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ENTRAPMENT AND RELEASE OF CAPTOPRIL AND PROPRANOLOL HYDROCHLORIDE FROM DIFFERENT LIPOSOMAL FORMULATIONS

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ABSTRACT

Different types of liposomes composed of a variety of lipids have been compared for their ability to incorporate captopril and propranolol hydrochloride. Multilamellar liposomes (MLV) prepared from egg phosphatidylcholine (EPC) allowed only low levels of entrapment for both drugs, but the entrapment was increased by inclusion of cholesterol or charged lipids into the vesicle bilayer. The freeze - thawing vesicles (FTV) showed higher encapsulation efficiency and lower permeability properties than MLV. The influence of surface charge, presence of trehalose and ionic strength of the medium on the encapsulation efficiency of FTV 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 and by the presence of trehalose. Producing liposomes by the reverse - phase evaporation technique (REV) resulted in more than 2-fold increase in liposomal uptake of both drugs compared to MLV of the same composition. The rate of drug efflux from liposomes was determined in-vitro and was dependent upon bilayer composition and the method of preparation.

INTRODUCTION

Since the discovery of lamellar structure in liposomes by **Bangham et al.** [1], many researchers have studied liposomes extensively as a model of biological membranes or as carriers for drugs [2-9]. 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) preparation can be carried out on a large scale, 4) sterilization can be carried out easily and 5) stability is sufficient to guarantee the quality for an appropriate storage period [10]. Various methods such as the hydration thin film method [1], detergent removal method [11] and ether injection method [12] have been utilized by many researchers and their comparative properties and practical usefulness discussed [13]. 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 these problems, the freeze - thawing method [14] has been devised for preparing liposomal formulations which are applicable for therapeutic use.

In order to investigate the *in-vivo* behaviour of liposomes, it is necessary to study the *in-vitro* release rate of an entrapped drug. The rate of release of a molecule from liposomes is governed by the material's physico-chemical properties [15]. Liposomes are freely permeable to water, but cations are released at a slower rate than anions [1], whereas aqueous hydrogen bonding may determine the efflux rate of non-electrolytes [16]. The degree of disorder of the lipid bilayer determines the permeability of liposomes. Phospholipids in the liquid crystalline state are more permeable to entrapped material than when they are in the gel state. Thus, loss of entrapped material is temperature dependent, generally being greatest around the phospholipid phase transition temperature (Tc) [17]. At Tc, rapid efflux of material has been attributed to passage through regions of high bilayer disorder, where gel and liquid crystalline states temporarily coexist. The incorporation of cholesterol into liposomal bilayers decreases the rotational freedom of the phospholipid hydrocarbon chains. At 50 mole % cholesterol the phase transition is lost, the efflux rate of cations is decreased, and the release rate exhibits little temperature dependence [18]. It is apparent that water soluble drugs are poorly incorporated into liposomes compared to more hydrophobic compounds [19]. However, the presence of charged lipid species electrostatically increases the spacing between phospholipid bilayers [20] causing an increase in the volume of the aqueous compartments and thus in the amount of hydrophilic drug entrapped [21].

The purpose of this research was to study the characteristics of captopril (CAP) and propranolol hydrochloride (PPL) liposomal formulations. The entrapment efficiency and drug release rates were studied in the absence and presence of cholesterol and charged lipids.

MATERIALS AND METHODS

MATERIALS :

Captopril (CAP) was a gift from Bristol Mayersquibb Company U.K. Propranolol hydrochloride (PPL) was obtained from Kahira for pharmaceuticals and Chemical Industries Company, Cairo, Egypt. Egg phosphatidylcholine (EPC, about 90%) was obtained from BDH Chemicals Ltd, U.K. and subsequently purified as described by **Bangham et al** [22]. L-α-dimyristoyl phosphatidylcholine (DMPC), L-α-dipalmitoyl phosphatidylcholine (DPPC), cholesterol (Chol; 99%), stearylamine (SA), dicetyl phosphate (DCP) and trehalose dihydrate were purchased from

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Sigma Chemical Co., St. Louis, MO, USA. Aluminium oxide, active, neutral, Brochman grade I was supplied by BDH Chemicals Ltd, U.k. All other chemicals and solvents were Analar grade and obtained from El-Nasr Company for Pharmaceutical Chemicals, Cairo, Egypt.

