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EFFECT OF HUMAN PLASMA ON THE STABILITY OF LARGE MULTILAMELLAR LIPOSOMES WITH DIGITOXIN

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ABSTRACT

The stability of multilamellar liposomes with digitoxin in human plasma at 37º C is studied "in vitro". It is noted that as plasma/liposomal suspension ratio is increased, the porcentage of drug retained in the liposomes decreases. Just so, independently from the dosage form, the efflux rate is maximum during the first hour and then falls gradually and in a non linear way. The physical state of the bilayer is a conditioning factor in the release of the encapsulated drug. The dosage forms of egg yolk phosphatidylcholine (EYPC) and of dimiristoylphosphatidylcholine (DMPC) quickly release digitoxin; while dipalmitoylphosphatidylcholine (DPPC) retains 54 % of the entrapped drug after 24 hours incubation with 80 % of plasma at 37º C. The inclusion of cholesterol (CHOL) and dicetylphosphate (DCP) in the liposomal matrix neither aids in the incorporation of digitoxin to the liposomes, nor augments the stability of the system in the human plasma.

INTRODUCTION

Many works have been published (1,2) since Gregoriadis (3), at the beginning of the seventies, suggested the use of liposomes as a possible alternative system to the conventional dosage forms of drugs.

The firts problem faced when used as vectors is their stability. The combination of liposome-drug must reach the target organ keeping not only its structure, but also the quantity of drug inicially incorporated. Those liposomes capable of retaining the drug in a isotonic medium at the physiological pH, are, however, instable when in contact with biological fluids. In the case of plasma the instability is atributed to the denaturating action of the lipoproteins, and more exactly, to the high density lipoproteins (HDL). It is a bidirectional phospholipid transfer between the liposomes and the HDL, which does not necessarily imply the loss of entrapped substance (4). The interaction of liposomes with biological fluids depends, amongst other factors on the nature of the phospholipid which make up the bilayer, on the size of the vesicles, on the presence of esters (5) or other substances, etc...

Permeability in plasma or serum of small size unilamellar liposomes with encapsulated hydrosoluble drugs or markers, has been widely studied. This does not occur with multilamellar liposomes (6) nor with drugs which are incorporated to the bilayer such as digitoxin (7). On the grounds of the aforesaid and trying to achieve an effective digitoxin liposomal formulation, which avoids the terapeutic risks of conventional preparations, the stability of digitoxin multilamellar liposomes in human plasma at 37º C has been studied "in vitro". The effects produced on the permeability by the proportion of plasma and the nature of the phospholipids employed in the preparation (egg yolk phosphatidylcholine or syntetic lecithins: dimiristoylphosphatidylcholine or dipalmitoylphosphatidylcholine) and the presence of

other lipids in the liposomal matrix (cholesterol or dicetyl-phosphate) are analysed.

MATERIALS AND METHODS

Natural phosphatidylcholine (EYPC) was isolated from fresh chicken egg yolk (8). Its oxidation state and degree of purity was checked, respectively, by stectrophotometry and thin layer chromatography on silicagel plates (DC-Alufolien Kieselgel 60, Merck), which were developed in the solvent chloroform:methanol:acetic acid:water (25:15:4:2). The spot was visualized with iodine vapors. L- α dimiristoylphosphatidylcholine (DMPC), DL- α dipalmitoyl-phosphatidylcholine (DPPC) and dicetylphosphate were supplied by Sigma Chem. Co.. Digitoxin (DGT) and cholesterol (CHOL) were purchased from Merck. Lecithins were stored as a chloroform solution,under nitrogen in the darck, at -20º C.

The isotonic buffer solution pH=7.2 was composed of NaCl 0.15 M and $NaPO_4H_2/Na_2PO_4H$ 5 mM, also coming from Merck.

The human plasma was obtained at the "Hospital de la Cruz Roja" in Madrid, and it was stored at -20º C until used.

Multilamellar liposomes (MLV) with digitoxin were prepared following Bangham's procedure (9). The drug was added to the lipidic solution in the flask of the rotary evaporator. The molar lecithin:digitoxin ratio was 10:0.4 or 10:0.1 mM, except for those in which the bilayer also had 1 mM of DCP, in whose case, the proportion of lecithin was 9 mM. Those of cholesterol were comprehended between 0 and 5 mM.

