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IMPROVEMENT OF ENCAPSULATION EFFICIENCY OF TIMOLOL MALEATE IN LIPOSOMES BY THE FREEZE - THAWING METHOD

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ABSTRACT

The Freeze-thawing (FT) technique was adopted in order to improve the encapsulation efficiency of timolol maleate in liposomes. The results were compared with those obtained by the conventional hydration (HY) method. The FT liposomes showed higher encapsulation efficiency and lower permeability properties than the HY liposomes. The influence of surface charge, drug and lipid concentration, presence of sucrose and ionic strength of the medium on the encapsulation efficiency of FT liposomes was also investigated. The results revealed that the formation of large liposomes by this technique which probably results from fusion of vesicles is strongly inhibited by increasing the ionic strength, by the presence of sucrose and by increasing the lipid concentration of liposomes.

INTRODUCTION

Since the discovery of lamellar structure in liposome by Bangham et al (1), many researchers have studied liposomes extensively as a model of biological membranes or as a carrier material for drugs (2,3).

However,in order for liposomes to be used more widely for therapeutic purposes, the preparation process should satisfy the following standards: (1) a high degree of drug encapsulation; (2) organic solvent or detergent can be completely removed; (3) sterilization can be carried out easily; (4) the final product is obtainable by simple procedure; (5) preparation can be carried out on a large scale; and (6) stability is good enough to guarantee the quality for an appropriate storage period.

So far, various methods such as the solvent vaporization method (4), detergent removal method (5) and ether injection method (6) have been proposed by many researchers and their comparative properties and practical usefulness have been discussed (7). However, almost all of these methods require the use of organic solvents or detergents and do not meet the above criteria. In order to overcome the above problems, the freeze-thawing (FT) method (8,9) has been devised for preparing liposomal formulations which are applicable for therapeutic use. The ability of ß blockers to interact with liposomal bilayers has been previously studied (10,11). The encapsulation of propranolol, a lipophilic drug and atenolol, a hydrophilic drug was measured in multilamellar and unilamellar liposomes (12).

In this study, timolol maleate was used as a model drug to measure the encapsulation efficiency and release characteristics from freeze-thawed liposomes. In addition, the effects of ionic strength, drug concentration, and phospholipid concentration on the properties of the freeze-thawed liposomes were investigated and compared with those prepared by the conventional thin film method.

EXPERIMENTAL

MATERIALS:

Timolol maleate (TM) was a gift from the Egyptian International Pharmaceutical Industries CO. (EPICO). Egg phosphatidylcholine (EPC; about 90%, BDH, U.K.) was subsequently purified as described by Bangham et al (13), cholesterol (Chol;99%) and dicetylphosphate (DCP) were obtained from Sigma (U.S.A.).

APPARATUS:

Eppendorf netheler-hinz centrifuge(west Germany), Shimadzu spectrophotometer 260 (Japan) and Buchi rotavapor (Switzerland).

METHODS:

Preparation of Liposomes

Hydration (HY) Method:

HY liposomes were prepared by the conventional method originated by Bangham et al. (1) with slight modifications. mixed lipid films of, pure EPC, cholesterol (50 mole %)/pure EPC and dicetylphosphate (DCP, 5,10 and 20 mole %)/pure EPC were prepared. Sufficient lipid, drug and aqueous phase were mixed to form 5 ml of a liposome suspension containing 20 mg ml^{-1} lipid and 10 mg ml^{-1} drug.

Freeze Thawing (FT) method:

FT liposomes were prepared essentially as described by Ohsawa et al (14). 2 ml of HY liposomes were taken in a glass vial, frozen at-20°C by incubation in a freezer and kept at that temperature for 24h. the frozen mixture was thawed at room temperature and subsequently shaken with a vortex mixer for 20 min.

Determination of TM Entrapment by Liposomes

Two ml samples were centrifuged at 14,000 r.p.m. for 30 min. The supernatant was separated from the liposome pellet and prepared for assay of free drug. Each result is the mean of three determinations (±S.D.).