METHODS :

Preparation of liposomes :

1. Multilamellar vesicles (MLV)

The method of preparation of MLV has been published extensively [19]. Briefly, the required amount of phospholipid, with charged lipid and /or cholesterol if required, was weighed into a 50 ml quick fit round -bottom flask and dissolved in a small volume of chloroform. Organic solvent was slowly removed under reduced pressure on a rotary evaporator at 40°C such that a thin film of dry lipid was deposited on the inner wall of the flask. 5 ml of the aqueous phase containing either CAP or PPL was added at 40°C for EPC liposomes and at a temperature 15°C in excess of the lipid Tc for synthetic lipid liposomes (i.e. DMPC = 38°C; DPPC = 56°C). The flask was maintained at that temperature for 1 hr, then shaken on a mechanical agitator for 2 min, to produce MLV which were annealed for 1 hr at a temperature of lipid film hydration [23].

2. Freeze - Thawing Vesicles (FTV)

FTV were prepared according to the method of **Pick** [14]. 2 ml of MLV were introduced in a glass vial, frozen at - 20°C by incubation in a freezer and kept at that temperature for 24 hr. The frozen mixture was thawed at room temperature and shaken using vortex mixer for 20 min.

3. Reverse - phase evaporation vesicles (REV)

REV liposomes were prepared according to the method of **Szoka and Papahadjopoulos** [24]. Lipid components were weighed into 100 ml long - necked quick fit round bottom flask and dissolved in chloroform : diethylether (1: 1). Aqueous phase containing the drug was added to the organic phase in a ratio of 1: 6 . The mixture was mixed for 10 min using vortex mixer. A stable emulsion was produced, from which the organic solvent was slowly removed at 45°C using a rotary evaporator. The liposomes were annealed for 1 hr at a temperature exceeding the phospholipid Tc.

Determination of drug entrapment in liposomes

Aliquots of 2 ml liposome dispersions were withdrawn and centrifuged at 14,000 r.p.m. for 30 min. The supernatant containing the free drug was separated and assayed spectrophotometrically at 265 and 318 nm for CAP and PPL respectively. The amount of entrapped drug was determined by difference between the total drug concentration and the concentration of the drug in the supernatant. The amount entrapped was expressed as mg drug/ 100 mg lipid.

Assessment of drug leakage rates

The leakage of CAP and PPL was assessed by periodic centrifugation of samples following a 1: 100 dilution of the initial preparation with saline. Diluted preparations were shaken in a water bath maintained at 37°C. Duplicate, 3 ml samples were centrifuged at 14,000 r.p.m for 30 min and the supernatant assayed at 265 nm and 318 nm for CAP and PPL, respectively.

RESULTS AND DISCUSSION

1. Entrapment and Release of CAP and PPL from Multilamellar vesicles (MLV)

The effect of cholesterol incorporation on the entrapment of CAP and PPL in EPC MLV is shown in Table 1. Inclusion of 33 and 50 mole % cholesterol into liposomes resulted in an increase in entrapment by 16.5 and 63% for CAP and by 13 and 45% for PPL. The increase in drug entrapment in liposomes in the presence of cholesterol is a function of the cholesterol - induced increase in vesicle size. The inclusion of cholesterol into bilayers at greater than 30 mole % was reported by **Johnson** [25] to increase the diameter of liposomes. The efflux profiles for PPL from EPC and EPC/ Chol (1: 1) MLV liposomes are shown in Fig. 1. Further studies with EPC vesicles were not undertaken due to the observed rapid release. The efflux of entrapped material is temperature dependent. Increasing temperature increases the degree of bilayer disorder. Molecules of EPC are in the fluid liquid crystalline state at 37°C, consequently drug loss is rapid through the highly disordered liposomal phospholipid bilayers. Incorporation of cholesterol into EPC MLV progressively increased the retention of PPL within the diluted liposomes. Cholesterol modulates membrane fluidity by restricting the movement of the relatively mobile hydrocarbon chains, which is associated with a loss of bilayer permeability [9].

