TOPICAL DELIVERY SYSTEM OF LIPOSOMALLY ENCAPSULATED VOLATILE OIL OF *ANETHUM GRAVEOLENS*

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Abstract

An essential step in the characterization of liposomal systems as drug vectors is one that analyzes the release rate of incorporated substances. *In vitro* release tests are used not only to characterize various formulations of therapeutic use, but also to predict their *in vivo* behavior. The objective of this work was to formulate and *in vitro* characterize hydrogels containing liposomal suspension of *Anethum graveolens* volatile oil for topical application, capable of efficient release of the incorporated active principle.

Liposomes composed of phosphatidylcholine and cholesterol and incorporating *Anethi aetheroleum* were prepared by thin film hydration method and dispersed into two types of hydrogels (0.5%, respectively 1% carbopol). For the *in vitro* release kinetics assessment of the entrapped volatile oil we used an adapted Frank diffusion cell. The experimental data were processed to verify different kinetic models, and the obtained results were used to assess the kinetic parameters of the designed hydrogels. Studies have shown that the designed hydrogels follow a 0 order kinetic model after a period of three hours from the beginning of a kinetic experiment. This reinforces the conclusion that liposomes can act as reservoir systems for the controlled release of the entrapped volatile oil.

Rezumat

O etapă esenţială în caracterizarea sistemelor lipozomale ca vectori medicamentoşi este cea care analizează viteza de cedare a substanţelor încorporate. Testele de cedare *in vitro* sunt utilizate nu numai pentru caracterizarea diverselor formulări de uz terapeutic, ci şi pentru a prevedea comportarea lor *in vivo*. Obiectivul studiului întreprins a fost de a formula şi caracteriza *in vitro* hidrogeluri ce conţin suspensii lipozomale cu ulei volatil de *Anethum graveolens* pentru aplicaţii locale, capabile să elibereze eficient principiul activ încorporat.

Lipozomii compuşi din fosfatidilcolină şi colesterol şi încorporand ulei volatil de *Anethum* graveolens au fost preparaţi prin metoda hidratării filmelor lipidice subţiri şi dispersaţi în două hidrogeluri (0.5%, respectiv 1% carbopol). Pentru evaluarea cineticilor de cedare *in vitro* a uleiului volatil înglobat în lipozomii experimentali am folosit un dispozitiv tip celulă de difuzie Frantz adaptată. Datele experimentale au fost prelucrate pentru a verifica diferite modele cinetice, iar rezultatele obţinute, au servit la evaluarea

parametrilor cinetici ai hidrogelurilor proiectate. Studiile au arătat că pentru hidrogelurile proiectate se respectă un model cinetic de ordin 0 după un interval de timp de trei ore de la începerea unui experiment cinetic. Acest lucru întăreşte concluzia că lipozomii pot acţiona ca sisteme rezervor pentru eliberarea controlată a uleiului volatil încapsulat.

Keywords: *in vitro* release, hydrogel, liposomes, volatile oil.

Introduction

Change of consumer demands, increase of public interest, awareness of health requirements, and not at least, the possibility of successfully use of essential oils from plants have opened a new page in modern phytomedicine.

Dill (*Anethum graveolens L.*, Apiaceae) is a common cultured herb, found especially in areas with warm and tropical climates. It is mainly cultivated due to the edible leaves and fruits, but also because of the medical uses. The performed experimental studies demonstrated the antimicrobial, stomachic, antioxidant, carminative properties of dill [3, 7, 15, 18]. Regarding the volatile oil of *Anethum graveolens*, it is used in many fields: aromatherapy, cosmetics and perfumes, food, cuisine, etc. Many researches were conducted in order to demonstrate the antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant properties of essential oils in general and volatile oil of dill in particular. However, there are often significant differences in the reported antimicrobial activity for the same type of volatile oil. This variability may be due to geographical areas, time of harvest, genotype, climate, and harvested plant part; all these factors can influence the chemical composition and relative proportions of components of plant's volatile oil [2, 4, 5, 11, 12, 16].

