International Journal of Pharmaceutics, 99 (1993) 145–156

© 1993 Elsevier Science Publishers B.V. All rights reserved 0378-5173/93/$06.00

IJP 03206

5-Fluorouracil: various kinds of loaded liposomes: encapsulation efficiency, storage stability and fusogenic properties

M. Fresta [a], A. Villari [b], G. Puglisi [a] and G. Cavallaro [c]

[a] *Istituto di Chimica Farmaceutica e Tossicologica, Facoltà di Farmacia Università di Catania, Viale A. Doria 6, 95125 Catania (Italy),* [b] *Dipartimento Farmaco-Chimico Università di Messina, v. le Annunziata 98010 Messina (Italy) and* [c] *Dipartimento di Chimica e Tecnologie Farmaceutiche, Università di Palermo, via Archirafi 32, 90125 Palermo (Italy)*

(Received 5 November 1992)
(Accepted 14 January 1993)

Key words: 5-Fluorouracil; Multilamellar vesicle; Stable plurilamellar vesicle; Large unilamellar vesicle; Storage stability; Particle size; Drug leakage

Summary

This paper describes the optimization of 5-fluorouracil (5-FU) loaded liposome formulations. Four different preparation procedures were carried out, obtaining two types of multilamellar vesicles (MLVs), stable plurilamellar vesicles (SPLVs) and large unilamellar vesicles (LUVs). In this study various phospholipids were used to prepare liposomes. The lipid mixtures containing dipalmitoylphosphatidylserine seemed the best for biological 5-FU delivery by presenting better encapsulation efficiency parameters, serum and storage stability, and fusogenic properties, which are an important prerequisite for in vivo liposome-cell interaction. The presence of cholesterol in the liposome composition was an important factor thereby ensuring serum and storage stability of the various vesicular systems. The most suitable liposome preparation was the SPLVs, that showed both good drug loading and stability parameters, compared to LUVs which had the highest loading capacity but low serum and storage stability.

Introduction

The therapeutic effect of a pharmaceutically active substance is closely linked to pharmacokinetics. An ideal drug should: (i) reach the site of action; (ii) arrive rapidly and in sufficient quantity; (iii) remain for a sufficient length of time; (iv) be excluded from other sites; and (v) be removed from the target site at the right time, as reported elsewhere (Notari, 1974; Lindell et al., 1978). These properties could be achieved by chemical modification of the drugs. Another way may be to transport the drug directly by a delivery device to the target organ and hold it there for as long as necessary. By means of liposomes, drugs can be delivered to the target organ and can reach a high local concentration (Jurima-Romet et al., 1990; Schenk et al., 1990).

This kind of drug delivery system could be successfully used in malignancy treatment by means of cytotoxic drugs (Gabizon et al., 1982, 1986; Mayhew and Rustum, 1985; Amselem et al., 1990). In fact, the chemotherapeutic treatment of solid tumours is still far from satisfactory, since the specificity of commonly used cytostatic

Correspondence to: G. Puglisi, Istituto di Chimica Farmaceutica e Tossicologica, Facoltà di Farmacia Università di Catania, Viale A. Doria 6, 95125 Catania, Italy.

drugs against tumours is insufficient. To develop an adequate means of treatment with higher antitumour activity and less toxic side effects, the tissue distribution pattern of the cytostatics in the tumour-bearing host should be targeted (Ackerman, 1986; Eriksen et al., 1986; Sigurdson et al., 1987; El Hag et al., 1990).

5-Fluorouracil (5-FU) is one of the most widely used pyrimidine antimetabolites in the treatment of solid tumours in many organs. Although it has been used in clinical practice for more than 20 years and remains an important antitumour agent, it has a severe drawback in that it has serious side-effects such as gastrointestinal and bone marrow toxicity (Chabner, 1982). These problems are the dose-limiting factor in its clinical and experimental use; for these reasons, as with almost all cytostatic agents, its therapeutic index needs improvement.

Low lipophicility of 5-FU gives rise to various delivery problems resulting in the reduction of bioavailability especially when it is administered orally or rectally; in fact, the only possible administration route for this drug is parenterally. Several efforts have been made to overcome these delivery problems as well as to reduce the toxic side-effects by chemical modification of 5-FU, developing transient derivatives (prodrug) with enhanced physicochemical properties in terms of delivery (Mollgaard et al., 1982; Kundu and Schmits, 1982; Yamashita et al., 1982; Buur and Bundgaard, 1984), and particulate or vesicular drug carriers (Kreuter and Hartman, 1983; Sasaki et al., 1987; Ozer and Talsma, 1989). In fact, positive results were obtained for 5-FU delivered by liposomes.