Table (1): Entrapment of CAP and PPL in EPC MLV liposomes.

Lipid composition (mole ratio)	Entrapment (mg mg^{-1}%)	
	CAP	PPL
EPC	3.75 (0.08)	1.85 (0.05)
EPC/Chol (1: 0.5)	4.37 (0.02)	2.09 (0.07)
EPC/Chol (1:1)	6.12 (0.07)	2.69 (0.12)

Each result is the mean of 3 determinations (± S. D.)

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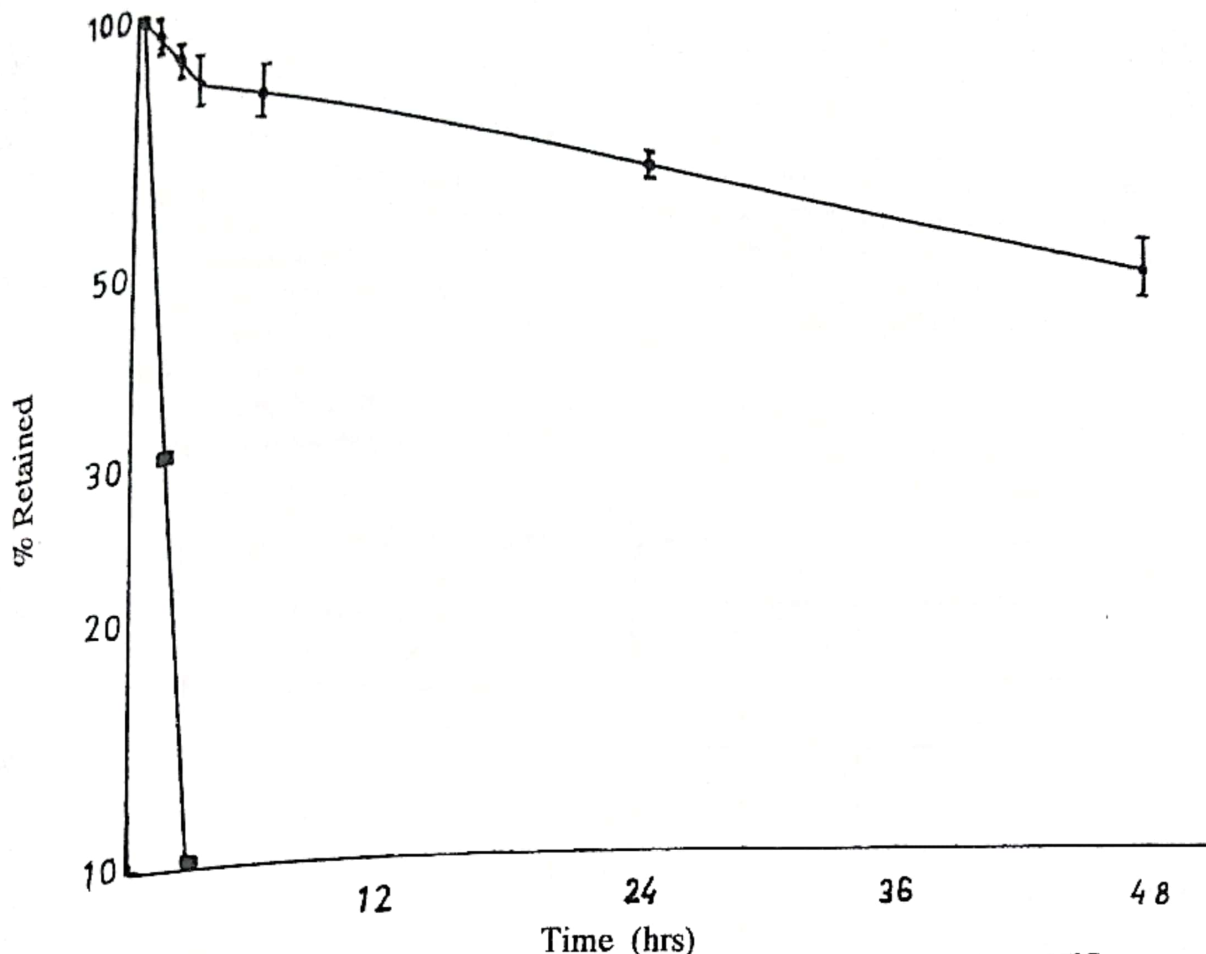