In order to test the stability of EYPC liposomes with DGT (10:0.4 mM) as a function of plasma/lipid ratio, adecuate volumes of plasma and liposomal suspension were mixed to attain a final volume of 2 ml, in which the porcentages of plasma varied from 5 to 80 per cent. The entireness was kept at 37º C $\pm$ 0.2 for 24 h. in a mild shaking thermomised bath. After 1, 4, 8 and 24 hours incubation, aliquots of this plasma/MLV suspension (0.4 ml) were extracted to analyse the DGT yielded from liposomes. The separation

was performed by centrifugation at 21,000 g for 15 min. at 5º C. The supernatant was removed and proteins were precipitated by adding absolute ethanol. After a new centrifugation at 4,000 r.p.m. for 10 min., DGT was measured spectrofluorometrically by the U.S.P. XXI procedure (10) and by difference with the drug concentration in the liposomal suspension the amount retained in the multilamellar vesicles is calculated.

In the same way, stability of MLV in isotonic buffer pH=7.2 was checked for avoid the effect of liposome dilution on the efflux of DGT by human plasma.

Independently, suspensions of plasma/liposomes, of 80 % (v/v) of the three lecithins alone and with different portions of CHOL and/or DCP, were prepared and suffered the same treatment. The DGT concentration employed in all these formulations was 0.1 mM; lower than the maximum incorporation ratio of the drug in the DPPC liposomes, which resulted in being the one with the lowest power of captation (11). The purpose of this is to avoid that non encapsulated durg forms a precipitate, due to its low water solubility which would make it impossible to know, by difference, the amount of DGT retained in the liposomes. However, in each and everyone of the cases it was checked, by optical microscopy, that did not exist any crystals of the drug in the liposomal suspensions before putting them in contact with the plasma. Moeover, all the preparations were observed through the microscope after 24 h. incubation.

RESULTS AND DISCUSSION

Figure 1 shows the percentage of DGT retained by EYPC vesicles with reference to the plasma or buffer percentage, for the different incubation times. It can be observed that when both (time and plasma or buffer) increases, the portion of drug retained in the liposomes decreases; but the effect of dilution is lesser than that due to the plasma constituents. Figure 2 shows the percentage of DGT yielded as function of plasma ratio, after avoiding the leakage due to the liposome dilution. Furthermore, it is observed

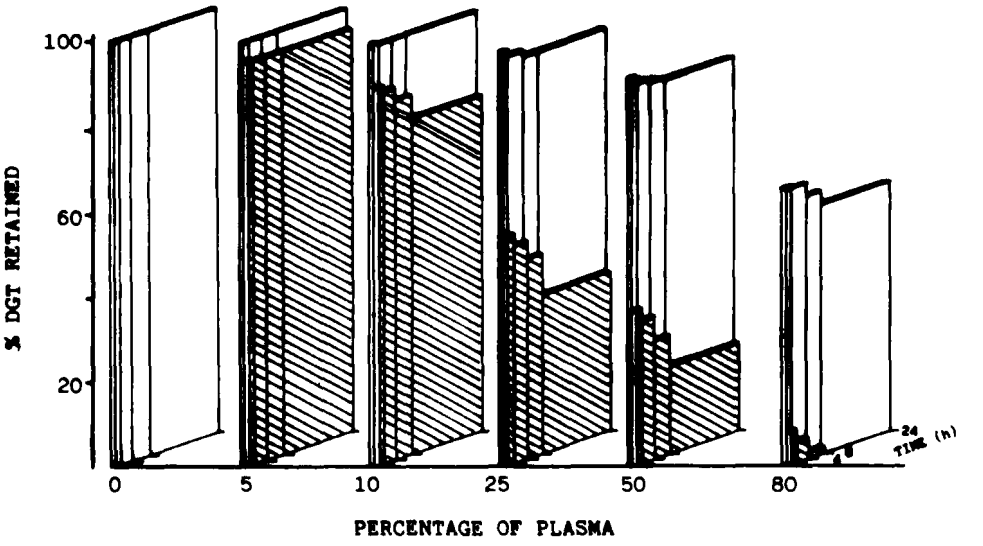

FIGURE 1

Effect of plasma (ruled lines) or buffer percentage and incubation time on retention of DGT by EYPC vesicles.

that the destabilizing effect of the plasma is almost immeadiate; the differences between the amounts of drug yielded after an hour, and after 24 hours are, regardless of the plasma portion, of little significance (fig. 1). Other autors (6) have noticed the same effect with encapsulated substances in the aqueous phase: a fast permeability increment of the liposomal membrane, which falls gradually in a non-linear way.

Table I shows the efflux average rate of DGT (% yielded DGT/h) from EYPC multilamellar liposomes, 10:0.4 mM molar ratio, at 37º C and different percentage of liposomal suspension in isotonic buffer or human plasma, for every incubation period. It was expresed as the ratio between the percentage of yielded DGT and the past time.