Because *Anethi aetheroleum* can cause photosensitivity and / or contact dermatitis in some human subjects, it is preferable to incorporate it into an adequate vehicle (system). A convenient method would be the entrapment of the volatile oil in liposomes, which are vehicles with the ability to incorporate both hydrophilic and lipophilic substances [6].

Liposomal formulations are widely used in the pharmaceutical field as drug delivery systems due to their versatility and clinical efficacy; they have been used to administer drugs by oral, parenteral, and topical routes [1]. Applied on the skin, liposomes may act as a solubilizing matrix for poorly soluble drugs, penetration enhancer as well as local depot, at the same time diminishing the side effects of these drugs. Topical liposome formulations could be more effective and less toxic than conventional formulations [1]. However, topical formulations, like all other formulations, must be formulated, manufactured and conditioned in order to ensure quality

regarding bioavailability, chemical and physical stability, and absence of microbial contamination. To meet all these conditions, liposomes should be incorporated in an adequate drug delivery and release system. Studies have shown that incorporation of liposomes in hydrogels for topical or transdermal administration has a number of advantages, such as handling ability, ease of application, appropriate rheological properties and thus higher retention time at the application site, good compatibility with tissues, improvement of their stability [10, 13].

Given these considerations, the main aim of this work was conditioning of *Anethum graveolens* volatile oil extracted from plants grown in Romania, with antifungal activity demonstrated in a previous study [8], in a formulation for topical administration.

Materials and Methods

Phosphatidylcholine (PC) was purchased from Sigma – Aldrich and cholesterol p.a. (Chol) from Fluka. Carbopol 940 and glycerin were purchased from Alpha Pharm, and triethanolamine (TEA) from Fluka. All solvents used were of analytical or HPLC grade and were purchased from Merck. *Anethum graveolens* volatile oil (VO) was extracted in our laboratory and assessed by gas chromatography. All other materials were analytical grade or equivalent.

Preparation of Liposomes

Multilamellar vesicles (MLV) were prepared according to the thin film hydration method. Lipid solutions were prepared by dissolving precise amounts of phosphatidylcholine, cholesterol and essential oil in chloroform. 5.0 mL of each solution was introduced in a 100 mL round-bottomed flask. The solvent was evaporated in a Heidolph Laborota 4000 rotaevaporatory**,** at 35°-40°C, under reduced pressure (13-14 mm Hg). The obtained dry lipid film was hydrated with 5 mL distilled water. The mechanical stirring of the lipids in aqueous medium was performed with the rotaevaporatory equipment at 37°C and by manual stirring in the water bath, for 2 h, at the same temperature. This suspension was allowed to hydrate for 2 h in order to anneal any structural defects. Unilamellar vesicles (SUV) were obtained by sonication of the MLV liposomes, in a bath-type sonicator (Sono Swiss SW 6L) for 30 min. (6x5min.). The optimal formulation of volatile oil entrapped liposomes was prepared, based on an earlier conducted study [8]. Two lots of liposomes were prepared, the final composition of the liposomes used in gel preparation, expressed in mg/mL, were PC : Chol : VO = 19.5 :2.6 :9, 39 : 2.6 : 9 respectively (L1, respectively L2).

Entrapment efficiency

The compound incorporated vesicles were separated from the unincorporated compounds by centrifugation. Vesicular dispersions were spun in a laboratory centrifuge Hettich Universal 320 R, at 10°C, 10000 rpm, for 60 min. The supernatant was removed and the liposomes were reconstituted with 5 mL distilled water. The quantity of volatile oil encapsulated in SUV vesicles was spectrophotometrically determined using a Perkin-Elmer Lambda 2 Spectrophotometer and the entrapment efficiency of the volatile oil was calculated.

Preparation of liposomal gels

G1: approximately 0.5 g Carbopol 940 were dispersed in water under mild stirring and allowed to swell for 24 h. Then, 12 g of glycerin were added. The obtained dispersion was neutralized with 0.4 g TEA to pH = 7. The preparation was filled up to 100g with 7.2 g of distilled water. G2: the 1% Carbopol 940 hydrogel was similarly prepared.

