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# Ethosomes for skin delivery of ammonium glycyrrhizinate: In vitro percutaneous permeation through human skin and in vivo anti-inflammatory activity on human volunteers

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**Abstract**

The aim of this work was the evaluation of various ethosomal suspensions made up of water, phospholipids and ethanol at various concentrations for their potential application in dermal administration of ammonium glycyrrhizinate, a useful drug for the treatment of various inflammatory-based skin diseases. Physicochemical characterization of ethosomes was carried out by photon correlation spectroscopy and freeze fracture electron microscopy. The percutaneous permeation of ammonium glycyrrhizinate/ethosomes was evaluated in vitro through human stratum corneum and epidermis membranes by using Franz's cells and compared with the permeation profiles of drug solutions either in water or in a water–ethanol mixture. Reflectance spectrophotometry was used as a non-invasive technique to evaluate the carrier toxicity, the drug permeation and the anti-inflammatory activity of ammonium glycyrrhizinate in a model of skin erythema in vivo on human volunteers. Ethosomal suspensions had mean sizes ranging from 350 nm to 100 nm as a function of ethanol and lecithin quantities, i.e., high amounts of ethanol and a low lecithin concentration provided ethosome suspensions with a mean size of ~100 nm and a narrow size distribution. In vitro and in vivo experiments were carried out by using an ethosome formulation made up of ethanol 45% (v/v) and lecithin 2% (w/v). The ethosome suspension showed a very good skin tolerability in human volunteers, also when applied for a long period (48 h). Ethosomes elicited an increase of the in vitro percutaneous permeation of both methylnicotinate and ammonium glycyrrhizinate. Ethosomes were able to significantly enhance the anti-inflammatory activity of ammonium glycyrrhizinate compared to the ethanolic or aqueous solutions of this drug. Some

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in vivo experiments also showed the ability of ethosome to ensure a skin accumulation and a sustained release of the ammonium glycyrrhizinate.


*Keywords:* Ethosomes; In vitro percutaneous permeation; Methyl nicotinate; Ammonium glycyrrhizinate; In vivo evaluation

## 1. Introduction

*Glycyrrhiza glabra* L. is a perennial, herbaceous shrub, belonging to the family of Leguminosae. The plant is endemic to Mediterranean countries, such as Spain, Greece and southern Italy [1], and contains triterpene saponins (3–5%), mainly glycyrrhizic acid (a derivative of glycyrrhetic acid), and flavonoids (1–1.5%) [2,3].

Triterpene saponins present an anti-inflammatory activity, due to an indirect strengthening of the glucocorticoid activity [4,5]. Recent studies demonstrate that liquorice extracts are useful in the treatment of dermatitis, eczema, and psoriasis, with an efficacy comparable to that of corticosteroids [6,7]. In particular, the ammonium salt of glycyrrhizic acid is characterized by an anti-inflammatory activity [8,9]. The application of this compound as a potential topical anti-inflammatory drug can be further improved by using certain drug delivery systems, e.g., ethosomes, which can enhance the permeation through the skin stratum corneum [10] and hence promote the dermal pharmacological action [11].

Ethosomes are an interesting and innovative vesicular system that have appeared in the fields of pharmaceutical technology and drug delivery in recent years [12]. This carrier presents interesting features correlated with its ability to permeate intact through the human skin due to its high deformability. In fact, ethosomes are soft, malleable vesicles tailored for enhanced delivery of active agents. It has been shown that the physicochemical characteristics of ethosomes allow this vesicular carrier to transport active substances more efficaciously through the stratum corneum into the deeper layers of the skin than conventional liposomes [13]. This aspect is of great importance for the design of carriers to be applied topically both for topical and systemic drug administration. Furthermore, the ethosomal carrier is also able to provide an effective intracellular delivery of both hydrophilic and lipophilic molecules [14] and also the penetration of an antibiotic peptide, i.e., bacitracin, within fibroblast cells was facilitated [15].

In this paper, ethosomes made up of phospholipids and ethanol at various concentrations were prepared to investigate the potential application of ethosomes for dermal delivery of ammonium glycyrrhizinate, useful for the treatment of various inflammatory-based skin diseases. The percutaneous permeation of ammonium glycyrrhizinate/ethosomes was evaluated in vitro through human stratum corneum and viable epidermis membranes and compared with the permeation profiles of drug solutions either in water or in a water–ethanol mixture and a physical mixture of free ethosomes incorporated in a water–ethanol drug solution. We also carried out some experiments to evaluate both the in vivo efficacy of the ammonium glycyrrhizinate/ethosomes and the prolonged release properties of this carrier.

## 2. Materials and methods

### 2.1. Chemicals

Phosphatidylcholine (Phospholipon 90®) was kindly provided by Natterman Phospholipid GMBH, Germany, and contained 93 ± 3% phosphatidylcholine. Absolute ethanol (Ph.Eur. analysis reagent) was purchased from Carlo Erba (Milan, Italy). Ammonium glycyrrhizinate (Glycamil) was a kind gift of Indena (Milan, Italy). Methyl-nicotinate (purity >99%) was a Sigma–Aldrich product (Germany). All other chemicals used throughout this investigation were of analytical grade and no additional purification was carried out. Double-distilled water was used throughout the study.