Fig. (1) : Efflux of propranolol hydrochloride from EPC MLV into saline at 37°C .
EPC (■) and EPC/ CHOL (1 : 1) (●).

Table (2) shows the effect of incorporation of DCP into EPC / Chol (1: 1) MLV liposomes on entrapment of PPL. Increasing the DCP component of the lipid phase to 1,10 and 20 mole % was accompanied by a proportionately greater uptake of PPL. DCP incorporates into the lipid bilayer and is routinely used for conferring a negative charge for liposomes [13]. In this study, it is likely that the improved entrapment of PPL is due to the formation of a lipophilic ion-pair between PPL and DCP, which partitions into the bilayers. Incorporation of SA, a positively charged lipid into EPC/Chol (1:1) MLV liposomes resulted in an increased uptake of CAP (Table 3). Stearylamine is commonly incorporated into bilayers to confer a positive charge on liposomes [19] . Electrostatic repulsion of adjacent bilayers increases liposome size and the size of the internal aqueous compartments [25] .

Table (2): Entrapment of PPL in EPC/Chol MLV liposomes containing dicetylphosphate.

Lipid composition (mole %) EPC/Chol	DCP	Entrapment (mg mg^{-1}%)
100	0	2.69 (0.12)
95	5	3.89 (0.04)
90	10	4.71(0.09)
80	20	5.95 (0.11)

Each result is the mean of 3 determinations (± S. D.)

Table (3) : Entrapment of CAP in EPC / Chol MLV liposomes containing stearylamine

Lipid composition (mole ratio)	Entrapment (mg mg^{-1}%)
EPC/ Chol (1:1)	6.12 (0.07)
EPC/Chol/SA (1:1: 0.02)	7.23 (0.04)
EPC/Chol /SA (1:1:0.22)	8.74 (0.09)

Each result is the mean of 3 determinations (± S. D.)

2. Entrapment and Release of CAP and PPL from Freeze-Thawing Vesicles (FTV)

Table (4) summarizes the encapsulation efficiencies of FTV and MLV of varying lipid composition for CAP and PPL respectively.

Liposomes prepared by the freeze - thawing technique resulted in more than 2 fold increase in CAP and PPL entrapment compared to MLV prepared by the conventional method. A mechanism outlining the events during FT cycle has been proposed by **Ohsawa et al.,** (26). 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.

Table (4): Comparison of encapsulation efficiencies of FTV and MLV of varying lipid composition for CAP and PPL.

Lipid composition (mole ratio)	Entrapment (mg mg -1 %)			
	CAP		PPL	
	MLV	FTV	MLV	FTV
EPC	3.75 (0.08)	8.20 (0.11)	1.85 (0.05)	4.10 (0.02)
EPC/ Chol (1:1)	6.12 (0.07)	13.80 (0.06)	2.69 (0.12)	7.20 (0.12)
DPPC/Chol (1:1)	6.81 (0.05)	14.20 (0.03)	2.91 (0.07)	7.61 (0.06)
DMPC/Chol (1:1)	6.52 (0.10)	13.60 (0.09)	2.70 (0.03)	6.90 (0.04)

Each result is the mean of 3 determinations (± S. D.)

