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CDP-CHOLINE ENTRAPMENT AND RELEASE FROM MULTILAMELLAR AND REVERSE-PHASE EVAPORATION LIPOSOMES

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ABSTRACT

In this work it was reported the determination of CDP-choline entrapment efficiency in MLVs liposomes constituted of neutral or negatively charged phospholipids. The encapsulation capacity (EC) increases with using charged phospholipids; moreover, also the presence of cholesterol into the vesicle bilayers enhances the EC values. Liposomes, prepared with reverse-phase evaporation method, entrapped greater amounts of drug than multilamellar liposomes of the same composition. The size of liposomes so like as the polidispersity index values were determined by light scattering analysis. The rate of CDP-choline

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efflux from liposomes was determined "in vitro" and was dependent upon bilayer composition and the method of preparation.

INTRODUCTION

For many years work has been in progress to find methods of directing drugs and other therapeutic molecules to specific sites in the body in order to achieve tissue-specific treatment of various conditions.

The goal of any drug delivery system involves altering of the pharmacokinetics and physiological disposition of the drug in the question in order to obtain a higher therapeutic index. This can be accomplished either by decreasing the toxicity of the drug or by increasing its efficiency. The most suitable drug device to obtain such effects is the liposomial system.[1-2]

Liposomes have an advantage as "in vivo" carrier in that they may be formed from natural molecules which can be easily methabolised in the body. They has been used to include chelating agents[3], antibiotics[4-5], anti-tumor drugs[6-7] and hormones.[8]

CDP-choline is a therapeutic agent widespreadly used in treating parkinsonism, extrapiramidal diseases and consciousness disorders in brain injury; furthermore, it may contribute to repair the membranous

structures of brain cells that has been broken down by cerebrovascular accidents and also to improve energy methabolism of mitochondria in the remaining brain cells, thus accelerating a functional reorganization.[9-11]

Unfortunally, the limiting factors in CDP-choline therapy were the transport through cell membranes[12] and the bioavailability;[13] in fact, its polar nature justifies the scarce ability to cross over the blood brain barrier and the small amounts, about 0.25 % of the total administered dose, that reach the active site.[14] For this reason a possible strategy in order to enhance the CDP-choline absorption across cell membranes, to increase the quantity of drug in central nervous system, could be the incorporation into liposomes.

In this paper we study how the liposome composition and the preparation methods could affect the encapsulation efficiency, besides of the drug leakage rates from liposomes. In fact a knowledge of the "in vitro" release of an entrapped drug is a necessary prerequisite to investigation of the "in vivo" behaviour of a liposomial drug delivery system.

MATERIALS

1,2-Dimyristoyl-sn-glycero-3-phosphocholine monohydrate (DMPC), 1,2-Dipalmitoyl-sn-glycero-3-phospho-

choline monohydrate (DPPC), 1,2-Dipalmitoyl-sn-glycero-phosphate disodium salt (DPPA) were commercial products (Fluka Chemicals co. Buchs, Switzerland); Dipalmitoyl-DL-α-Phosphatidyl-L-serine (DPPS) and Cholesterol (CHOL) were purchased from Sigma Chemicals co. (St. Luis, USA).

The purity of phospholipids was greater than 99 % as assayed by bidimensional thin-layer chromatography.[15]

CDP-choline was a gift from Cyanamid Italia. The purity was greater than 99 % by HPLC. All others materials and solvents were of analytical grade and deionized water was used.

METHODS

Preparation of Liposomes

All liposome preparation had a total lipid concentration of 20 $mg \cdot ml^{-1}$. The required amount of phospholipids and their mixtures was weighed into a quickfit round-bottom flask and dissolved in the smallest possible volume of chloroform. Organic solvent was slowly removed at 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. Aqueous phase (CDP-choline in 0.1 M NaCl) was added at

a temperature 15 °C more than the phase transition temperature (Tm) from gel to liquid crystal. The flask was maintained at that temperature for 1 h, then shaken on a mechanical agitator for 3 min to produce Multi-Lamellar Vesicles (MLVs).

Reverse-phase Evaporation Vesicles (REVs) were prepared by the method of Szoka and Papahadjopoulos.[16] Lipid components were weighed into a long-necked 100 ml quickfit round-bottom flask and dissolved in chloroform / diethyl ether (1:1). CDP-choline in 0.1 M NaCl was added such that the organic to aqueous phase ratio was 5:1. The flask was sealed under nitrogen and the mixture first shaken by a vortex, then sonicated for 30 min at 55 °C in an ultrasonic bath to get a fine emulsion from which organic solvent was slowly removed at 35 °C with a rotary evaporator under a nitrogen stream to produce the liposomes. The flask remained on the evaporator until organic solvent could not be detected by olfactory means.

Following production all liposomes were maintained for 1 h at a temperature exceeding the phospholipid Tm to anneal the liposome structure.