### 2.2. Preparation of ethosome colloidal suspensions

Ethosome colloidal suspensions were prepared as elsewhere reported [12] and were made up of 1–3%

(w/v) Phospholipon 90®, 30–45% (v/v) ethanol, active molecules as described and water to 100% (w/v). Typically, the lecithin component was poured into a Pyrex® glass vial and solubilized with ethanol under mixing at 700 rpm with a magnetic stirrer (Midi MR1 Digital Ikamag®; IKA-WERKE GMBH and Co., Staufen, Germany). The glass vial was hermetically sealed and connected with a Teflon tube to a syringe system (Alitea AB. type S1, Solkraftsvägen, Stockholm, Sweden) to allow the addition of water and to avoid, as much as possible, ethanol evaporation. Following the solubilization of Phospholipon 90®, double-distilled water was added at a flow rate of 200 μl/min, to obtain the ethosome colloidal suspensions. Ethosome suspensions were finely homogenized (15,000 rpm) for 1 min by means of an Ultra-Turrax T 25 equipped with an S25N-8G homogenizing probe (IKA-WERKE). All the procedures for ethosome preparation were carried out under a nitrogen atmosphere at 25.0 ± 0.1 °C (GR 150 thermostat, Grant Instruments Ltd., Cambridge, UK). The final milky suspension of ethosomes was left at room temperature for 30 min under continuous stirring (Orbital Shaker KS 130 Control, IKA-WERKE). Ammonium glycyrrhizinate/ethosomes were obtained by solubilizing the drug in ethanol to obtain a final concentration of 0.3% w/v. In the case of methylnicotinate/ethosomes, the molecule was solubilized in the aqueous phase.

### 2.3. Ethosome size analysis

The mean size of the ethosome colloidal suspension was determined by photon correlation spectroscopy (Zetamaster, Malvern Instruments Ltd., Spring Lane South, Worcs, England). The experiments were carried out using a 4.5 mW laser diode operating at 670 nm as a light source. Size measurements were carried out at a scattering angle of 90°. To obtain the mean diameter and polydispersity index of colloidal suspensions, a third-order cumulant fitting correlation function was performed by a Malvern PCS sub-micron particle analyzer [16,17]. The samples were suitably diluted with filtered water–ethanol mixtures at the same ratio used for ethosome preparations (Sartorius membrane filters 0.22 μm) to avoid multiscattering phenomena and they were placed in a quartz cuvette. Typically, the size determination of ethosome colloidal suspensions consists in the measurement of the mean size of five different batches (30 measurements per batch), and the result is the average ± standard deviation.

### 2.4. Ethosome analysis by freeze fracture transmission electron microscopy

For vesicle characterization, the samples, after centrifugation at 30,000×*g* for 30 min at room temperature (Microcentrifuge Ole Dich. Denmark), were examined by means of the freeze fracture microscopy technique: samples were impregnated with 30% glycerol and then frozen in partially solidified Freon 22, freeze fractured in a freeze fracture device (−105 °C, 10–6 mm Hg) and replicated by evaporation from a platinum/carbon gun. The replicas were extensively washed with distilled water, picked up onto Formvar-coated grids and examined with a Philips CM 10 transmission electron microscope.

### 2.5. Entrapment efficacy of ethosomes

The amount of ammonium glycyrrhizinate entrapped within various ethosome colloidal formulations was determined by ultracentrifugation to separate the untrapped aliquot. The ethosomal suspensions were stored overnight at 4 °C and then poured into 15 ml polycarbonate eppendorf tubes. The centrifugation procedure was carried out at 4 °C using an Avanti 30 Centrifuge (Beckman, Fullerton, CA) equipped with a fixed angle rotor Beckman mod. F1202. The centrifugation speed was 28,000 rpm (56,000×*g*) for 45 min. The supernatant was removed from the ethosomal pellet and the drug amount was determined by HPLC (see Section 2.7). The entrapment efficiency was calculated with the following equation:

$$\frac{D_E}{D_E + D_S} \times 100$$

where $D_E$ is the amount of ammonium glycyrrhizinate determined in the ethosome pellet and $D_S$ is the amount of drug determined in the supernatant. The results are expressed as a mean value of five different ethosome preparations ± standard deviation.

### 2.6. In vitro permeation experiments through human skin

Samples of healthy adult human skin (mean age $29 \pm 4$ years) were obtained from abdominal reduction surgery. Membranes of the stratum corneum and viable epidermis (SCE) were isolated as previously reported [18]. Subcutaneous fat was surgically removed by means of a scalpel and the skin was immersed in distilled water at $60 \pm 1$ °C for 2 min. Then SCE was peeled off and immediately used for the various permeation experiments. The barrier integrity of SCE was checked by determining the tritiated water permeability coefficient ($K_p = 1.5 \pm 0.3 \times 10^{-3}$ cm $h^{-1}$), that resulted consistent with values previously reported [19]. Skin permeation studies were carried out by using Franz-type diffusion cells [20]. The cell had a diffusional surface area of 0.75 $cm^2$ and a volume of 4.75 ml. Human skin was mounted horizontally with the stratum corneum side up, dividing the cell into two compartments: i.e., the donor and the receptor compartments. The donor compartment was covered with Parafilm® in order to achieve occlusive conditions. The receptor was filled with pH 7.4 isotonic phosphate-buffered solution. The receptor fluid was constantly stirred with a small magnetic stirring bar in order to ensure its homogeneity. Each formulation (200 μl) was applied in the donor compartment and the amount was sufficient to maintain steady state conditions. The applied dose of methyl-nicotinate and ammonium glycyrrhizinate with the various formulations were not sufficient to saturate the receptor buffer (solubility of drugs >0.1 g/ml) and sink conditions were maintained throughout the duration of the permeation experiments. The duration of experiments was 24 h. At prefixed intervals, 500 μl of the receptor phase was withdrawn and analyzed by HPLC to determine the amount of permeated methyl-nicotinate or ammonium glycyrrhizinate. The withdrawn volume was replaced with fresh medium and a correction for dilution was carried out. The recovery for both methyl-nicotinate and ammonium glycyrrhizinate at the end of permeation experiments was higher than 98.5% of the applied dose. Six different permeation experiments were carried out for each formulation and the results are expressed as the mean value ± the the standard deviation.