The incorporation of the liposomes into gels was achieved by slow mechanical mixing (25 rpm), using a Velp Scientifia type BS stirrer, for 10 min. The first experimental gel was obtained by mixing 92g 0.5% Carbopol base hydrogel and 10 mL liposomal suspension L1, respectively L2 (G1L1, G1L2). G2 experimental gel was similarly prepared, using 1% Carbopol base hydrogel and 10 mL liposomal suspension L1, respectively L2 (G2L1, G2L2).

***In vitro* release kinetics study of *Anethi aetheroleum* from experimental gels.**

The tested systems are represented by four hydrogels containing liposomes L1 and L2.

For evaluating the *in vitro* release kinetics of volatile oil entrapped in experimental liposomes we used an adapted Franz diffusion cell. Receptor solution was a mixture of 7.4 pH phosphate buffer: ethanol in a volume ratio 3:1. Artificial membrane was soaked before use in receptor solution for 24h and then fitted on the adapted Franz diffusion cell, thickness being determined with a Starret lever micrometer (0.07 mm). An accurately weighed quantity of gel (1 g) was placed on the membrane in each case.

At appropriate time, 5 mL of the sample was withdrawn from the receptor compartment and the same amount of fresh solution (same temperature as receptor solution) was added to keep the volume constant. The samples were analyzed spectrophotometrically, using a UV-Vis Perkin-Elmer spectrophotometer. Each data point represented the average of three determinations. The experimental data were processed to verify different

kinetic models and the results have been used for evaluation of the kinetic parameters of designed hydrogels. *In vitro* release studies were recorded for a 24-hour period.

Results and Discussion

The entrapment efficiency (E%) of tested liposomes was calculated by:

$$E = \frac{T - S}{T} 100$$

where: T – the total amount of volatile oil from supernatant and sediment (measured after disruption of liposomes with methanol) and S – the quantity of volatile oil from supernatant.

The spectrophotometric method was validated in a previous study [15]. The carvone amount from *Anethi fructus* volatile oil (carvone is the main component of *Anethi fructus*, 75.21% concentration) was estimated at λ = 236 nm. The calculated values of the entrapment efficiency for the two lots of experimental liposomes were: L1: 85%, respectively L2: 86.5%.

Liposome gels were subjected to a rheological study, which allowed us to select those with a non-Newtonian, pseudoplastic, thixotropic behavior at both considered temperatures (23°C, temperature of the hydrogels storage, respectively 37°C, temperature for kinetic experiments). This type of rheological behavior indicates an appropriate spreadability in case of topical administration of the tested hydrogels [9].

Kinetic analysis completes the complex problem of the study of the active principle release from topical formulations, by the possibility of highlighting the active substance release mechanism from designed system and the availability for cutaneous absorption.

In order to determine the influence of formulation type on the release rate, experimental tests were conducted for all proposed liposomal gels, ie G1L1, G2L1, G1L2, G2L2. Sampling was done every half hour in the first phase, then every hour. Samples were spectrophotometrically analyzed to determine the released mass of carvone, respectively volatile oil. Data obtained, *i.e.* the cumulative amount of released substance at time t_0 and time t were processed for fitting to the following kinetic models: zero order, first order, Higuchi, Korsmeyer – Peppas, in order to evaluate the kinetic parameters characteristic to the release of *Anethum fructus* volatile oil from liposomal formulations from designed gels. The processed data were used for plotting the release profiles for each kinetic model and for the evaluation of the kinetic equations and corresponding correlation coefficients for all the experimental gels.

The analysis of the graphs showed that all formulations are fitting to a Higuchi kinetic model over a period of 7.5 hours (figures 1-4). We also found a value close to 0.5 of the release exponent of Korsmeyer-Peppas model, indicating linearity between the released quantity of drug/unit area and $t^{1/2}$, according to Fick diffusion.