Determination of CDP-choline

The separation of unentrapped fraction of CDP-choline was carried out by a centrifugation (30' at

8000 RPM for MLVs and 13000 RPM for REVs at 10 °C) with a centrifuge Beckman Mod. J2-21 equipped with a Beckman JA-20 rotor. After the centrifugation and the elimination of the unencapsulated drug fraction, to know the real quantity of drug entrapped in the vesicles, we added 2 ml of $CHCl_3$ to destroy the liposomal structure to make the encapsulated CDP-choline free. This suspension was washed with 3 ml of 0.1 M NaCl for three times to extract all the drug. After each washing it was necessary to centrifugate the mixture to completely separate the organic from the aqueous phase. The concentration of CDP-choline loaded liposomes was calculated from the UV-absorbance of the aqueous phase at 280 nm detected by a UV-Vis Perkin-Elmer 300 spectrophotometer, using a 0.1 M NaCl solution as reference. The CDP-choline determination was also performed by HPLC.[17]

Quantitative Expression of Entrapment

The expression of the amount of material (CDP-choline) entrapped in liposomes is often express as the percentage of starting material which becomes liposomally associated. This has become very misleading in comparing results from different workers for the following reason. When the proportion of lipid used to make liposomes is increased compared with the volume of

aqueous phase, the fraction of the aqueous phase enclosed between the bilayers, and hence the entrapment, will increase. Careful consideration will make it evident that this will not cause greater entrapment per mg liposomal lipid, whereas when expressed as a percentage of starting material entrapment is considerably increased. We know that true entrapment is a function of the entrapped aqueous volume. If the entrapment of a particular molecule exceedes the value expected by this criterion, then factor such as electrostatic or hydrophobic interaction with the lipid bilayers are contributing to the amount of material which becomes associated with the liposomes. We thus propose that the entrapment of material in liposomes should be expressed as a function of liposomal lipid and be related to the volume of the internal aqueous space.

For this reason, according to Benita,[18] we use the encapsulation capacity (EC) value as a parameter to relate the volume of encapsulated aqueous space per millimole of lipid. EC values for the various phospholipid systems were calculated by the ratio between the final CDP-choline concentration (mmol/ml) found in the vesicles and the product of the initial CDP-choline concentration (mmol/ml) added during vesicle formation with the lipid concentration (mmol/ml) in the liposome suspension.

The molar fraction (MF) of the drug entrapped per mole of lipid was calculated by the ratio of the molar concentration of drug to the molar concentration of lipids determined in the liposomes. The different values reported are the average of five experimental measurements.

Determination of Liposome Size

Vesicles size was determined by light scattering (LS) analysis. The apparatus consisted of a He-Ne Spectra Physic mod. 120 laser (7 mW), a holding sample cell PC 8 Malvern thermostated at 24 °C by a Haake F3-R and eqipped with a Microcontrol precise mechanical goniometer and a optical system Melles-Griot f. 150; the employed photomultipliers were Hamamazu R 1333 and RCA 8852. In LS analysis we performed two methods: Photon Correlation Spectroscopy (PCS) and Elastic Light Scattering (ELS).

Measurement of particle size by PCS is based on the theory that the observed time dependence of the fluctuations in intensity of scattered light from a colloidal dispersion is a fuction of the size of the scattering particles. The autocorrelation fuction, $g(\tau)$, of the scattering intensity can be expressed, for a monodisperse system, as a fuction of the decacy rate

(Γ) and the correlation delay time (τ).

Eq. 1 $g(\tau) = \exp(-\Gamma\tau)$

The decacy rate can be expressed as $\Gamma = DK^2$, where D is the particle traslational diffusion coefficent, $K = (4\pi/l)\, n \cdot \sin(\theta/2)$, is the magnitude of the scattering vector, n is the refractive index of the solution and θ the scattering angle. For a polydisperse systems, g(τ) consists of a sum or distribution of single exponentials:

Eq. 2 $$g(\tau) = \int_0^\infty G(\Gamma) \exp(-\Gamma\tau) d\Gamma$$

The z average diffusion coefficent and the equivalent z-average particle diameter (dz) may be calculated from the mean decacy rate (Γ), obtained by expression of Eq. 2,[19] and the variance of the distribution is given by:

Eq. 3 $$Q = \mu_2 / \Gamma^2$$

where Q is known as the quality parameter or polydispersity index (PI).[20] All the values of PCS analysis were correlated by a Malvern 4700 C particle analyzer connected to a Olivetti 240 computer. The scattering angles were 20° and 40°.

Measuring the intensity profile by ELS method, or rather the intensity scattered by the sample at different observation angles, it was obtained radius values in agreement with the PCS values.

CDP-choline Leakage Rates from Liposomes

To monitor the release rate of CDP-choline from MLVs and REVs, periodic centrifugations of CDP-choline loaded liposome dispersions following a 1 to 50 dilution of the initial preparation with 0.1 M NaCl were carried out. Diluted preparations were stored in a water bath at 37 °C under shaking. At predetermined time intervals one diluted sample was taken and the ratio free to liposome-associated CDP-choline determined, after centrifugation, at 280 nm. Total release of drug was determined from the concentration of CDP-choline in diluted preparations.

RESULTS AND DISCUSSION

Entrappment of CDP-choline in Liposomes

CDP-choline entrapmet within liposomes is found in the internal aqueous compartment of these particles owing to its low affinity and solubility in the lipids which costitute the liposomal structure; in fact, how investigated by us in a previous work,[21] the only interaction which take place among the drug and the

phospholipids is along their polar heads, no or negligible interaction, depending on the liposome composition, was detected with the hydrocarbon core of the phospholipid bilayer. Thus, a good entrapment of CDP-choline requires encapsulation of large aqueous spaces within liposomes.

