples were taken at specified time intervals from the receptor fluid and analyzed by HPLC. All experiments were performed in triplicate (n=3).

Statistical analyses

Tests for significant differences between means were made by analysis of variance (ANOVA). Reference to significant difference in the below text denotes that the test was carried out at level $p<0.05$.

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