The composition of the aqueous phase used to prepare FTV was varied to investigate the effect of ionic strength on the encapsulation of CAP and PPL in EPC/Chol (1: 1) liposomes. As shown in Table 5, the entrapment of both drugs rapidly decreased as the concentration of sodium chloride increased from O-0.4 M in the aqeous phase. **Ohsawa et al.,** (26) suggested that in the presence of sodium chloride , the aqueous

Table (5): Influence of ionic strength of aqueous phase on entrapment of CAP and PPL in EPC/Chol (1:1) FT liposomes.

Sodium chloride concentration **M**	Entrapment (% of total)	
	CAP	**PPL**
0.00	11.04 (0.06)	14.40 (0.12)
0.05	9.81 (0.03)	12.20 (0.08)
0.10	7.12 (0.08)	10.40 (0.06)
0.20	6.21 (0.12)	9.10 (0.04)
0.30	5.18 (0.02)	7.80 (0.05)
0.40	4.03 (0.05)	5.90 (0.09)

Each result is the mean of 3 determinations (± S. D.)

phase remains unfrozen, because the eutectic point of sodium chloride (-21°C) is below the freezing temperature (-20°C). Thus the lipid particles are dispersed in a larger space during the freezing process and aggregates large enough to give high entrapment do not occur.

The effect of trehalose on the encapsulation of CAP and PPL in EPC/Chol FTV is shown in Table 6. Increasing trehalose concentration from 0-4 g trehalose / g lipid in the aqueous medium resulted in a marked decrease in drug entrapment . This probably may be due to the ability of trehalose to prevent aggregation and fusion of liposomes at very low temperatures by forming hydrogen bonds with the lipid phosphate head groups. (27).

Table (6): Influence of the addition of different concentrations of trehalose on entrapment of CAP and PPL in EPC/Chol (1:1) FT liposomes.

Sugar concentration (g trehalose / g lipid)	Entrapment (% of total)	
	CAP	**PPL**
0.00	11.04 (0.06)	14.40 (0.12)
0.50	9.80 (0.21)	12.10 (0.31)
1.00	7.30 (0.19)	10.60 (0.08)
2.00	5.90 (0.07)	8.20 (0.12)
3.00	4.60 (0.03)	7.10 (0.11)
4.00	3.90 (0.13)	5.80 (0.05)

Each result is the mean of 3 determinations (± S. D.)

Incorporation of stearylamine, a positively charged lipid into EPC/ Chol (1:1) FT liposomes resulted in an increased uptake of CAP (Table 7). This may be due to the formation of a lipophilic ion-pair between CAP and SA which partitions into the liposome bilayer.

Table (7) : Entrapment of CAP in EPC/Chol (1:1) FT liposomes containing stearylamine.

Lipid composition (mole ratio)	Entrapment (mg mg-1%)
EPC/ Chol (1:1)	13.8 (0.06)
EPC/Chol/SA (1:1:0.02)	15.1 (0.09)
EPC/Chol /SA(1:1:0.2)	16.8 (1.20)
EPC/Chol /SA (1:1:0.5)	18.2 (1.51)

Each result is the mean of 3 determinations (± S. D.)

Table (8) shows the effect of incorporation of DCP into EPC/Chol (1:1) FT liposomes on the entrapment of PPL. Increasing the DCP component of the lipid phase gave proportionately greater up take of PPL.

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The mechanism by which DCP enhances PPL incorporation has been discussed before.

Table (8): Effect of DCP concentration on the entrapment of PPL in EPC/ Chol (1:1) FT liposomes.

Lipid composition (mole ratio)	Entrapment (mg mg^{-1}%)
EPC / Chol (1:1)	7.20 (0.12)
EPC Chol / DCP (1:1:0.02)	8.91 (0.07)
EPC Chol / DCP (1:1:0.2)	10.20 (0.05)
EPC Chol / DCP (1:1:0.5)	11.60 (0.08)

Each result is the mean of 3 determinations (± S. D.)