### 2.7. HPLC determination of methyl-nicotinate and ammonium glycyrrhizinate

The concentration of methyl-nicotinate in the receptor compartment of Franz's cells was determined by a Hewlett–Packard model 1050 liquid chromatographic system (Hewlett–Packard, Milan, Italy) equipped with a 20 μl Rheodyne model 7125 injection valve (Rheodyne, Cotati, CA). The chromatographic analysis was carried out with a reverse phase Nucleosil 100-5 C18 column (5 μm, $250 \times 4.6$ mm i.d.; Applied Biosystems) at room temperature. Methyl-nicotinate detection was carried out at $\lambda_{max}$ 218 nm. The mobile phase was a mixture of acetonitrile/water (35:65 v/v) with a flow rate of 0.6 ml/min.

The HPLC determination of ammonium glycyrrhizinate was carried out with a reverse phase LiChrospher 100 RP-18 column (5 μm, $125 \times 4$ mm i.d.; Merck, Darmstadt, Germany) at room temperature. Ammonium glycyrrhizinate was detected at $\lambda_{max}$ 247 nm. The mobile phase was a mixture of methanol/acetonitrile/0.1 N phosphoric acid (60/30/10 v/v/v) and was delivered at a flow rate of 1 ml/min.

### 2.8. In vivo tolerability on human volunteers

Skin acceptability of topical formulations was evaluated on human volunteers with reflectance spectrophotometry, which is a non-invasive technique. The reflectance spectrophotometer, SP60 (X-Rite Incorporated, USA), having 0° illumination and 45° viewing angle, was calibrated with a supplied white standard traceable to the National Bureau of Standard's perfect white diffuser. The spectrophotometer was connected to a personal computer, which performed all colour calculations from the spectral data by means of the program supplied with the instrument. Reflectance spectra were obtained over the wavelength range 400–700 nm by using illuminant C and 2° standard observer.

Twelve healthy volunteers were fully informed about the nature of the study and the procedures involved and they gave their written consent. Subjects did not suffer from any ailments, were not on medication (for at least 1 week) and rested for 30 min before the experiments at room conditions ($22 \pm 2$ °C and 40–50% r. h.).

Six sites on the ventral surface of each forearm were randomly defined using a circular template (1 $cm^2$). The distance between sites was at least 2 cm in order to avoid any possible interference. The induced erythema was monitored for a variable period. Erythema index (EI) baseline values were taken at each designated site before application of ethosomal suspensions and then the formulations were applied on two sites using Hill Top chambers (Hill Top Research, Inc. Cincinnati, Ohio). Before reflectance spectrophotometric readings, the chambers were removed and the skin surface was gently washed with water to remove the applied formulation and the skin was allowed to dry for 15 min. The possible induced erythema (as EI) was monitored at various times, 6, 24 and 48 h and was calculated according to the following equation [21]:

$$EI = 100\left[\log 1/R_{560} + 1.5(\log 1/R_{540} + \log 1/R_{580}) - 2(\log 1/R_{510} + \log 1/R_{610})\right]$$

where $1/R$ is the inverse reflectance at a specific wavelength (510, 540, 560, 580, 610).

### 2.9. In vivo evaluation of methyl-nicotinate erythema

To evaluate the in vivo permeation profiles of methyl-nicotinate/ethosomes through the human skin with respect to aqueous or water–ethanol solutions of methyl-nicotinate, we monitored chemically induced erythema following the topical application of the three formulations. The intensity of the induced erythema is directly proportional to the amount of methyl-nicotinate permeated through the human skin [22].

### 2.10. In vivo anti-inflammatory activity of ammonium glycyrrhizinate

To evaluate the efficacy of ammonium glycyrrhizinate/ethosome suspensions in reducing the chemically induced erythema in comparison with simple aqueous or water–ethanol solutions of ammonium glycyrrhizinate, the following procedure was carried out: eight sites on the ventral surface of each forearm were randomly defined using a circular template (1 $cm^2$) and demarcated with permanent ink. The distance between sites was at least 2 cm in order to avoid any possible interference. For each volunteer (a total of six), all the sites were treated with 100 μl of an aqueous methyl-nicotinate solution (0.2% w/v) for 15 min using Hill Top Chambers. After the topical treatment with methyl nicotinate, the chambers were removed and the skin surface was gently washed with water to remove the applied formulation and then 200 μl of the various formulations (ethosomes, aqueous or water–ethanol solutions) containing ammonium glycyrrhizinate (0.3% w/v) were applied. As a blank 200 μl of a saline solution (NaCl 0.9% w/v) was used. The induced erythema was monitored until its disappearance.

In addition, to evaluate the in vivo controlled release properties of ethosomes another experimental protocol was carried out. Six sites on the ventral surface of each forearm were randomly defined using a circular template (1 $cm^2$) and demarcated with permanent ink (a distance of at least 2 cm was also respected between sites). For each volunteer, two sites were pretreated with 200 μl of one formulation (ethosomes, aqueous solution or water–ethanol solution) containing ammonium glycyrrhizinate (0.3% w/v) for several intervals of time (1, 3 and 5 h) using Hill Top chambers (1 $cm^2$). After the pretreatment period, the chambers were removed, the skin surface was gently washed with water to remove the applied formulation and a Hill Top Chamber containing 100 μl of methyl-nicotinate (0.2% w/v) was applied for 15 min. At the end of the treatment, the chambers were removed and the skin surface was gently washed with saline solution to remove the applied formulation and allowed to dry for 15 min. The induced erythema was then monitored for a period of 4 h.