Considering that the different methods of liposome preparation could greatly influence the biological activity of the drugs, e.g., as a chemotherapeutic agent (Fountain et al., 1985; Gruner et al., 1985), it was our intention to study the effect of different lipidic mixtures upon the 5-FU-loaded vesicle formulation, in order to control the dose-response effect, and the release of the entrapped drug. The effect of the liposomal preparation process on the pharmaceutical formulation parameters was also studied.

Materials and Methods

Chemicals

5-Fluorouracil (5-FU), dipalmitoyl-DL-α-phosphatidyl-L-serine (PS), cholesterol (CH) and tocopherol succinate were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine monohydrate (MC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine monohydrate (PC) and 1,2-dipalmitoyl-*sn*-glycerophosphate disodium salt (PA) were purchased from Fluka Chemical Co. (Buchs, Switzerland). Before each experiment, the purity of phospholipids had to be greater than 99%, as assayed by two-dimensional thin-layer chromatography (Castelli et al., 1989).

Bio-Beads (spherical, macroreticular polystyrene-divinylbenzene, 20–50 mesh) were a commercial product (Bio-Rad Laboratories, Richmond, CA). For the release experiments, heat-inactivated foetal calf serum (Mescia Brunelli, Italy) was used. Sephadex G-50 fine was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden).

All other materials and solvents were of analytical grade and sterile double-distilled water was used.

Liposome preparation

Four different procedures were carried out for liposome preparation. Two preparation processes gave multilamellar vesicles (MLVs), the other two gave stable plurilamellar vesicles (SPLVs) (Gruner et al., 1985) and large unilamellar vesicles (LUVs). Liposome preparations had a final lipid concentration of 40 mg/ml.

As a preventative measure to avoid degradation reactions of both the drug and lipids, e.g., hydrolysis, photochemical decomposition and peroxidation, 5-FU was always stored in the dark at 4°C and all stages during liposome preparation were carried out under reduced oxygen pressure.

In storage stability experiments, tocopherol succinate was included in the lipid phase to improve the chemical shelf-life stability of lipids.

MLV preparation Two different methods were used: method A reported by Bangham et al.

(1965) and method B proposed by Amselem et al. (1990). Both preparations were passed through 1.2 μm filters (Sartorius Italia, s.r.l.).

Method A The required amount of lipids was weighed into a round-bottomed flask and dissolved in a chloroform-methanol mixture (70:30 v/v). The organic solution was then removed at 30°C on a rotary evaporator under reduced pressure and then stored overnight under high vacuum. Thus, a thin film of dry lipid was deposited on the inner wall of the flask. Liposomes were prepared by adding to the flask 1 ml of an isotonic phosphate buffer solution containing 1 mg/ml of 5-FU. The aqueous dispersion was heated to a temperature 15°C higher than the gel to liquid crystalline phase transition temperature (T_m) of the selected lipid mixture. The flask was maintained at that temperature for 1 h to allow full hydration of the samples. Then, to form MLVs, the suspension was shaken twice on a mechanical agitator for 10 min. The liposome dispersion formed was left for 3 h at room temperature for the annealing and swelling of phospholipids.

Method B The lipid components were dissolved in chloroform in a 100 ml round-bottomed flask containing 40 g of glass beads (2–3 mm mean size) (Carlo Erba, Italy). The scope of the glass bead addition was to increase the surface area of the dried lipid film, thus enhancing the phospholipid contact with the buffer solution containing the pharmaceutically active substance. In this way the hydration and dispersion of lipid were easier. The final volume of lipid solution was adjusted to 35 ml with chloroform. The organic solution was dried and a thin film coating the flask and the beads was obtained. Possible residual organic solvent was eliminated by connecting the flask to a lyophilizer (Edwards Freeze Dryer Modulyo, equipped with an Edwards high vacuum pump model Serial E 2M8 42810) overnight at a pressure of 150 Torr. MLVs were formed by means of mechanical stirring for 3 h of the lyophilized lipids with the drug solution.

SPLV preparation The lipid films (obtained by MLV preparation) were dissolved in 5 ml diethyl ether; in some cases, dropwise addition of methanol was necessary to increase lipid solubility. Successively, 0.4 ml of the isotonic phosphate solution containing 1 mg/ml of 5-FU was added to the organic solution. Aqueous and organic solutions were emulsified in a sonicator bath (Bransonic model 2200), resulting in a homogeneous opalescent dispersion. During the emulsification process, the mixture was flushed under a gentle stream of nitrogen. This was carried out for approx. 15 min until the ether had mostly evaporated and could not be smelled. The resulting viscous gel was resuspended in 3 ml of phosphate buffer by swirling the fluid.