Assay for TM

A UV assay was employed. A calibration curve of UV absorbances at 292 nm versus concentration was constructed for solutions containing 10 to 100 $\mu g\ ml^{-1}$ of TM in water or 0.9% sodium chloride.

Assessment of drug efflux rates from liposomes

The efflux of TM was determined according to the method of Alpar et al (15). The equilibrated liposomes were centrifuged at 14,000 r.p.m. for 30 min. The supernatant was discarded and the pellet of liposomes resuspended in 25 ml 0.9% sodium chloride. The suspension was placed in a stoppered glass flask in a shaking water bath at 37°C. At zero time and consequently at 1,2,4,6,8 and then at 24 h intervals, aliquots were centrifuged as before and the supernatants assayed at 292 nm. Total release of drug was determined from the concentration of TM in diluted preparations estimated in the presence of absolute ethanol. The first-order efflux rate constants (K, hr^{-1}) for TM from liposomes were calculated from least squares linear regression analysis of plots of ln latency versus time (16).

RESULTS AND DISCUSSION

Evaluation of Encapsulation Efficiency (EN%) of FT Liposomes

Liposomes prepared by the FT technique resulted in more than two fold increase in TM entrapment compared to HY liposomes (Table 1). A mechanism outlining the events during FT cycle has been proposed by Ohsawa et al (17). In freezing, drugs and liposomes are concentrated, particles are closely packed in contact with each other and consequently fusion of liposomes takes place. During thawing, large aggregates which include the drug in its inner space are formed. After shaking, liposomal particles are formed entrapping efficiently the drugs.

Effect of Surface Charge on The Encapsulation Efficiency of FT Liposomes

Increasing the DCP component of the lipid phase to 5, 10 and 20 mole% gave proportionately greater uptake of TM at equilibrium (Table 2). DCP is commonly incorporated into bilayers to confer a negative charge into liposomes. In this study it was apparent that DCP substantially improved uptake of TM by ion-pair formation to form a lipophilic moiety (16).

Effect of Drug Concentration on Encapsulation Efficiency of FT Liposomes

Increasing TM concentration from 5mg ml^{-1} to 20 mg ml^{-1} resulted in an increase in drug entrapment expressed per 100 mg lipid (EU) corresponding to a nearly constant value for the

proportion of total aqueous volume encapsulated (EN%) within the liposomes (Table 3). TM is soluble in the aqueous phase and insoluble in the lipid phase, probably therefore existing in the aqueous phase before the generation of liposomes by thawing (18). As the percentage drug encapsulation (EN%) depends upon the amount of the aqueous phase enveloped in the phospholipid agglomerates during the thawing process, the result that EN% scarcely changed with increasing drug concentration suggests that the enveloped aqueous phase was almost the same irrespective of drug concentration.

Effect of Total Lipid Concentration on Encapsulation Efficiency of FT Liposomes

The influence of total lipid concentration on the amount of TM encapsulated in EPC/Chol(1:1) FT liposomes is shown in Table 4. EN% increased from 10.8% to 16.7% as the lipid concentration was increased from 10 mg ml^{-1} to 30 mg ml^{-1}. However, the amount of encapsulated TM per 100 mg lipid(EU) decreased as the lipid concentration increased. This means that the fraction of lipid taking part in encapsulation decreases as the concentration of lipid increases. A similar decrease in asparaginase entrapment expressed as(mg per one gram Yolk phospholipid) was previously observed when the lipid concentration of FT liposomes was increased from 2.5% to 20% (18).

Effect of Ionic Strength of The Suspension Medium on Encapsulation Efficiency of FT Liposomes

The composition of the aqueous phase used to prepare FT liposomes was varied to investigate the effect of ionic strength on the encapsulation of TM in EPC/Chol (1:1). As shown in Table 5, the entrapment of TM rapidly decreased as the concentration of sodium chloride increased from 0-0.2 M in the aqueous phase.