### 2.11. Statistical analysis

Statistical analysis of the various experimental results was performed by using one-way ANOVA. A posteriori Bonferroni $t$-test was carried out to check the ANOVA test. A $p$ value $< 0.05$ was considered statistically significant. All the values are reported as the average ± standard deviation.

## 3. Results and discussion

In this study, we evaluated ethosomes as carriers for the topical application of a natural anti-inflamma-

tory agent such as ammonium glycyrrhizinate. For this purpose, we prepared some formulations at different ethanol and soybean phosphatidylcholine percentages to evaluate the effect of the composition on the mean size distribution of ethosomes. Various formulations were submitted to light scattering analysis to choose the most suitable formulation to be tested in vitro and in vivo. As shown in Table 1, ethosomes showed a narrow particle size distribution, in particular the ethosomal formulation prepared with the greatest amount of ethanol. In the interval of ethanol concentration investigated in this paper, the size of ethosomes decreased with increasing ethanol concentration. While, the concentration of lecithin used for ethosome preparation influenced the vesicle mean size in a different way, namely the higher the lecithin concentration the larger the ethosome mean size. These data are in perfect agreement with previous findings [12]. In particular, the largest vesicles were observed in the formulation C (342 ± 31 nm), while the smallest vesicles were observed in the formulations G and H (~100 nm). Photon correlation spectroscopy analysis was also carried out on methylnicotinate and ammonium glycyrrhizinate/ethosomes (data not reported). In both cases, at the concentrations investigated in this paper, no significant ($p > 0.05$) size variation with respect to unloaded ethosomes was observed, thus showing that the presence of the drugs was not a determinant factor capable of influencing the mean size of the various ethosome colloidal suspensions.

The transmission electron micrograph of ammonium glycyrrhizinate/ethosomes after freeze fracture, prepared according to the composition H (Table 1), is reported in Fig. 1. The sample was the one chosen for percutaneous permeation experiments and, as it is possible to observe, the vesicles show a monolayer structure and their size is quite uniform.

Drug entrapment within a vesicular carrier is an important parameter to be defined to really evaluate the delivery potentiality of the system. For this reason, the entrapment efficiency of ammonium glycyrrhizinate within the formulations A-I was evaluated in an attempt to investigate the influence of ethosome composition, i.e., the quantity of ethanol and lecithin, on the drug loading capacity. As shown in Table 1, both ethanol and lecithin amounts, used for ethosome preparation, positively influenced the entrapment efficiency of the colloidal carrier. Namely, the higher the amount of ethanol and lecithin, the greater the ammonium glycyrrhizinate entrapment within the ethosomes. In agreement with previously reported findings [12,13], the values of entrapment efficiency for ammonium glycyrrhizinate are higher than those expected for conventional vesicle formulations [23]. This fact can be explained by the presence of ethanol, which increases the ammonium glycyrrhizinate solubility in the polar phase of the colloidal formulations of ethosomes.

The loading capacity and the mean size of a colloidal carrier are important parameters that are able to influence the percutaneous permeation of an incorpo-

Table 1
Influence of the amount of ethanol and Phospholipon 90® used for the preparation of ethosomes on vesicle suspension mean size, polydispersity index and ammonium glycyrrhizinate entrapment efficiency[a]

| Ethosome Formulation | Composition | | Size (nm)[b] | PI[c] | Entrapment efficiency |
|---|---|---|---|---|---|
| | Ethanol (%, v/v) | Lecithin (%, w/v) | | | |
| A | 30 | 1 | 147 ± 25 | 0.399 ± 0.052 | 38.3 ± 3.7 |
| B | 30 | 2 | 186 ± 37 | 0.317 ± 0.039 | 51.7 ± 4.1 |
| C | 30 | 3 | 342 ± 31 | 0.415 ± 0.041 | 60.8 ± 4.3 |
| D | 40 | 1 | 115 ± 23 | 0.371 ± 0.052 | 43.1 ± 3.8 |
| E | 40 | 2 | 131 ± 13 | 0.163 ± 0.017 | 59.0 ± 4.1 |
| F | 40 | 3 | 287 ± 24 | 0.084 ± 0.011 | 72.5 ± 4.7 |
| G | 45 | 1 | 102 ± 5.5 | 0.417 ± 0.007 | 63.4 ± 3.4 |
| H | 45 | 2 | 109 ± 3.7 | 0.109 ± 0.003 | 78.9 ± 4.4 |
| I | 45 | 3 | 220 ± 11 | 0.091 ± 0.013 | 81.7 ± 4.2 |

[a] The experimental data represent the average of five different ethosome batches ± standard deviation.
[b] Photon correlation spectroscopy experiments were carried out immediately after ethosomes preparation.
[c] Polydispersity index.

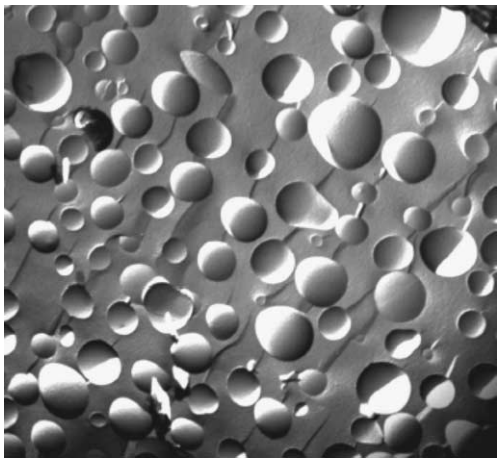

Fig. 1. Freeze fracture electron micrographs of ethosome formulation H.

rated drug. For this reason, we chose formulation H, which is characterized by small vesicle size, a homogenous vesicle size distribution (Table 1) and a high entrapment efficiency of ammonium glycyrrhizinate (Table 1). Considering that the percutaneous penetration enhancement is mostly determined by the ethosomal system per se rather than the real loading of the drug, formulation H was used for in vitro and in vivo experiments without separating the unloaded drug from the loaded drug.