The release profiles of CAP from MLV and FTV positively charged liposomes are shown in Fig 2. Dilution of EPC/ Chol/ SA (1: 1: 0.22) MLV preparation produced a biphasic release profile with a large rapid release of drug following dilution. The release profile of CAP from EPC/ Chol / SA FTV also showed an apparent biphasic release process but CAP latency was maintained more efficiently by FTV than MLV. This is presumably due to the increased lamellarity of FTV compared to MLV due to aggregation and fusion of the vesicles. Each lamella of lipid represents a barrier to the diffusion of materials from liposomes. The Linearity of the log efflux plots indicate that the release proceeds by first-order kinetics once an initial phase of rapid loss has terminated.

The release profiles of PPL from MLV and FTV negatively charged liposomes showed the same trend (Fig 3).

3.Entrapment and Release of CAP and PPL from Reverse - Phase Evaporation vesicles (REV)

Producing liposomes by the REV technique resulted in more than two fold increase in CAP and PPL entrapment compared to MLV produced by the conventional method (Table 9). **Sammour and Hassan** [6] reported that electron micrographs of REV liposomes revealed the presence of large unilamellar vesicles each having a large internal aqueous core relative to its diameter, and this was responsible for the efficient entrapment of the aqueous volume.

Table (9): Comparison of encapsulation efficiencies of REV and MLV for CAP and PPL.

Lipid composition (mole ratio)	Entrapment (mg mg^{-1}%)			
	CAP		PPL	
	MLV	REV	MLV	REV
EPC / Chol (1:1)	6.12 (0.07)	12.60 (0.07)	2.69 (0.12)	5.83 (0.12)
DPPC / Chol (1:1)	6.81 (0.05)	14.10 (0.06)	2.91 (0.07)	6.50 (0.06)

Each result is the mean of 3 determinations (± S.D.).

The release profiles of CAP from DPPC/Chol (1:1) MLV and REV liposomes are shown in Fig 4. Dilution of REV preparations produced a biphasic release profile with a phase of rapid drug loss lasting for about 8 h . At 8 h post dilution 43% of entrapped CAP was lost from REV liposomes compared to a loss of about 19% from MLV liposomes.

The release profiles of PPL from DPPC/Chol (1:1) MLV and REV liposomes showed the same trend (Fig. 5). At 6 h post dilution, 36 and 18% of entrapped PPL were lost from REV and MLV liposomes respectively. This phase of rapid losses may be due to the rapid release of drug from unilamellar vesicles present in REV formulations since large unilamellar vesicles have a larger surface area to volume ratio than MLV and possess only a single lipid bilayer barrier to hydrophilic drug diffusion.

Dilution of liposome suspensions leads to the efflux of drug across liposome membranes until equilibrium is re-established. The driving force for drug efflux is then the concentration gradient across the liposome bilayers. As efflux progresses, the concentration gradient is reduced, and hence efflux rate is reduced, indicating first-order release kinetics, which is confirmed in Figs. (1-5) by the linearity of the log efflux plots, following an initial phase of rapid release.

CONCLUSIONS

The preceding study was an attempt to evaluate the potential use of liposomes as a sustained delivery system for captopril and propranolol hydrochloride. Evaluation *in-vitro* of liposome-drug systems is an important pre-requisite before *in-vivo* administration. An optimum formulation encapsulating a high percentage of available drug which could be released at a controllable rate *in-vitro* over a prolonged period of time, could then be considered for use in a clinical evaluation.