Hunt (12) points out that liposomes previously incubated with a small amount of plasma, offer a higher resistence to a second exposition. From these results, he postulates various compartimental

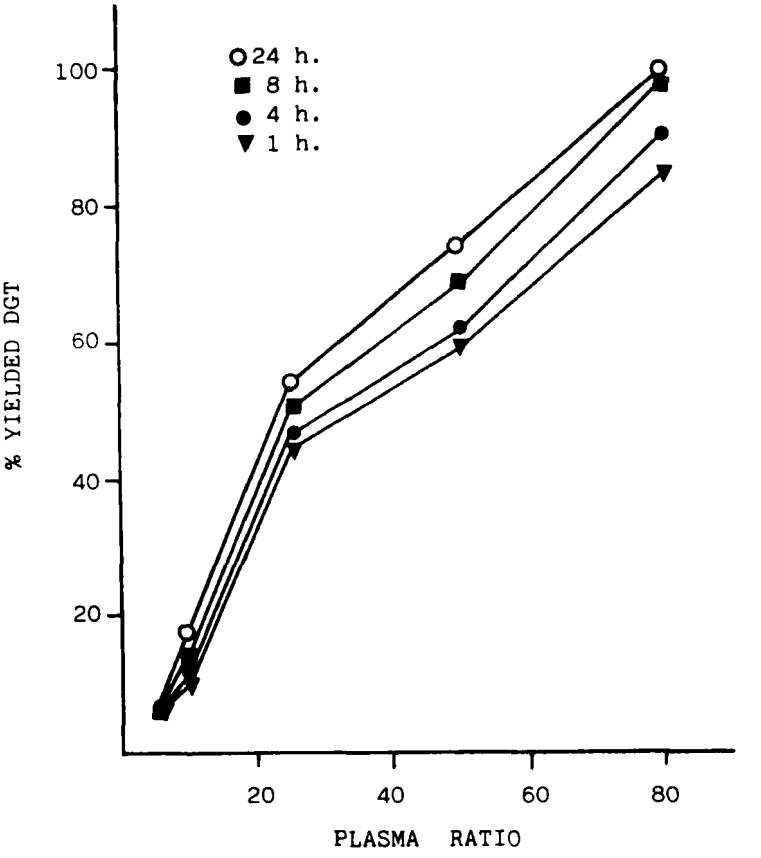

FIGURE 2

Percentage of DGT yielded as function of plasma ratio,expresed as: % yielded by plasma - % yielded by tampon.

interaction models, which may converge in a simpler one, being one and only aqueous compartment with a single, although variable, permeability constant. When putting in contact liposomes and plasma a fast protein adsorption on the surface of liposomes (13) takes place, which casuses the appearance of new tension forces in the bilayer and the increase of the permeability in that region. It also produces a rearrangement of the lipids, which determines the formation of a more stable surface configuration (14), with which,

TABLE I

Efflux average rate of DGT from EYPC liposomes

% LIPOSOMAL SUSPENSION	INCUBATION PERIOD (HOURS) 0 - 1		1 - 4		4 - 8		8 - 24	
	Buffer	Plasma	Buffer	Plasma	Buffer	Plasma	Buffer	Plasma
100	n.d.	——	n.d.	——	n.d.	——	n.d.	——
95	0.03	5.24	n.d.	0.15	n.d.	n.d.	n.d.	n.d.
90	0.98	11.26	0.11	0.48	0.07	0.70	n.d.	0.34
75	2.84	46.37	0.56	0.73	0.33	1.21	n.d.	0.20
50	8.61	62.95	0.84	1.13	0.21	1.48	n.d.	0.32
20	33.57	90.32	0.43	1.21	0.87	1.26	n.d.	a

n.d. = no detectable ; a = all DGT has been leakaged

after a short period in which the DGT quickly diffuses, comes the second phase of considerable reduction of the permeability.

On the other hand it is seen that the destabilizing effects of the plasma tend to reach a maximum. This is probably reached when the surface area avaiable for the interchange or the adsorption of lipids and plasmatic proteins is saturated. Therefore, if the plasma/liposomes proportion is low, the avaiable area of the vesicles is enough so that saturation is not reached and the destabilizing effects are minimum (figure 2). Like this, the uptake of liposomal lipid by the HDL particle is, also, saturable (4).