LUV preparation LUVs were prepared using a detergent solubilization method (Foldvari et al., 1990, 1991). The lipid film was solubilized in 5-FU phosphate buffer solution which contained Triton X-100. The phospholipid Triton X-100 ratio was 1:2 (w/w). This aqueous solution was treated overnight with 350 mg/ml of moist SM-2 Bio-Beads (capacity 70 mg Triton X-100/100 mg beads) by changing the Bio-Beads once after 2 h of incubation (Vainstein et al., 1984). The Bio-Beads were washed with methanol and filtered water (0.2 μm Millipore) five times before addition to the aqueous solution. As the detergent was removed, the micelles coalesced and the phosphlipid adopted the bilayer configuration resulting in scaled LUVs. After liposome preparation, Bio-Beads were separated from the suspension by means of a Whatman 42 filter paper.

Separation of free 5-FU

Free 5-FU was removed from the liposomal suspension by centrifugation. It was necessary to adapt the centrifugation process to the kind of liposome. Thus, MLVs prepared via both methods A and B were centrifuged for 30 min at 9000 rpm, whereas SPLVs and LUVs were centrifuged at 15 000 and 20 000 rpm, respectively (Beckman model J2-21 centrifuge, with a Beckman JA-20.1 fixed angle rotor).

The experimental results on 5-FU encapsulation efficiency, obtained by centrifugation, were compared to those using a gel filtration method, in order to assess the validity of the former. It is essential that the centrifugation of liposomes is carried out at the appropriate rate, since as reported previously (Fresta et al., 1993), this pa-

rameter influences the amount of drug entrapped. Therefore, free 5-FU was also removed by passing 1 ml of the liposome suspension through a fine Sephadex G-50 column (35 × 1 cm).

The experimental data thus obtained were comparable to those of centrifugation (data not reported).

Determination of 5-FU

After separation of the free 5-FU fraction, the amount encapsulated could be determined. 3.5 ml of a CH_2Cl_2-CH_3OH (2:1 v/v) mixture were added to the pelleted liposomes to destroy the phospholipid bilayer structures, freeing the drug. This organic solution was poured into a long-necked 10 ml quickfit round-bottomed flask and made up to 10 ml with methanol. 10 μl of this solution was submitted to 5-FU determination by HPLC analysis. The chromatographic apparatus consisted of a Varian model 9010 solvent delivery pump (Varian Associates Inc., Walnut Creek, CA, U.S.A.), a Rheodyne model 7125 syringe loading injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 10 μl loop, a Varian model 9050 variable wavelength ultraviolet UV-Vis detector operating at 261 nm and a Varian model 4400 reporting integrator. Chromatography was carried out on a Hypersil ODS reverse-phase C_{18} analytical column (5 μm, 160 mm × 4.6 mm i.d., Shandon Southern, Runcorn, U.K.). The mobile phase, constituted of 10 mM *t*-butylammonium hydroxide-methanol (70:30 v/v), was delivered at 1.5 ml/min. The solvent mixture was filtered through 0.2 μm microporous PTFE membrane filters (Millipore, U.S.A.) and degassed by ultrasonication prior to use. Chromatography was carried out at room temperature. Results were calculated from linear regression of an external standard of 5-FU, relating peak area and concentration.

As reported alsewhere (Benita et al., 1984; Puglisi et al., 1992), the amount of 5-FU encapsulated in liposome was expressed as encapsulation capacity (EC) values calculated from the ratio between the concentration of drug entrapped (mmol/ml), and the product of the added drug concentration (mmol/ml) and lipid concentration (mmol/ml) of the liposome suspension. Another parameter linked to drug encapsulation is the molar fraction (MF) determined from the ratio between entrapped drug (mmol/ml) and lipids (mmol/ml) × 10^2.

Partition coefficient

The partition coefficient of 5-FU was determined either with octanol/0.1 M buffer or chloroform/0.1 M buffer at 20°C. The buffer solutions were phosphate (pH 4, 6 and 7.4) and pH 5 acetate. A 1 mg aliquot of 5-FU was added to 10 ml of octanol or chloroform plus 10 ml of buffer, these presaturating each other. The samples were immersed in a thermostated water bath at 20°C and mixed thoroughly by shaking for at least 3 h. The phases were separated by centrifugation at 500 × *g* for 5 min. Aliquots were removed from the upper and lower phases and then assayed using the above HPLC method (see previous section). The partition coefficient was expressed as the logarithm of the ratio of the amount of 5-FU in the organic phase to that in the aqueous phase (log *P*).

Release from liposomes

The liposome pellet was made up to 5 ml with pH 7.4 isotonic phosphate buffer or with serum attaining a final phospholipid concentration of 30 mg/ml, and submitted to dialysis. The dialysis donor compartment was a Spectrapor/por 2 membrane (Mol. Wt cut-off 12 000–14 000; Spectrum, Medical Industries, Inc., Los Angeles, CA). The dialysis bag was immersed in the receiver compartment containing 350 ml of the buffer solution or serum. Experiments were performed at 37°C in a thermostated bath (I.S. Co. model BTU 6). At predetermined time intervals, 2-ml samples were removed from the receiver compartment and the 5-FU content was determined by HPLC analysis. When the release experiment was carried out with serum, HPLC determination of 5-FU was performed as reported by Stetson et al. (1985).