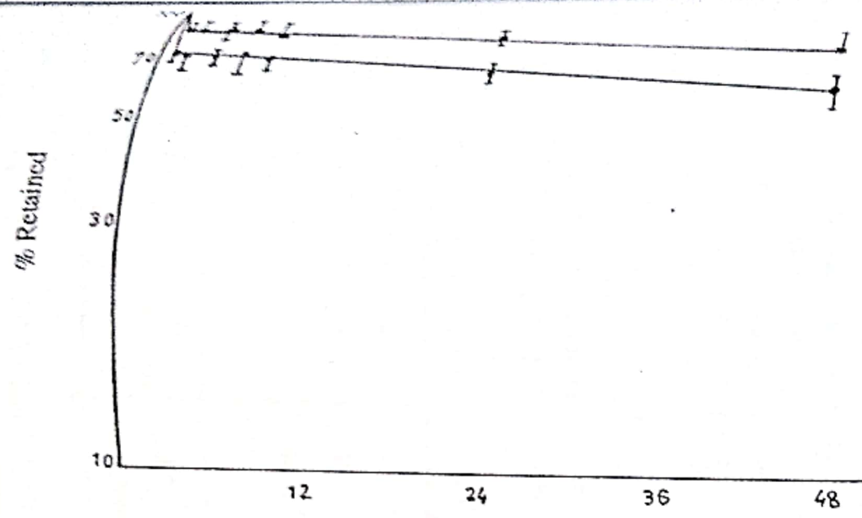

Fig. (3) : Efflux of propranolol hydrochloride from EPC/ CHOL/ DCP (1 : 1 : 0.22) MLV and FTV liposomes into saline at 37°C . MLV (●) and FTV (■).

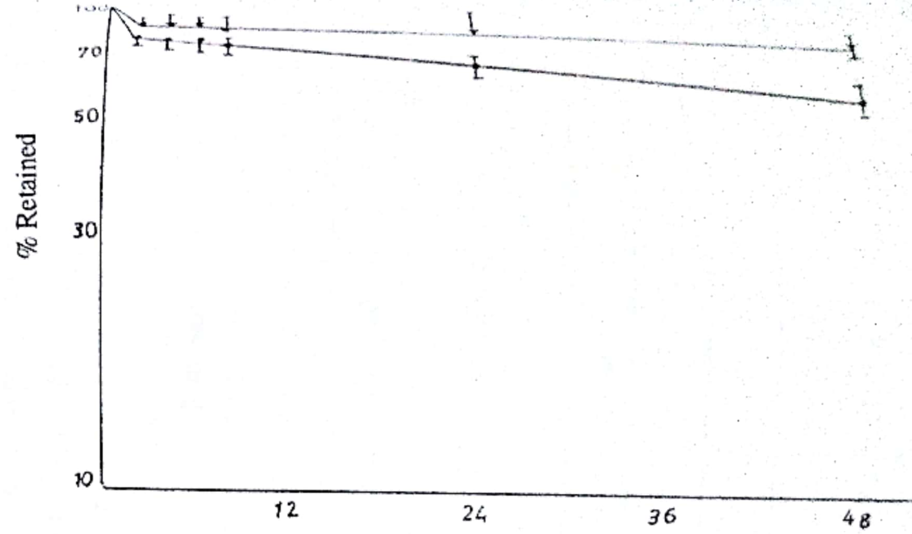

Fig. (2) : Efflux of captopril from EPC/ CHOL/SA (1 : 1 : 0.22) MLV and FTV liposomes into saline at 37°C . MLV (●) and FTV (▼).

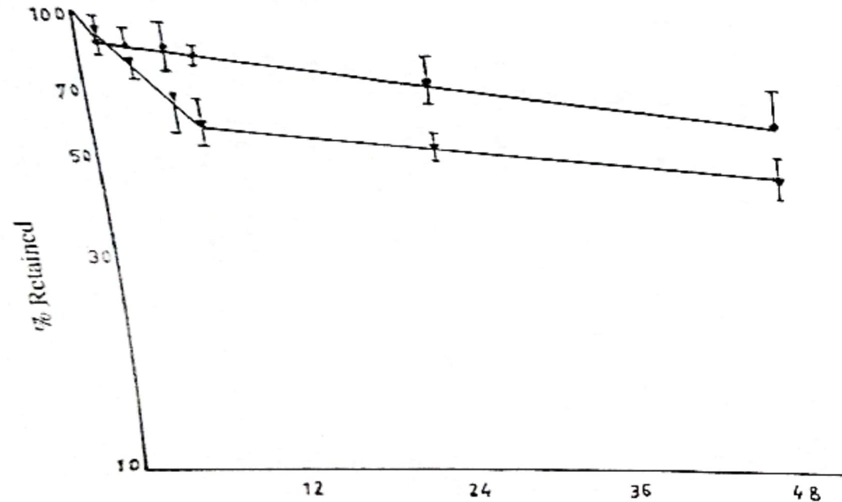

Fig (4) : Efflux of captopril from DPPC/ CHOL (1 : 1) MLV and REV liposomes into saline at 37°C . MLV (●) and REV (▼).