Ohsawa et al (19) suggested that in the presence of sodium chloride, the aqueous phase remains unfrozen, because the eutectic point of NaCl (-21°C) is below the freezing temperature (-20°C). Thus the lipid particles are dispersed in a larger space during the freezing process than in the case without any Nacl and aggregates large enough to give high entrapment do not occur. As a conclusion, the ionic strength of the suspension medium should be as low as possible in order to obtain liposomes of high encapsulation efficiency by the FT method.

Effect of Sucrose on the Encapsulation Efficiency of FT Liposomes

The effect of sucrose on the encapsulation of TM in EPC/Chol(1:1) FT liposomes is shown in Table 6. Increasing sucrose concentration from 0-4 g sucrose/g lipid in the aqueous medium resulted in a marked decrease in drug entrapment. This probably may be due to the ability of sucrose to prevent aggregation and fusion of liposomes at very low temperatures by forming hydrogen bonds with the lipid phosphate head groups (20).

Efflux of TM from Liposomes

Fig 1 shows leakage of TM from HY and FT liposomes composed of equimolar EPC and Chol. The liposomes were prepared to contain the same concentrations of lipid. The release profile of TM from FT liposomes shows an apparent biphasic release process with a rapid release of drug following dilution. The phase of rapid loss may be due to desorption of TM bound to the liposome surface. TM latency post 3h was maintained more efficiently by FT than HY liposomes such that efflux half-lives were 127.4 h and 46.2 h for FT and HY liposomes respectively. This is presumably due to the increased lamellarity of FT compared to HY liposomes due to aggregation and fusion of the vesicles. Each lamella of lipid represents a barrier to the fusion of materials from liposomes.

The chief virtues of the FT technique compared with other procedures which are capable of high encapsulation efficiency are its mildness and simplicity. Moreover, multilamellarity has the advantage of decreasing the rate of loss of diffusible entrapped solutes. The only disadvantage of the method is that sugars and high ionic strengths have to be avoided during the freezing and thawing procedure since they interfere with the fusion process and reduce the trapping capacity.

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تحسين معدل تحوصل عقار ماليات التيمولول في الليبوزومات المحضرة بطريقة التجميد والتسييح

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تم في هذا البحث تحضير الليبوزومات بطريقة التجميد والتسييح بهدف زيادة معدل تحوصل عقار ماليات التيمولول في الليبوزومات وعندمقارنة الليبوزومات المحضرة بطريقة التجميد والتسييح بالليبوزومات المحضرة بالطريقة التقليدية وجد أن هذة الليبوزومات لها قدرة عالية علي احتواء العقار ولكن معدل انطلاق العقار منها كان أقل من مثيلتها المحضرة بالطريقة التقليدية .
كما تم دراسة تأثير الشحنة الكهربائية وتركيز كل من العقار والمواد الدهنية وتأثير وجود مادة السكروز وكذا تأثير القوة الأيونية للوسط علي معدل تحوصل الليبوزومات المحضرة بطريقة التجميد والتسييح وقد وجد أن تكوين ليبوزومات كبيرة الحجم نتيجة لالتحام الحويصلات قد تم تثبيطة بدرجة كبيرة نتيجة لزيادة القوة الايونية ولوجود مادة السكروز وأيضا نتيجة لزيادة تركيز المادة الدهنية في الليبوزومات.

Table 1. Comparison of Encapsulation Efficiency for TM of FT Liposomes with that of HY Liposomes.

Lipid Composition (mole ratio)	Entrapment (% of total) HY	FT
EPC	3.10 (0.06)	7.1 (0.11)
EPC/Chol (1:1)	5.91 (0.16)	14.2 (0.12)

Table 2. Effect of DCP Concentration on the Entrapment of TM in FT Liposomes.

Lipid composition(mole %)		Entrapment
EPC	DCP	(% of total)
100	0	7.1 (0.11)
95	5	10.5 (0.09)
90	10	13.1 (0.15)
80	20	16.4 (0.21)

Table 3. Effect of TM Concentration on the Amount of Drug Encapsulated in EPC/Chol(1:1) FT Liposomes.