The erosion caused by plasma may possibly leave bare the most hydrophobous regions of the bilayer, where the cardiotonic genine would be anchored. This, together with the fact that PC at the physiological temperature is found in the liquid-crystalline (fluid) state, would be responsible for the scare retention power of these vesicles. Opposing, at 37ºC, egg yolk phosphatidylcholine multilamellar liposomes retained the DGT loaded at the beginning, onset after 24 hours incubation. This fate reinforces the idea that DGT is associated in the liposomal matrix.

TABLE II

Percentage of DGT retained by liposomes in contact with human plasma at 37º C.

LIPIDIC COMPOSITION	DRUG LOADING % ± S.D.(n=3)	INCUBATION TIME (HOURS) 0.25	1	4	8	24
EYPC:CHOL:DCP						
10 : — : —	89.83 ± 2.01	9.42	3.99	1.26	——	——
10 : 0.5: —	81.38 ± 3.39	8.59	2.44	0.86	——	——
10 : 1.0: —	75.55 ± 1.37	11.25	3.90	0.98	——	——
10 : 2.5: —	63.61 ± 1.07	17.59	6.44	1.07	——	——
10 : 5.0: —	44.54 ± 2.97	18.70	7.90	3.44	——	——
9 : — : 1	60.15 ± 4.82	16.80	6.48	1.18	——	——
9 : 0.5: 1	59.80 ± 1.45	19.51	4.51	0.98	——	——
9 : 1.0: 1	50.68 ± 2.59	17.54	9.19	1.91	——	——
9 : 1.5: 1	35.28 ± 4.15	27.15	11.45	1.21	——	——
DMPC:CHOL:DCP						
10 : — : —	91.61 ± 1.27	3.20	0.22	——	——	——
10 : 0.5: —	85.22 ± 1.89	3.09	0.15	——	——	——
10 : 1.0: —	85.48 ± 2.72	4.49	0.17	——	——	——
9 : — : 1	78.65 ± 1.61	1.15	——	——	——	——
9 : 0.5: 1	68.81 ± 4.95	2.25	0.68	——	——	——
9 : 1.0: 1	58.86 ± 4.17	9.65	2.89	——	——	——
DPPC:CHOL:DCP						
10 : — : —	83.60 ± 1.58	86.29	71.11	68.98	67.92	54.15
10 : 0.5: —	79.44 ± 3.10	84.46	59.07	50.79	50.01	36.92
10 : 1.0: —	75.46 ± 3.53	85.05	60.96	51.34	46.54	37.54
9 : — : 1	71.63 ± 1.71	84.72	47.20	33.80	33.00	26.30
9 : 0.5: 1	69.71 ± 1.30	77.76	44.60	33.03	30.65	18.82

The following experiences were designed on the basis of keeping the plasma/liposomal suspension ratio constant (80% of plasma and 20% of MLV suspension) and analyzing the influence of the lipidic composition upon the capacity of retention of the drug. The results obtained are shown on table II. They confirm that the physical state of the bilayer is the factor which conditions the release of the encapsulated drug. Both PC and DMPC have a transition temperature which is lower than the incubation one. At 37º C the laxer packing of these phospholipids in the bilayer is responsible for the fact that liposomes produced with them are more instable. On the contrary, those of DPPC maintain, after 24 hours, 54% of the incorporated drug. In the proximities of the phase transition temperature (Tc), gel state and liquid-cristalline state molecules coexist, causing irregularities on the surface, through which plasmatic proteins can penetrate. Following the appointed, liposomes produced with DPPC, whose it has been observed (4) that at a temperature equal to its Tc, DMPC is more sentive, when in contact with plasma, than DPPC at is own. Molecules of this latter one are found more packed in the transition phase than those of DMPC.

Lelkes and Friedman (6) detected a stabilizing effect of plasma in multilamellar liposomes of DPPC with carboxifluoresceine and that the release of marker is directly related to the transference from phospholipids to plasmatic proteins. As the interaction between these ones with liposomes is restricted in the gel satate (15), the stability of multilamellar vesicles of DPPC is higher than that of the other two lecithins.

Table III shows the efflux average rate of DGT from DPPC liposomes in contact with plasma at 37º C. It can be seen that, regardless of the formulation, the efflux rate is maximum for the firts hour, as in previous experiences (Table I), and that the CHOL and the DCP increase the leakage of DGT. A first order process can be takes place for the firts eight hours, as shows figure 3, with a efflux rate constant quite similar in all cases. While the leakage lightly increase at the 8 to 24 h incubation period.

TABLE III

Efflux average rate of DGT from DPPC liposomes.