The manufactoring process used in this study yielded, after sonication and removal of unencapsulated solute, big MLVs, as confirmed by LS analysis, and REVs.

MLVs showed a narrow range of size distribution, how it was detected by PCS analysis that gave polidispersity index values of 0.5 ± 0.3, which underlined the sample homogenity; that is the presence of liposome suspension almost monodispersed. Main particle size of MLVs containing CDP-choline varied from 1.12 μm for the electrically charged mixture DPPA/DPPS to 0.8 μm for DPPC, a neutral phodspholipid (Table 1).

REVs are in the size range 0.35 - 0.42 μm and show a polidispersity index higher than 1 (from 1.75 to 2.39). In this way we obtain liposomal suspensions caracterized by particle with a main diameter smaller than MLVs, that are heterodispersed with a more or less large range of size distribution (Table 1). All this is due to the fact that the REVs method produces both

TABLE 1

Size and Polidispersity Index of MLVs and REVs Liposomes Entrapping CDP-choline.

LIPOSOME COMPOSITION	MLVs		REVs	
	SIZE[a] (μm)	PI[b]	SIZE[a] (μm)	PI[b]
DPPC	0.80	0.73	0.36	1.75
DMPC	0.81	0.81	0.35	1.79
DPPA	0.94	0.50	0.41	2.30
DPPC-DPPA (1:1)	0.85	0.49	0.39	2.19
DPPC-DPPS (1:1)	0.93	0.62	0.40	2.27
DPPC-DPPS (3:1)	0.86	0.57	0.38	2.03
DPPA-DPPS (1:1)	1.12	0.37	0.42	2.39

a - Mean diameter was determined by LS analysis
b - Polidispersity Index

unilamellar and oligolamellar liposomes that present a significant difference in their size; anyway, with our preparation procedure we obtain polidispersity index values better than those obtained in the past.[18]

Moreover it is noteworthy that for the REVs systems the higher PI values (Table 1) are related to

charged liposomes, which present oligolamellar vesicles with larger diameter than neutral liposomes, resulting in a slight enhancement of the main vesicle diameter and a real increase in the PI values. It must be underlined that our liposome suspensions posses a reasonable stability: in fact, during a week, no significative variation in the mean diameter and PI was observed.

Being our intention to carry out some "in vivo" experiments, we thought to investigate the influence of different CHOL molar fraction vs the loading capacity. In fact, it must be considered that the addition of CHOL in the liposomes minimize the interaction of lipoproteins with liposome phospholipids.[22] By this way liposomes could maintain their integrity.

We observed an enhancement of the encapsulation parameter values linearly to the increase of CHOL molar ratio with respect to DPPC (Table 2). This was due to the fact that CHOL caused a strong reduction in permeability of those liposome systems for which an interaction of the phospholipids with CHOL was demonstrated. Furthermore, the enhanced CDP-choline entrapment capacity as a fuction of the CHOL molar ratio (Table 2), expecially for MLVs, is also explainable by the increased mean vesicle diameter in the presence of CHOL (Table 3).

TABLE 2

CDP-choline Encapsulation Parameters of MLVs and REVs Liposomes Constituted by DPPC with Different CHOL Molar Ratio

DPPC-CHOL AT DIFFERENT MOLAR FRACTION	MLVs EC	MLVs§ MF	REVs EC	REVs§ MF
0.0	2.84	0.59	29.00	6.29
0.1	2.95	0.63	29.31	6.36
0.2	3.00	0.65	29.46	6.39
0.3	3.51	0.75	29.51	6.40
0.4	3.92	0.83	29.68	6.44
0.5	4.14	0.87	29.75	6.47

§ Molar fraction of drug entrapped/mole of lipid · 10^2.

Therefore, CHOL not only plays such a barrier-membrane behaviour, but is also able to provide more physical stability and endurance against any mechanical strain; by this way, it is possible to reduce the CDP-choline loss during operations like centrifugation and sonication. This was demonstrated by submitting various DPPC liposome suspensions at different CHOL molar ratios, containing a known CDP-choline amount, to a set of centrifugations at different speed. Thus we was able

TABLE 3

Size and Polidispersity Index of MLVs and REVs Liposomes Constituted by DPPC with Different CHOL Molar Ratio.