TM Concentration	TM Entrapped	
(mg ml^{-1})	% of total (EN %)	mg mg^{-1}% (EU)
5	13.9 (0.08)	3.48 (0.02)
10	14.2 (0.12)	7.1 (0.06)
15	14.5 (0.61)	10.9 (0.43)
20	14.3 (0.21)	14.3 (0.21)

Table 4. Effect of Lipid Concentration on the Encapsulation of TM in ECP/Chol (1:1) FT Liposomes.

Lipid Concentration ($mg\ ml^{-1}$)	TM Entrapped	
	% of total (EN %)	$mg\ mg^{-1}$ % (EU)
10	10.8 (0.10)	10.8 (0.10)
20	14.2 (0.12)	7.1 (0.06)
30	16.7 (0.20)	5.6 (0.03)

Table 5. Influence of Ionic Strength of Aqueous Phase on Entrapment of TM in EPC/Chol(1:1) FT Liposomes.

Sodium Chloride Concentration (M)	Entrapment (% of total)
0.00	14.2 (0.12)
0.01	12.8 (0.02)
0.05	10.2 (0.06)
0.10	9.1 (0.04)
0.15	7.6 (0.11)
0.20	5.9 (0.08)

Table 6. Influence of the Addition of Different Concentrations of Sucrose on Entrapment of TM in EPC/Chol (1:1) FT Liposomes.

sugar concentration (g sucrose / g lipid)	Entrapment (% of total)
0.0	14.2 (0.12)
0.1	13.3 (0.09)
0.5	12.1 (0.42)
1.0	10.3 (0.67)
2.0	8.9 (0.21)
3.0	7.1 (0.11)
4.0	5.9 (0.05)

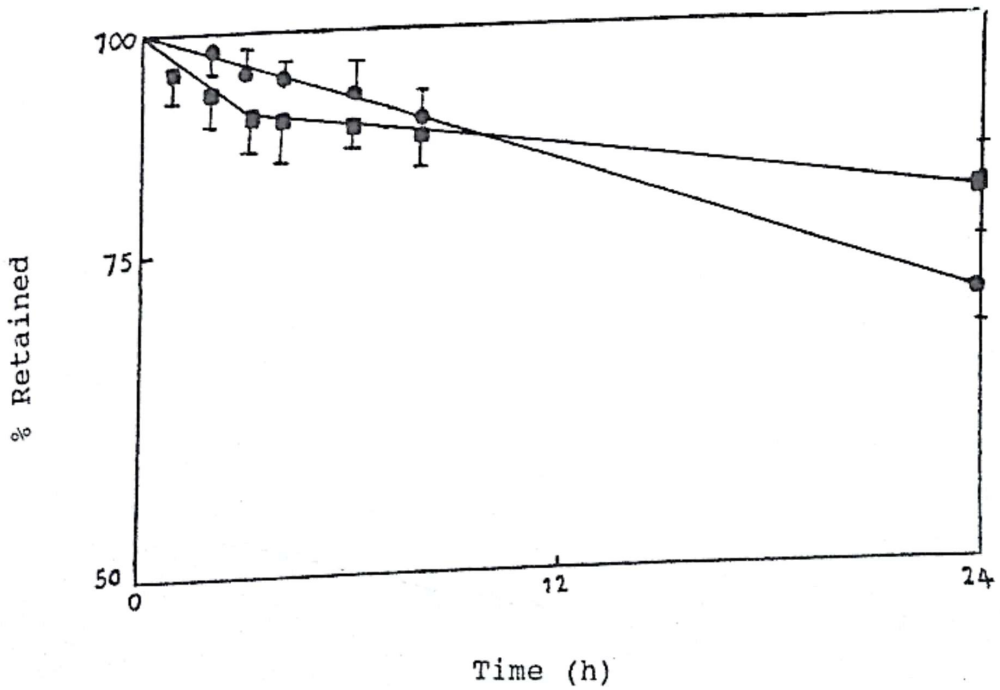

Fig 1: Efflux of TM from EPC/ Chol (1:1) HY(●) and FT (■) liposomes into 0.9% Nacl at 37^0 c