LIPIDIC COMPOSITION DPPC:CHOL:DCP	ICUBATION PERIOD (HOURS) 0 - 0.25	0.25 - 1	1 - 4	4 - 8	8 - 24
10 : — : —	54.84	20.24	0.71	0.26	0.86
10 : 0.5: —	62.16	33.85	2.76	0.19	0.82
10 : 1.0: —	59.80	32.12	3.20	0.45	0.75
9 : — : —	61.12	50.02	4.46	0.20	0.42
9 : 0.5: —	88.96	44.21	3.86	0.59	0.74

The state of the water-lipid interface is conditioning the susceptibility of lipid bilayers to interactions with plasma ptoteins. The incorporation of cholesterol to the bilayer offerds a higher stability to liposomes, as it reduces both its permeability and the interchange of lipids with HDL's (16). However, on table I it can be noticed a lower power of retention of DGT for those formulations which include CHOL in their composition.

Scherphof and cols. (4) have observed that the interaction between plasmatic HDL and cholesterol enriched unilamellar liposomes seems to be superficial and limited to the external monolayer. Any irregularity in the packing of the lipid molecules at the surface may effectivelly facilitate such interaction. These irregularities may be of various nature as the presence of foreing substances. It has been suggested (7) that cardiotonic heterosides such as digitoxin and digoxin, can join the liposomal matrix with the sugar moiety oriented towards the aqueous phase of the membrane near the polar head group, and the steroid part packed in the hydrocarbon region, but not deep into the fatty acyl cahins. Therefore, CHOL does not favour the stability of liposomes with DGT. Furthermore, it seems to exist a competition between them,

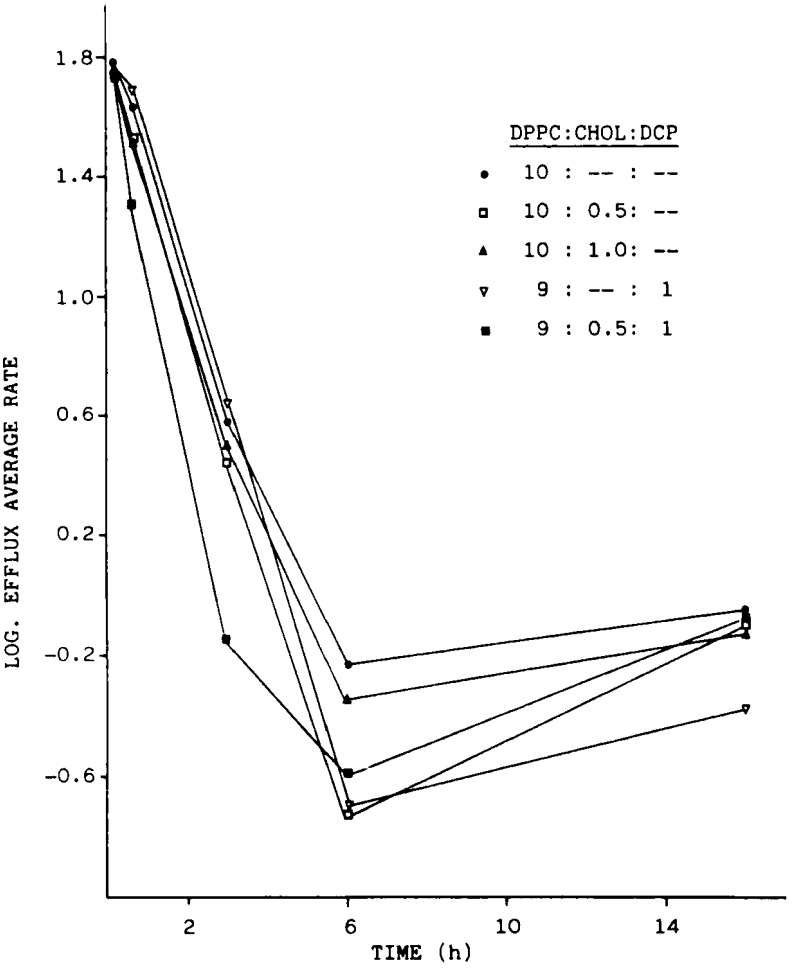

FIGURE 3

Plot of log. efflux average rate of DGT from DPPC liposomes incubated with plasma at 37º C against average incubation time.

as the increase of the steroid proportion disminishes the initial captation of the glycoside. With higher CHOL ratios than those indicated in table II, small DGT crystals appear when liposomal suspensions are seen through a microscope.

Dietylphosphate, as all charged lipids, increases the fluidity of the membrane and the instability of the system, in

regard to the drug retention; although the appearence of repulsive forces between the vesicles, of an electrostatic nature, produces a lower aggregation of them, both before and after the incubation with plasma, as it has been verified by optic microscopy.

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