DPPC-CHOL AT DIFFERENT MOLAR FRACTION	MLVs		REVS	
	SIZE[a] (μm)	PI[b]	SIZE[a] (μm)	PI[b]
0.1	0.81	0.71	0.35	1.75
0.2	0.83	0.59	0.36	1.81
0.3	0.86	0.61	0.37	1.83
0.4	0.88	0.53	0.39	1.87
0.5	0.90	0.47	0.39	1.93

a - Mean diameter was determined by LS analysis
b - Polidispersity Index

to recognize the different retain capacity of the various liposomal systems. Particularly, as Figures 1 and 2 show, we note that, by increasing the CHOL amount, there was a reduction of the extruded drug. This phenomenon is more marked for REVs than for MLVs. Of course, while MLVs were costituted of phospholipid multilayers, REVs were a liposomal suspension largely costituted of unilamellar vesicles; for this reason CDP-choline is able to reach the external aqueous space

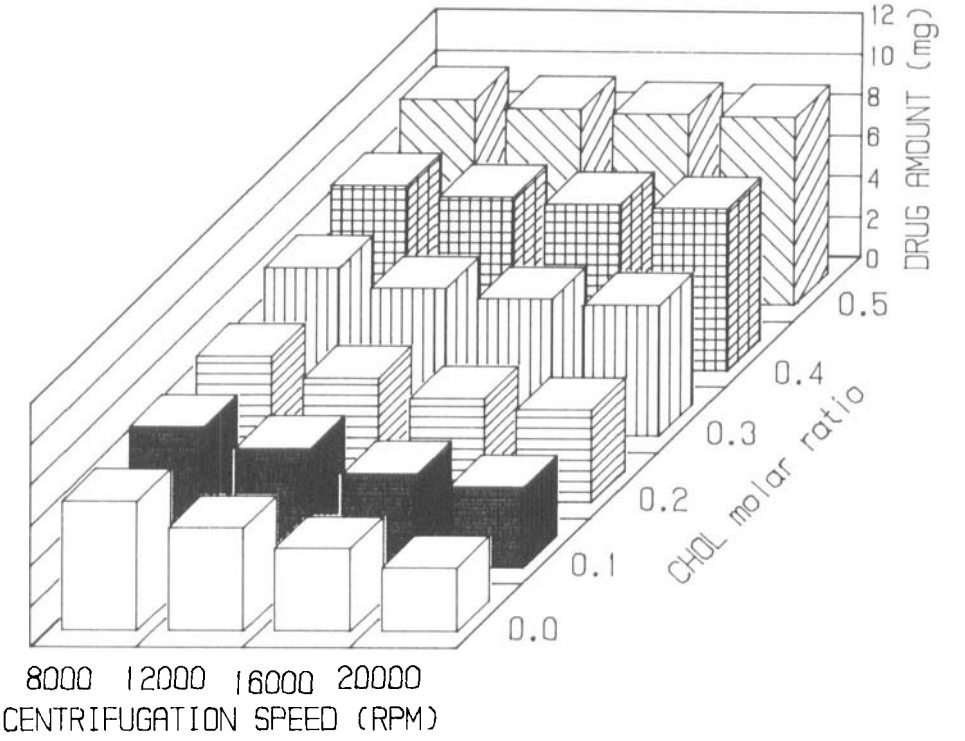

FIGURE 1

CDP-choline retention from MLVs of DPPC at different CHOL molar ratio as a fuction of centrifugation speed.

more easily when it is loaded in REVs than MLVs. As a consequence, the presence of CHOL affect much more the drug extrusion from REVs suspension.

Both REVs and MLVs reached the top concentration of encapsulated CDP-choline for DPPC-CHOL system (1:1 molar ratio), obtaining an EC values respectively of 29.75 and 4.14, which is twice than that of pure DPPC.

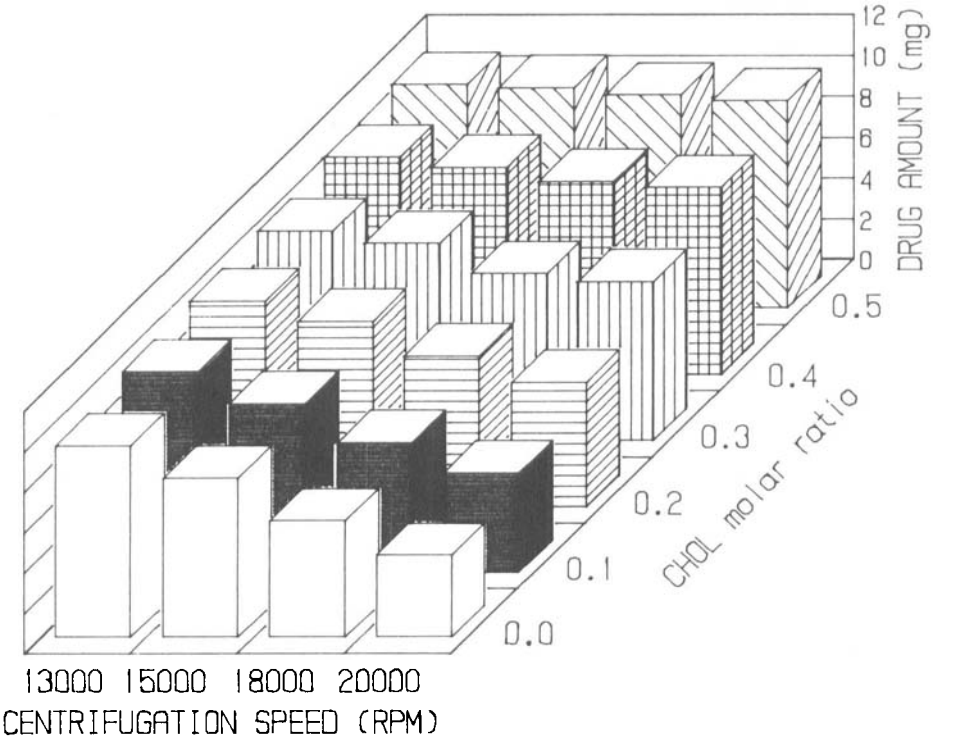

FIGURE 2

CDP-choline retention from REVs of DPPC at different CHOL molar fraction as a function of centrifugation speed.