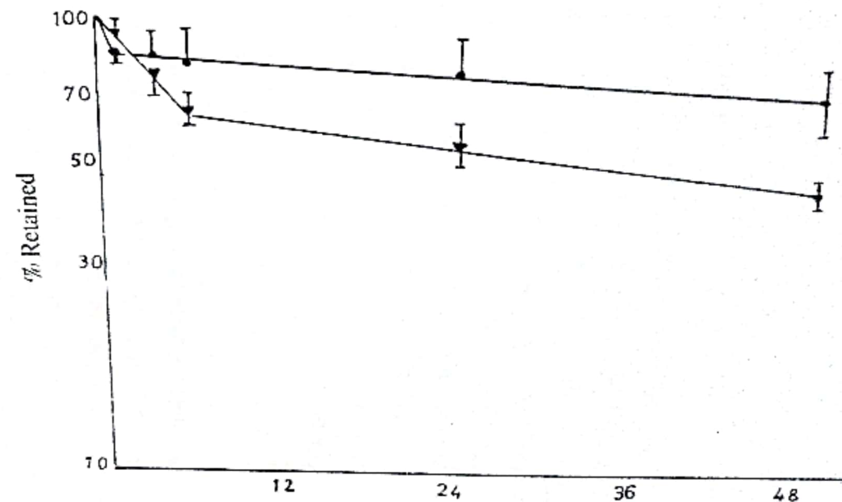

Fig (5) : Efflux of propranolol hydrochloride (1 : 1) MLV and REV liposomes into saline at 37°C MLV (●) and REV (▼)

evaluation.

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الملخص العربى

حوصلة وإنطلاق الكابتوبريل وهيدروكلوريد البروبرانولول من الليبوزومات المحضرة بطرق مختلفة

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تم فى هذا هذا البحث تحضير الليبوزومات بعده طرق وذلك لمقارنة مدى قدرتها على حوصلة الكابتوبريل وهيدروكلوريد البروبرانولول . ولقد وجد أن نسبة تحوصل العقارين داخل الليبوزومات عديدة الطبقات قليلة ولكن معدل التحوصل قد زاد بوجود كل من الكوليستيرول والشحنات الكهربائية على سطح الليبوزمات.

كذلك أثبتت الدراسة أن الليبوزومات المحضرة بطريقة التجميد والتسييح لها قدرة عالية على إحتواء العقارين مقارنة بالليبوزومات عديدة الطبقات . هذا وتم أيضا دراسة تأثير الشحنة الكهربائية وتأثير وجود مادة التريهالوز وكذا تأثير القوة الأيونية للوسط على معدل تحوصل الليبوزومات المحضرة بطريقة التجميد والتسييح وقد وجد أن تكوين ليبوزومات كبيرة الحجم نتيجة لالتحام الحويصيلات قد تم تثبيطة بدرجة كبيرة نتيجة لزيادة القوة الأيونية ولوجود مادة التريهالوز.

هذا وأوضحت الدراسة أيضاً أن تحضير الليبوزومات بطريقة التبخير بإنعكاس الوسط قد أدت إلى زيادة معدل التحوصل إلى الضعف مقارنه بالليبوزومات عديدة الطبقات التى لها نفس التركيب وعند دراسة التوافر المعملى للعقارين من الليبوزومات المختلفة وجد أن معدل الإنطلاق يعتمد على تركيب طبقات الليبوزومات وأيضاً على طريقة التحضير.