An important characteristic to be evaluated before the proposal of a drug carrier as a potential topical drug delivery system is its in vivo skin tolerability on human subjects. In this work, a non-invasive technique, i.e., reflectance spectrophotometry, to evaluate the possible erythema derived from the topical application of empty ethosomes was used (Fig. 2). The results of skin tolerability are expressed as the variation of the erythema index (ΔEI). The effect of empty ethosomes on human skin was compared with that induced by a 0.9% (w/v) NaCl solution or a water–ethanol mixture (55:45 v/v) at the same ratio used for the preparation of ethosomes. ΔEI data measured for empty ethosomes at the end of the various experiments showed values not significantly different with respect to an aqueous saline solution (control). On the other hand, the water–ethanol mixture showed a significantly different ($p<0.001$) behavior with respect to the ethosomal formulation and the saline solution; namely the mixture induced a remarkable skin erythema. These results demonstrate that ethanol present in the ethosomal formulation is not able to act as a skin erythema-inducing agent, even though it is present at a high concentration.

In order to evaluate the permeation profile characteristics of this colloidal carrier to be used for topical administration, in vitro percutaneous experiments using Franz-type cells were carried out. The percutaneous permeation of both methyl-nicotinate and ammonium glycyrrhizinate from various formulations through human skin stratum corneum was carried out (Figs. 3 and 4). The permeation profile of methyl-nicotinate/ethosomes was compared with those obtained from an aqueous solution and a water–ethanol solution of methyl-nicotinate at the same percentage of ethanol (45% v/v) used for ethosome preparation to evaluate the influence of the organic solvent on the percutaneous permeation (Fig. 3). The aqueous solution showed a permeation profile characterized by a lag time of 3 h and a cumulative amount of methyl-nicotinate permeated after 24 h of 25.2% of the applied dose. The water–ethanol solution pre-

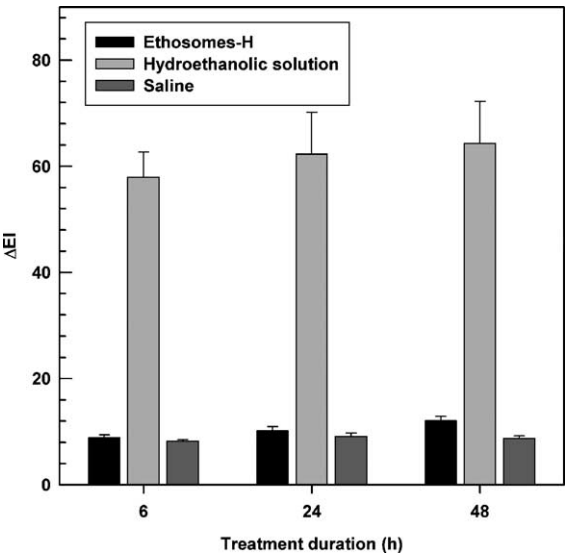


Fig. 2. In vivo human skin tolerability of various topical formulations after 6, 24 or 48 h of treatment. Results are expressed as a mean value of ΔEI ($n=6$) ± standard deviation. Legend keys: ethosomes-H—formulation H ethosomes containing 2% (w/v) Phospholipon 90® and 45% (v/v) ethanol; hydroethanolic solution—solution of water and ethanol at a volume ratio of 55:45; saline—control saline (0.9% w/v NaCl in water) solution.

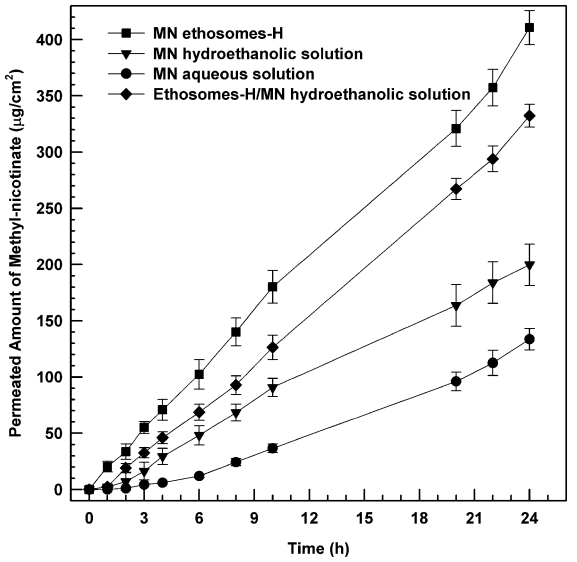


Fig. 3. In vitro percutaneous permeation through SCE membrane of various formulations containing methyl-nicotinate at a concentration of 0.2% (w/v). Legend keys: MN ethosomes-H—methyl nicotinate formulation H ethosomes containing 2% (w/v) Phospholipon 90® and 45% (v/v) ethanol; MN hydroethanolic solution—methyl nicotinate solution in a mixture of water and ethanol at a volume ratio of 55:45; MN aqueous solution—methyl nicotinate aqueous solution; ethosomes-H/MN hydroethanolic solution—a mixture of empty formulation H ethosomes containing 2% (w/v) Phospholipon 90® and 45% (v/v) ethanol with a methyl nicotinate solution in a mixture of water and ethanol at a volume ratio of 55:45. Each value is the average of six different experiments ± standard deviation.

sented a similar permeation profile with respect to the aqueous solution, but was characterized by an overall percutaneous permeation of a higher amount of methyl-nicotinate (37.5% of the applied dose) and a shorter lag time (2 h). In the case of the methyl-nicotinate/ethosome suspension, the permeation profile presented no lag time showing a significant amount of methyl-nicotinate after 1 h. This finding was probably due to a more rapid distribution of methyl-nicotinate from the formulation to the SCE membranes. In this case, the amount of methyl-nicotinate permeated (77.1% of the applied dose) was significantly ($p<0.001$) greater than those observed for both aqueous and hydroethanolic solution.