Variuos amount of phospholipids as DPPA or DPPS (anionic lipids) were included in the liposomal bilayer membranes, making the surface electrically charged. This resulted, for MLVs, in a repulsion and hence an increase in the distance between the different bilayers, causing as a consequence an enhancement of the vesicle size (Table 1).

For this reason, negatively charged liposomes gave larger amounts of CDP-choline and higher rates of

TABLE 4

CDP-choline Encapsulation Parameters of MLVs and REVs Liposomes Constituted by Neutral and Charged Phospholipids or their Mixtures.

LIPOSOME COMPOSITION	MLVs EC	MLVs[§] MF	REVs EC	REVs[§] MF
DPPC	2.84	0.59	29.00	6.29
DMPC	2.65	0.58	29.02	6.29
DPPA	9.80	2.04	30.36	6.59
DPPC-DPPA (1:1)	5.70	1.20	30.05	6.51
DPPC-DPPS (1:1)	9.35	1.99	30.19	6.56
DPPC-DPPS (3:1)	5.58	1.17	30.05	6.54
DPPA-DPPS (1:1)	11.24	1.60	30.37	6.61

§ Molar fraction of drug entrapped/mole of lipid · 10^2.

aqueous volume encapsulation than the neutral liposomes, confirming the previous hypothesis, as shown by the encapsulation parameters reported in Table 4.

Taking in count the results in Table 4, it is easy to note that the determinant parameter of a phospholipid to have a good encapsulation efficiency is its polar head rather than the nature of the hydrophobic carbonium chain. It should be noted that an

encrease in the charge membrane concentration, keeping costant all the others factors, enhanced the sequestration volume capacity and CDP-choline content of the liposomes, indicating that the interlamellar spacing within the MLVs depends on the extent of bilayer ionization; in fact, we obtained the top EC values for DPPA and DPPA-DPPS (1:1 molar ratio), entirely costituted by charged phospholipids.

Table 4 shows that REVs encapsulation efficiency values were less affected by the nature of the phospholipid heads, than the MLVs. This, of course, is an indisputable result because the REVs population was costituted of unilamellar or at most of oligolamellar liposomes and then the repulsive forces were unexistent or have a little role in the bilayer repulsion owing to the greater interbilayers aqueous spaces. This type of liposomes has a large internal aqueous core relative to its diameter and this was responsible for the more efficient entrapment of aqueous volume than MLVs.

Release of CDP-choline from Liposomes

By considering the incorporation effect of different amount of CHOL into DPPC liposomes on the drug leakage, it was noted that CDP-choline retention within the diluted MLVs suspension progressively increase as a function of the vesicle CHOL content