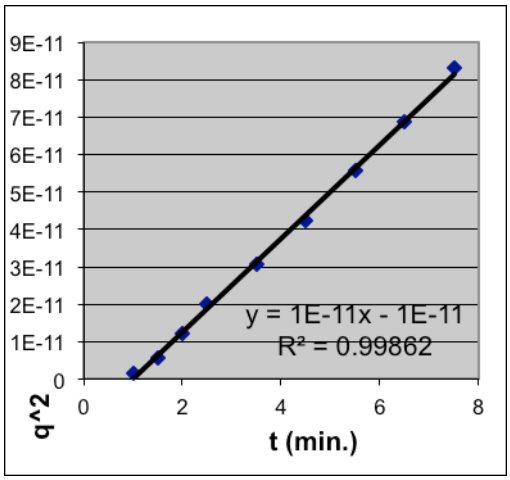

Figure 1
Higuchi model for G1L1

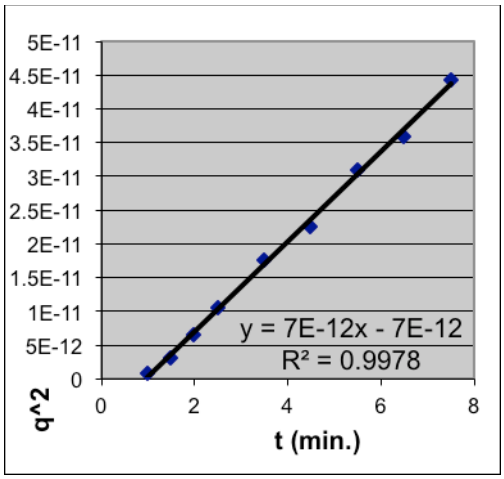

Figure 2
Higuchi model for G2L1

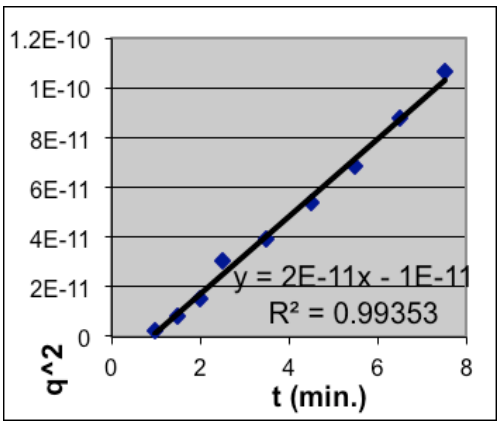

Figure 3
Higuchi model for G1L2

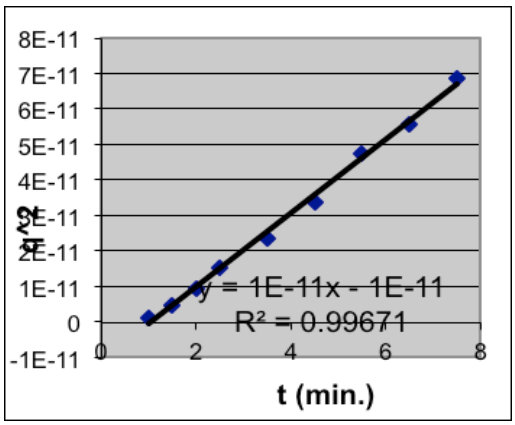

Figure 4
Higuchi model for G2L2

The release rates in case of Higuchi model, for each tested gel, show that there is a decrease of these tares with increased amount of carbopol from formulations. Table I presents regression equations, correlation coefficients and percentages of active substance released in 24h for formulations G1L1, G2L1, G1L2, G2L2.

Table I

Values of kinetic parameters in the release process of volatile oil from G1L1, G2L1, G1L2, G2L2

Gel type	G1L1	G2L1	G1L2	G2L2
Regression equations	10^{-11}x-10^{-11}	$7\cdot10^{-12}$x -$7\cdot10^{-12}$	$2\cdot10^{-11}$x -10^{-11}	10^{-11}x-10^{-11}
Release rate·10^{-11} g/h	1	0.7	2	1
Correlation coefficient R^2	0.9986	0.9978	0.9935	0.9967
Diffusion coefficient D (cm^2/s)	$2.21\cdot10^{-9}$	$1.55\cdot10^{-9}$	$4.18\cdot10^{-9}$	$2.10\cdot10^{-9}$
Released percentage in 24 h	18.48	13.39	20.12	15.84
Latency time (h)	1.03	1.46	0.81	1.08

Diagram $q^2 = f(t)$ plotting confirms the applicability of the Higuchi equation:

$$D = \frac{q^2}{4\,tC_0^2}\pi$$

where q - released quantity of drug/unit area.