Furthermore, to evaluate the influence of the drug entrapment on the SCE permeation process, experiments with empty ethosomes incorporated in an water–ethanol solution of methyl-nicotinate were also carried out. As shown in Fig. 3, empty ethosomes showed a significant ($p<0.001$) penetration enhancement effect of methyl-nicotinate compared to the simple water–ethanol solution of the drug. The permeation profiles of methyl-nicotinate from the mixture empty ethosomes/water–ethanol drug solution showed a lag time of 1 h and a cumulative amount of 62.3% of the applied dose. This finding showed that the drug encapsulation within ethosomes could positively influence the permeation of a molecule through human SCE at a certain extent, although the real contribution is difficult to evaluate due to the flexible and permeable nature of ethosomes that allows the rapid drug diffusion from the environment into ethosome compartments.

In Fig. 4 the permeation profiles of ammonium glycyrrhizinate through human SCE are reported. Ammonium glycyrrhizinate was able to permeate from aqueous solution and a water–ethanol (55/45

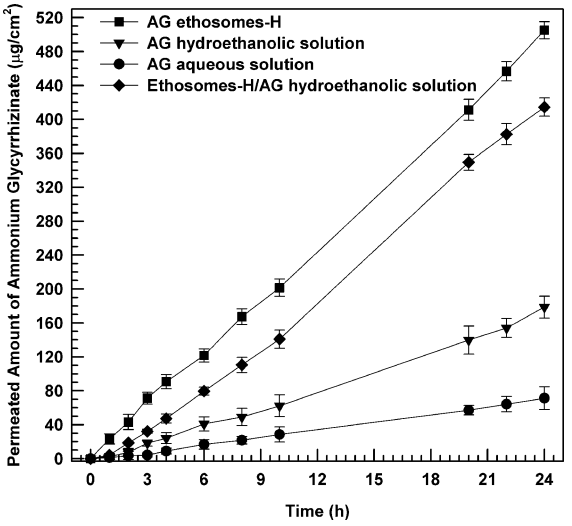


Fig. 4. In vitro percutaneous permeation through human stratum corneum and epidermis membrane of various formulations containing ammonium glycyrrhizinate at a concentration of 0.3% (w/v). Legend keys: AG ethosomes-H—ammonium glycyrrhizinate formulation H ethosomes containing 2% (w/v) Phospholipon 90® and 45% (v/v) ethanol; AG hydroethanolic solution—ammonium glycyrrhizinate solution in a mixture of water and ethanol at a volume ratio of 55:45; AG aqueous solution—ammonium glycyrrhizinate aqueous solution. Ethosomes-H/AG hydroethanolic solution, a mixture of empty formulation H ethosomes containing 2% (w/v) Phospholipon 90® and 45% (v/v) ethanol with an ammonium glycyrrhizinate solution in a mixture of water and ethanol at a volume ratio of 55:45. Each value is the average of six different experiments ± standard deviation.

v/v) solution through SCE membranes showing similar profiles of release, but the total amount permeated at the end of the experiment was significantly ($p<0.001$) higher for the hydroalcoholic solution (22.3% of the applied dose) compared with the aqueous one (8.9% of the applied dose). The ammonium glycyrrhizinate/ethosomes showed the greatest ($p<0.001$) cumulative amount of permeated drug (63.2% of the applied dose) with respect to other formulations. The permeation experiments with empty ethosomes incorporated in an water–ethanol solution of ammonium glycyrrhizinate showed a penetration enhancement effect of this drug in a similar way to that observed for methyl-nicotinate. Also in the case of permeation profiles of ammonium glycyrrhizinate, a difference in lag time was observed between the various formulations, i.e., 3 h, 2 h, 1 h and no lag time for aqueous solution, water–ethanol solution, mixture empty ethosomes/hydroethanolic drug solution and ammonium glycyrrhizinate/ethosomes, respectively.

The differences between methyl-nicotinate and ammonium glycyrrhizinate in the cumulative amount of drug permeated through human SCE from the same type of formulations were probably due to the different physicochemical properties of the two molecules. As expected [24] and references cited therein, ethanol had permeation-enhancing properties for both methyl-nicotinate and ammonium glycyrrhizinate (Figs. 3 and 4). However, the permeation enhancement observed for all ethosome-based formulations was much greater than that could be expected from ethanol alone. These findings, as previously reported [12], may be due to a synergistic mechanism between ethanol, phospholipid vesicles and skin lipids.

To evaluate a possible correlation between in vitro and in vivo behaviors, different skin penetrations of methyl-nicotinate from ethosomal suspension or aqueous or water–ethanol solutions were monitored in vivo by following the extent of the induced erythema derived from the topical application of various vehicles containing methyl-nicotinate. As previously reported in literature [22], methyl-nicotinate erythema depends on two principal factors: vehicle composition and duration of application. The induced erythema generated a vasodilatory effect, whose intensity was proportional to the methyl-nicotinate present in the dermis. Therefore, considering that the various formulations applied on the skin had the same amount of methyl-nicotinate and that the amount of applied formulation and the application time was absolutely the same for the various formulations any difference in the in vivo profiles of induced erythema between the various formulations was due to the vehicle and hence to its ability to promote the passage of methyl-nicotinate through human skin. The data are expressed as the variation of erythema index as a function of time.