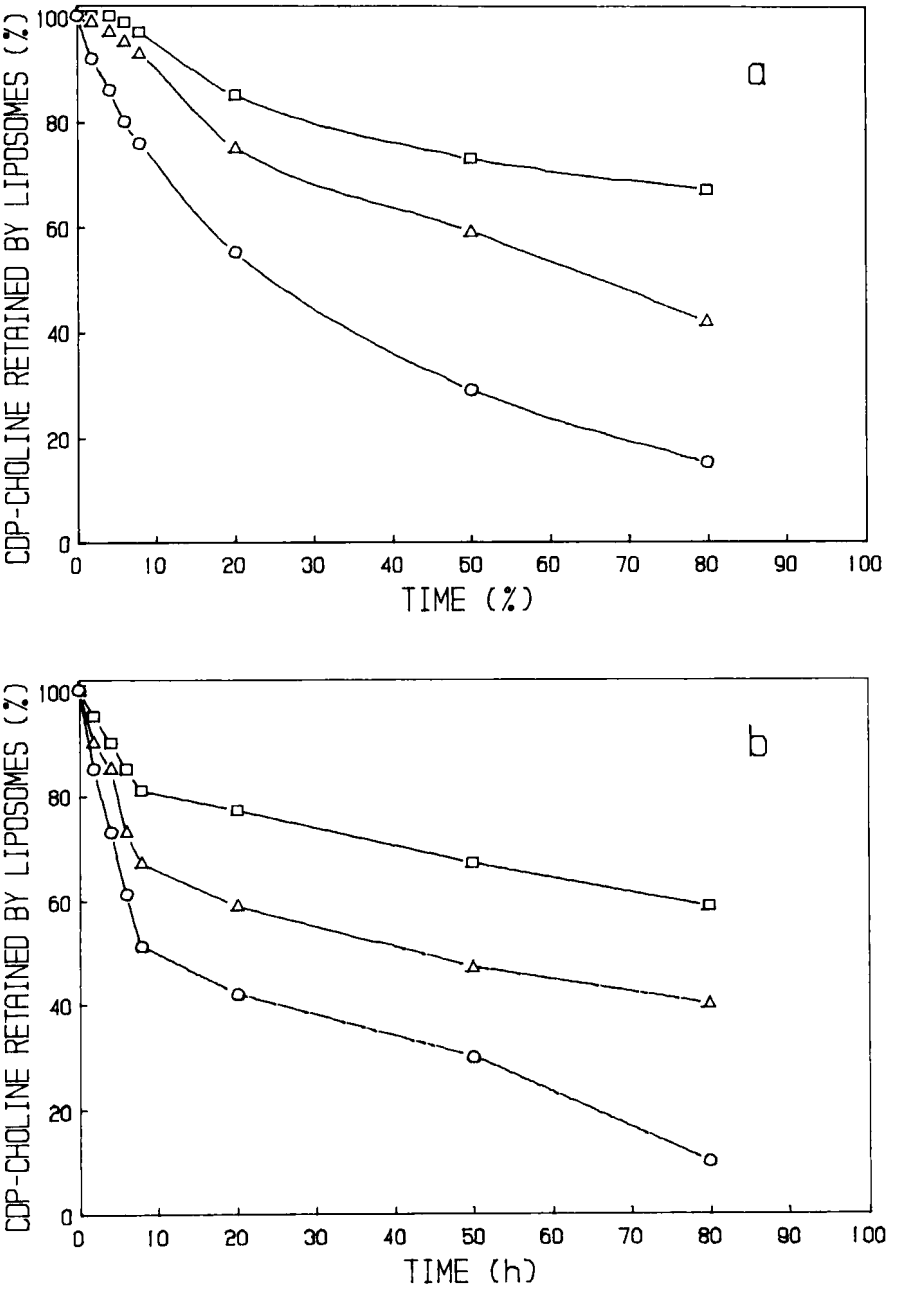

FIGURE 3

Release of CDP-choline from DPPC liposomes at different CHOL molar fraction (○ = 0.1; △ = 0.3; □ = 0.5) prepared by MLVs method (a) and REVs method (b). Each point is the mean for 5 preparations.

(Figure 3). According to the literature,[23] the decrease in permeability is proportional to the concentration of CHOL. The reduced permeability is explained to be due to the increased packing and decreased mobility of the hydrocarbon chains; in addition, CHOL also reduces the temperature-dependence of the permeability.

A confirm of this is show in Figure 4. All encapsulated CDP-choline was lost from DMPC liposomes within 35 h, whereas DPPC shows a slower release. The difference between DMPC and DPPC stands in the hydrocarbon chains which are responsible for the different thermotropic behaviour; while DMPC, that has a Tm of 25 °C, at the temperature of the leakage experiments (see experimental section) is in liquid crystal state, DPPC is in gel or better in ripple state (DPPC ripple Tm 36.9 °C, DPPC liquid crystal Tm 42.2 °C) and for this reason DMPC is more permeable than DPPC. Always in Figure 4 we note how DMPC REVs lost all the encapsulated CDP-choline in less time than MLVs owing to the presence of uni- or oligolamellar liposomes and then the drug was able to pass through the lipid barrier more easily and rapidly.

Concerning the release profile of CDP-choline from charged liposomes, it is interesting to observe (Figure 5) there is not any substantial difference

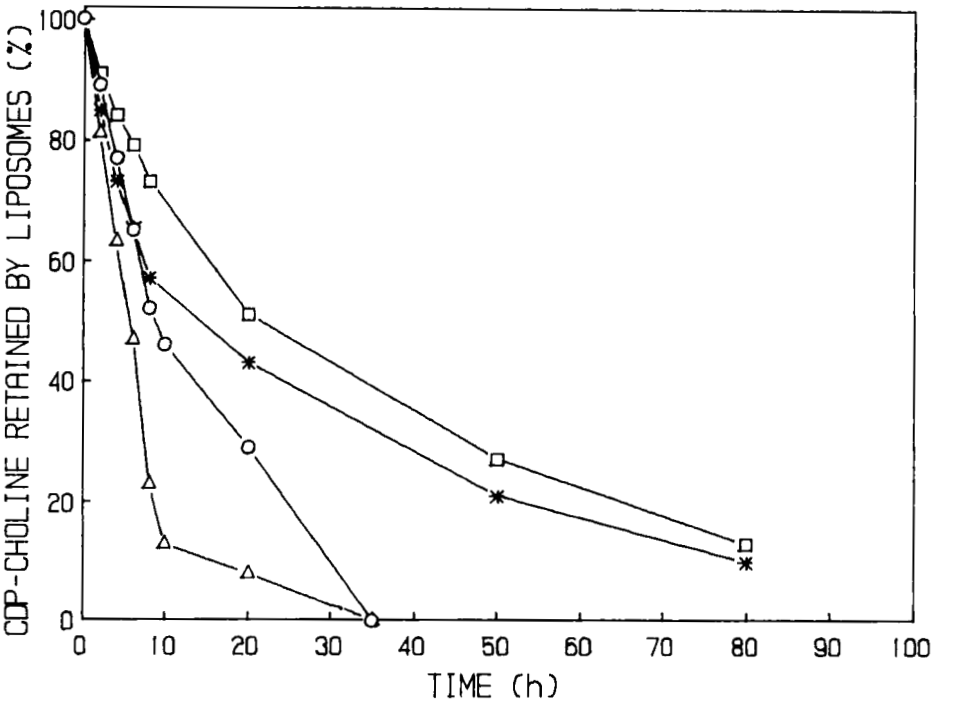

FIGURE 4

Release of CDP-choline from MLVs (○) and REVs (△) DMPC liposomes and MLVs (□) and REVs (✱) DPPC liposomes. Each point is the mean for 5 preparations.