This equation is valid when the released substance is less than 30% of the total amount present in the mixture of excipients.

Table I also presents the diffusion coefficients values, for each tested hydrogel, calculated by the above equation. Studies found in literature showed that most of the active molecules are absorbed through the skin by passive diffusion [14]. It was assumed that there was an initial period, preceding the appearance of active principle with constant release rate in the receptor compartment, latency time (T_l), which can be calculated using the equation:

$$T_l = \frac{h^2}{6D}$$

where: h = thickness of the membrane (0.007cm), D = diffusion coefficient.

The latency times mentioned in Table I were calculated based on this equation. It can be noticed the correspondence between calculated latency time and starting point of the straight lines corresponding to mathematical equations of Higuchi kinetic model.

Analysis of data in table I shows that the release rate of the volatile oil from analyzed gels is lower for formulations with higher concentration of carbopol for each type of liposomes. The same trend can be observed in both percentage of released active substance after 24 hours and diffusion coefficient from hydrogels. This behavior can be explained by the higher value of viscosity for G1 gel versus G2 gel.

For the 0 order kinetic model, after an interval of three hours from the beginning of a kinetic experiment, correlation coefficient values are very

close to or even higher than the ones in Higuchi model (Table II). This suggests that liposomes can act as reservoir systems for controlled release of encapsulated volatile oil.

To monitor the stability of prepared gels they were analyzed immediately after preparation, at 2 and respectively 4 weeks after preparation. Experimental gels were packed in plastic tubes and maintained during this period at 4 ° C. In this period there were no changes in the organoleptic properties of all tested formulations, the pH values of all gels was within the range of ± 0.3 pH units from baseline. Also, there were no major changes in the release profiles of volatile oil from experimental gels (Table II).

Table II

Kinetic parameters values for the tested formulations during 1 month of storage

Sample	Time	n	R^2	0 order kinetic model (3 – 7.5h)		Higuchi model	
				$k \cdot 10^5$	R^2	$K \cdot 10^{11}$	R^2
G1L1	Freshly prepared	0.53	0.9537	7	0.9971	1	0.9977
	2 weeks			6.8	0.9968	1	0.9973
	1 month			6.6	0.9969	1	0.9974
G2L1	Freshly prepared	0.54	0.8979	5	0.9963	0.7	0.9954
	2 weeks.			4.9	0.9978	0.6	0.9956
	1 month			4.8	0.9967	0.56	0.9975
G1L2	Freshly prepared	0.54	0.9142	9.8	0.9977	2	0.9854
	2 weeks.			9.6	0.9970	1.9	0.9958
	1 month			9.7	0.9975	1.7	0.9953
G2L2	Freshly prepared	0.56	0.9113	7.3	0.9986	1	0.9951
	2 weeks.			7.1	0.9985	1	0.9976
	1 month			6.9	0.9976	1	0.9954

Overall efficiency of a topical semisolid formulation, translated by adhesion to treated surface in required time in order to ensure compliance with patient and a convenient release rate of the active principle for a rapid start of the activity, depends both on the pharmacokinetics of the active principle and the properties of the vehicle. An optimal hydrogel formulation with *Anethi aetheroleum* should allow maximum thermodynamic activity of the active principle, ensuring a high flow of the

volatile oil [17]. Of all the formulations tested, gel G1L2 best meets the above requirements.

Conclusions

In vitro release studies showed that the designed hydrogels follow a 0 order kinetic model after a period of three hours from the beginning of a kinetic experiment (correlation coefficient values are very close to or even higher than the ones in Higuchi model). This reinforces the conclusion that liposomes can act as reservoir systems for controlled release of encapsulated volatile oil. Stability experiments showed that there were no major changes in the release of volatile oil profiles from experimental gels for a period of one month.

This study suggests that gels containing volatile oil entrapped in liposomes can confer optimal therapeutic effects, due to local controlled release, which leads to increased efficiency and compliance, in addition to increased stability of experimental topical formulations.

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