As shown in Fig. 5, erythematogen processes developed with different profiles. Methyl-nicotinate/ethosomes showed an early intensive erythema characterized by significantly ($p<0.001$) higher ΔEI values than those observed for the methyl-nicotinate aqueous solution or the water–ethanol solution. In agreement with the in vitro percutaneous permeation experiments through human skin, the intensity of the induced erythema was proportional to the amount of methyl-nicotinate that permeated through human skin and reached the dermis. In particular, the profile of the

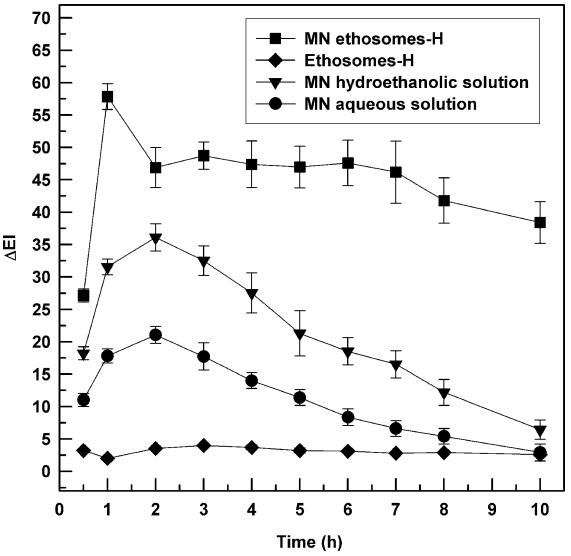


Fig. 5. Erythema index variation measured after topical application of various formulations containing methyl-nicotinate. The variation of ΔEI values as a function of time is directly proportional to the amount of methyl-nicotinate present in the human skin. Legend keys: MN ethosomes-H—methyl nicotinate formulation H ethosomes containing 2% (w/v) Phospholipon 90® and 45% (v/v) ethanol; MN hydroethanolic solution—methyl nicotinate solution in a mixture of water and ethanol at a volume ratio of 55:45; MN aqueous solution—methyl nicotinate aqueous solution. Results were expressed as mean ΔEI ($n=6$) ± standard deviation.

erythematogen response determined by the application of methyl-nicotinate/ethosomes presented a peak at the first hour, and then it was quite constant for a long period (10 h). This situation reflected the in vitro findings, namely ethosomes allowed a rapid passage through human epidermis of methyl-nicotinate thus triggering the immediate induction of erythema and the appearance of the peak observed in Fig. 5.

To evaluate the potentialities of ethosomes to be used as an in vivo carrier for skin delivery of ammonium glycyrrhizinate in the attempt to improve the anti-inflammatory action of this natural compound, another set of experiments was carried out (Fig. 6). Different skin sites were pretreated with a solution of methyl-nicotinate to cause an erythema, and then the formulations containing ammonium glycyrrhizinate were applied. The chemical-induced erythema followed a different profile as a function of the applied formulation (Fig. 6). Ammonium glycyrrhizinate/ethosome formulations were able to reduce the erythema more rapidly with respect to the ethosome free solutions of ammonium glycyrrhizinate. In particular, the induced erythema was not observed in the sites treated with ammonium glycyrrhizinate/ethosome formulations 3 h after the topical application of this formulation. While the sites treated with aqueous or water–ethanol solution of ammonium glycyrrhizinate still showed the presence of the induced erythema. In some experiments, empty ethosomes were used to verify the possible effect of the formulation components on the inhibition of the chemically induced erythema. No anti-inflammatory activity was observed for empty ethosomes (data not reported). These findings demonstrate that ethosomes are able to enhance the percutaneous permeation of ammonium glycyrrhizinate and hence to improve the therapeutic anti-inflammatory response.

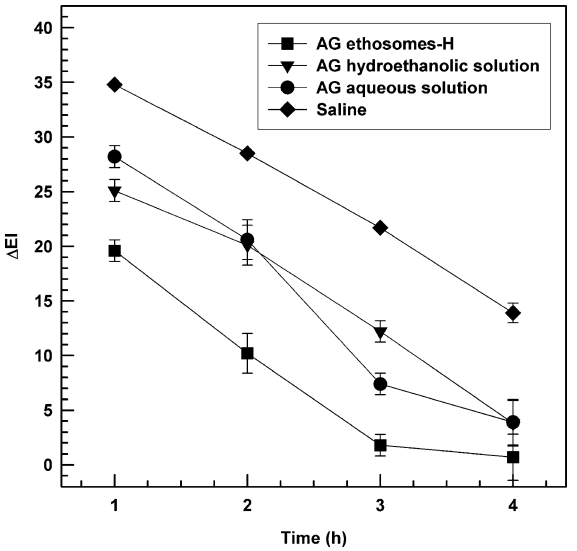


Fig. 6. In vivo anti-inflammatory activity in human volunteers of various formulations containing ammonium glycyrrhizinate (0.3% w/v) evaluated as the ability to reduce a skin erythema, chemically induced by pretreatment with an aqueous solution (100 μl) of methyl-nicotinate (0.2% w/v). Legend keys: AG ethosomes-H—ammonium glycyrrhizinate formulation H ethosomes containing 2% (w/v) Phospholipon 90® and 45% (v/v) ethanol; AG hydroethanolic solution—ammonium glycyrrhizinate solution in a mixture of water and ethanol at a volume ratio of 55:45; AG aqueous solution—ammonium glycyrrhizinate aqueous solution; saline—control saline (0.9% w/v NaCl in water) solution. Results are expressed as a mean value (six different volunteers) of the erythema index variation ± standard deviation as a function of the time.