among the various charged phospholipid systems, indicating the absence of preferential interactions with a particular phospholipid. The charged phospholipid systems have shown a release slower than that expected, probably due to the fact that all the negative charged liposomes are in the gel state[21] and CDP-choline, negatively charged, for an electrostatic repulsion phenomenon can not pass easily the negatively charged phospholipid bilayer.

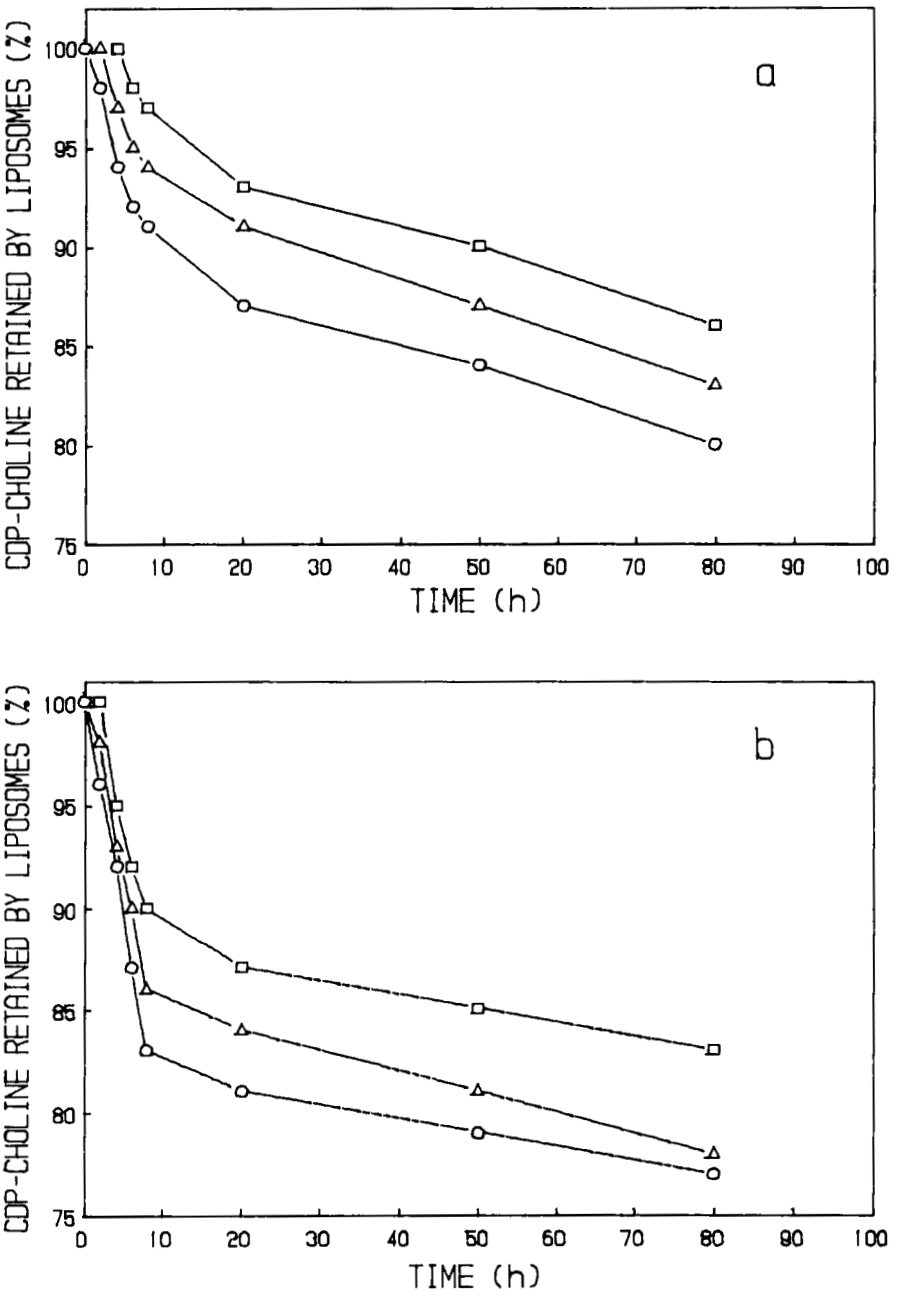

FIGURE 5

Release of CDP-choline from DPPC-DPPA [1:1] (○), DPPC-DPPS [1:1] (△) and DPPA-DPPS [1:1] (□) prepared by MLVs method (a) and REVs method (b). Each point is the mean of 5 preparations.

Analyzing the Figures 3b - 4 - 5b it is possible to observe that all REVs preparations are caracterized by a biphasic leakage: rapid drug release in the first period and then a more gradual drug loss.