The sustained effect of ammonium glycyrrhizinate/ethosomes with respect to the aqueous and water–ethanol solutions of ammonium glycyrrhizinate was evaluated by pretreating some forearm sites of healthy volunteers with the various formulations containing the same amount of ammonium glycyrrhizinate (0.3% w/v) for different times (1 h, 3 h and 5 h). Following the pretreatment, the various sites were treated with a methyl-nicotinate aqueous solution (0.2% w/v). The extent of inhibition of the induced erythema depended on the amount of ammonium glycyrrhizinate present at the level of the cutis and hence on the release rate from the various formulations to the skin.

The curves of erythema inhibition induced by methyl-nicotinate treatment are reported in Fig. 7. All the sites pretreated with ammonium glycyrrhizinate/ethosomes were able to antagonize the appearance of erythema, in particular when the pretreatment was carried out for 5 h. These data show that, in the case of the pretreatment with ammonium glycyrrhizinate/ethosomes, the amount of ammonium glycyrrhizinate that permeated through the skin was higher with respect to that observed with the solutions used as controls. In particular, following methyl-nicotinate application, the skin site pretreated with saline showed an erythema peak at 1 h, which was significantly ($p < 0.001$) higher than those observed for the other skin sites pretreated with formulations containing ammonium glycyrrhizinate. In this case, the

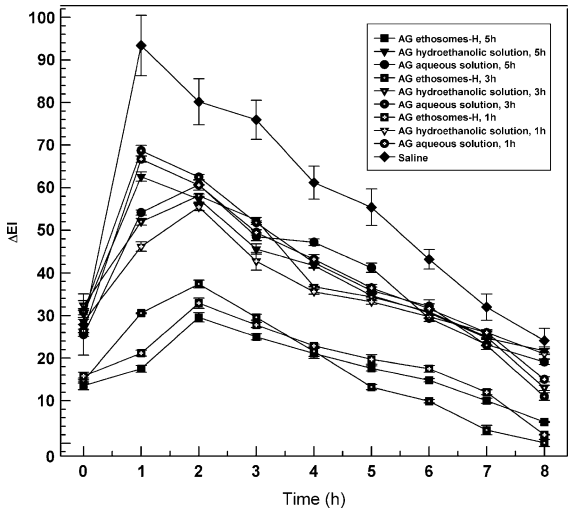


Fig. 7. Inhibition of a skin erythema due to a topical application of a methyl-nicotinate aqueous solution (0.2% w/v) elicited by pretreatment for different time (1 h, 3 h and 5 h) of human volunteers with various formulations containing ammonium glycyrrhizinate (0.3% w/v). Legend keys: AG ethosomes-H—ammonium glycyrrhizinate formulation H ethosomes containing 2% (w/v) Phospholipon 90® and 45% (v/v) ethanol; AG hydroethanolic solution—ammonium glycyrrhizinate solution in a mixture of water and ethanol at a volume ratio of 55:45; AG aqueous solution—ammonium glycyrrhizinate aqueous solution; saline—control saline (0.9% w/v NaCl in water) solution. Results are expressed as a mean value of ΔEI ($n=6$) ± standard deviation as a function of time.

erythema was also more intense with respect to other skin sites during all the duration of the experiments. Interestingly, the sites pretreated with ammonium glycyrrhizinate aqueous formulations showed an erythema peak at 1 h, whereas both water–ethanol and ethosomal formulations showed an erythema peak at 2 h. These findings were probably due to the presence of ethanol that can improve the skin accumulation of ammonium glycyrrhizinate. In the case of the ammonium glycyrrhizinate/ethosomes, this phenomenon was much more pronounced, namely the lowest extent of erythema responses was observed during all the duration of the experiments. Ammonium glycyrrhizinate/ethosomes, due to a combined effect of ethanol and phospholipids, could allow the sustained drug release that was probably determined by the formation of a reservoir of the drug in the skin. Therefore, the presence of ammonium glycyrrhizinate at the level of human skin for a longer period than the pretreatments was able to antagonize the appearance of erythema induced by the application of methyl-nicotinate. These findings are in good agreement with previous observation regarding the in vivo application of a cannabidiol ethosome formulation which produced a significant accumulation of this drug in mouse skin and in the underlying muscle [25].

## 4. Conclusions

The dimensions of empty and ammonium glycyrrhizinate/ethosomes depend on their composition, and in particular the mean size decreases with increasing ethanol concentrations whereas it increases as phospholipid concentrations decrease. This vesicular system was assayed to evaluate the applicative potentialities for clinical use, in this attempt empty ethosomes showed a very good tolerability in human volunteers as carriers for dermal delivery.

The ethosomes elicited an increase of the percutaneous permeation of ammonium glycyrrhizinate both in vitro and in vivo; thus improving the anti-inflammatory activity of this drug in an in vivo model of skin erythema. In addition, in vivo experiments showed that ammonium glycyrrhizinate/ethosomes can ensure a sustained release of the drug and hence a prolongation of its therapeutic activity, which can be related to an accumulation of ammonium glycyrrhizinate in the skin.

These findings are very encouraging and confirm that ethosomes are a very promising carrier for the topical administration due to the enhanced delivery of drugs through the skin thus prompting various opportunities for the development of suitable therapeutic strategies through the topical route.

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