Knowing the heterogeneous composition of the REVs preparation, costituted of unilamellar and oligolamellar vesicles, it is possible attribute the phase of rapid CDP-choline loss to the release of the drug from the unilamellar liposomes, since these kind of vesicles have a larger surface area to volume ratio than larger MLVs or oligolamellar vesicles and posses only a single lipid bilayer barrier to hydrophilic drug diffusion. After the first period the drug release is determined by the oligolamellar vesicles and then it is depending on the liposome phospholipid composition.

CONCLUSION

Choosing the correct experimental conditions appears to be extremely important for the quality of the CDP-choline containing liposome dispersions with respect to encapsulation efficiency, particle size and fraction of non-associated drug and therefore for the therapeutic index of this drug "in vivo". In fact, in a vesicular carrier system it is necessary to consider not only the drug amount loaded in the delivery device but also to know the release profile of the drug from the pharmaceutical formulation.

By considering all the parameters relative to the various liposomal systems it is possible to deduct what liposomal formulation is better to the "in vivo" experiments. Except DMPC system, which at the physiological temperature is in the liquid crystal state causing a rapid loss of the entrapped CDP-choline, all the other systems present favourable behaviour for the "in vivo" applications. Expecially the negative charged liposomes have both good EC values and release profiles; in addition, for these systems, particularly in the presence of DPPS, two type of process, i.e. phagocytosis and fusion, by which liposomes may enter cells was demonstrated.[24] A longer half-life in the circulation was observed for unilamellar as compared with multilamellar structures. Despite REVs present a first step of rapid CDP-choline loss compared to MLVs that, with the same phospholipid composition, show more drug retention.

By considering all this we can hypothesize that the DPPC-DPPS system owing to its EC values, release profile and thermotropic behaviour[21] is the most suitable liposomal system for our further "in vivo" experiments.

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REFERENCES

1. F. Szoka, F. Olson, T. Heath, W. Vail, E. Mayhew, D. Papahadjopoulos, Biochem. Biophys. Acta, 601, 559 (1980).
2. G. Gregoriadis, N. Engl. J. Med., 295, 704 (1976).
3. G. Gregoriadis, FEBS Lett., 36, 292 (1973).
4. R. Brasseur, G. Laurent, J.M. Ruysschaert, P. Tulkens, Biochem. Pharmacol., 33(4), 629 (1984).
5. T. Kimura, M. Yoshikawa, M. Yasuhara, H. Sezaki, J. Pharm. Pharmacol., 32, 394 (1980).
6. D.J.A. Crommelin, N. Sloats, L. van Bloois, Int. J. Pharm., 16, 79 (1983).
7. C. Nastruzzi, R. Gambari, E. Menegatti, P. Walde, P.L. Luisi, J. Pharm. Sci., 78, 672 (1990).
8. Y. Maitani, M. Nakagaki, T. Nagai, Int. J. Pharm., 64, 89 (1990).
9. H. Nagai, K. Sakano, T. Seguchi, Yakuri to Chiryo, 4, 1153 (1976).
10. G. Arienti, L. Corazzi, P. Mastrofini, I. Montanini, B. Tirillini, G. Porcellati, J. Biochemistry, 28(1), 39 (1979).
11. K. Shimamoto, Y. Aramaki, Takeda Kenkyusho Ho, 34, 189 (1975).
12. J.H. Charcosset, B. Sales, A. Jacquemin-Sablon, Biochem. Pharmacol., 32, 1037 (1983).

13. W. Scheler and I. Blank, In Kinetics of Drug Action, J.M. Van Rosum, ed Springer-Verlag Berlin, 1977.

14. A.B. Burlina, L. Galzigna, Rivista di Neurologia, 59, 26 (1989).

15. F. Castelli, G. Puglisi, R. Pignatello, S. Gurrieri Int. J. Pharm., 52, 115 (1989).

16. F. Szoka, D. Papahadjopoulos, Proc. Natl. Acad. Sci. USA, 75, 4194 (1978).

17. G. Puglisi, M. Fresta, C. La Rosa, C. Ventura, A.M. Panico, G. Mazzone, Die Pharmazie, in press.

18. S. Benita, P.A. Poly, F. Puisieux, J. Delatre, J. Pharm. Sci., 73, 1751 (1984).

19. S.J. Douglas, L. Illum, S.S. Davis, J. Kreuter, J. Colloid Interface Sci., 101, 149 (1984).

20. P.M. Pusey, D.E. Koppel, D.W. Schaefer, R.D. Camerini-Otero, S.H. Koening, Biochemistry, 13, 952 (1974).

21. C. La Rosa, D. Grasso, M. Fresta, C. Ventura, G. Puglisi, Thermochimica Acta, in press.

22. C. Kirby, J. Clarke, G. Gregoriadis, Biochem. J., 186, 591 (1980).

23. R.A. Demel, B. De Kruyff, Biochem. Biophys. Acta, 457, 109 (1976).

24. D.A. Tyrrel, T.D. Heath, C.M. Colley, B.E. Ryman, Biochem. Biophys. Acta, 437, 259 (1976).