Each sample removed was replaced by an equal volume of the dialysis liquid. Retention of the drug in the various liposome types was calculated from the following equation:

$$\% \text{ drug retention} = (C_0 - C_f) \times 100/C_0$$

where C_0 is the initial concentration of drug entrapped and C_f the free 5-FU concentration at various times.

Differential scanning calorimetry (DSC)

DSC scans were recorded using a Mettler DSC 12E instrument. Calibration of the temperature scale and energy (ΔH) were carried out using indium as standards. The plotting range as full scale deflection was set to 1 mW; the noise was < 20 μW. The thermometric and calorimetric sensitivity was 56 μV/°C and 3 μV/m, respectively. Each experimental measurement presented an accuracy of ± 0.4°C with a reproducibility and resolution of 0.1°C. The ΔH values were calculated from the peak area using Mettler system software (TA89E).

For DSC analysis, 40 μl of the liposomal suspension containing about 2.5 mg of phospholipids were sealed in an aluminium pan and submitted to calorimetric analysis. The thermograms of the liposomal suspension were recorded during both heating and cooling in the range 20–75°C at a scanning rate of 1°C/min.

For fusion experiments, the liposome samples were prepared 'heterogeneously' (La Rosa et al., 1992a): PC vesicles and phospholipid mixture vesicles were prepared separately and then mixed at a temperature below that of the gel to liquid crystalline phase transition (T_m) of the various components.

Size analysis

For size determination experiments, a photocorrelation spectroscopic (PCS) method was used. The PCS apparatus was a Spectraphysic model 120 He-Ne laser (7 mW), a Malvern PC 8 sample holding system thermostated at 23°C and a Hamamazu R 1333 photomultiplier positioned at 90° (Puglisi et al., 1992; Fresta et al., 1993).

Results and Discussion

In this study we have investigated only negatively charged liposomes, since positively charged or neutral liposomes are less efficient for drug delivery as well as having lower in vivo antitumour activity (Straubinger et al., 1983; Crommelin, 1984; Heath-Timoty et al., 1985).

It has been well established (Straubinger et al., 1985; Nicholas and Jones, 1986) that studies concerning the loading capacity and the release profile of drug loaded liposomes require a profound knowledge of the interaction occurring between host and guest in order for the correct interpretation of the data. A suitable procedure is DSC analysis, which is a powerful and non-perturbing thermodynamic technique for characterizing the thermotropic behaviour of lipid bilayers (La Rosa et al., 1992b).

As shown in Fig. 1 and Table 1, no particular interaction was detected among the liposome lipid

TABLE 1

Thermotropic behaviour values calculated either in the presence or in the absence of 5-FU during the gel to liquid-crystalline phase transition of MLVs with different lipid composition in pH 7.4 isotonic phosphate buffer [a]

Phospholipid composition	Without 5-FU			With 5-FU		
	T_m (°C)	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})	T_m (°C)	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})
PC-PS 3:1	44.3	8.9	28.0	44.5	8.7	27.4
PC-PA 3:1	48.9	7.1	22.0	48.6	6.9	21.5
MC-PS 3:1	29.5	6.5	21.5	30.0	6.4	21.1
MC-PA 3:1	31.5	6.1	20.0	31.0	6.3	20.7

[a] Each value was the average of six experiments.

components and 5-FU at a concentration of 1 mg/ml. In fact, no noticeable difference was observed in the ΔH and T_m values determined for all the lipid mixtures in either the presence or absence of the drug; moreover, the shapes of the transition-peaks were also similar. These findings, according to the partition coefficient of 5-FU (log P_{oct} −0.7), demonstrated that the drug was solubilized into the aqueous core of the vesicles and that none was incorporated into the bilayer structure or greatly adsorbed onto the polar-head liposomal surface.

The delivery of drug loaded liposomes into cells is linked to the ability of the lipid vesicles to interact with the cells. There exist various interaction pathways between liposomes and cells: stable absorption, lipid exchange or trasfer, fusion and endocytosis. Since not all of these routes can ensure the delivery of the drug to cells, the study of a phospholipid system with a high potential for injecting 5-FU into cells (fusion) was of great importance for the development of a biologically efficient delivery device. For this reason we studied fusion between the different phospholipid mixtures and PC membranes, which mimic most aspects of biological membranes. Among the various phospholipid mixtures, only systems comprising PS presented fusogenic properties. As shown in Fig. 2, the first DSC scan presented two peaks centred at 42 and 54.8°C for PC (a) and PC/PS (2:1 molar ratio) (b) vesicles, respectively. Subsequent cycles showed the disappearance of the former peak with a shift of the two signals toward a single peak centred at 49°C.

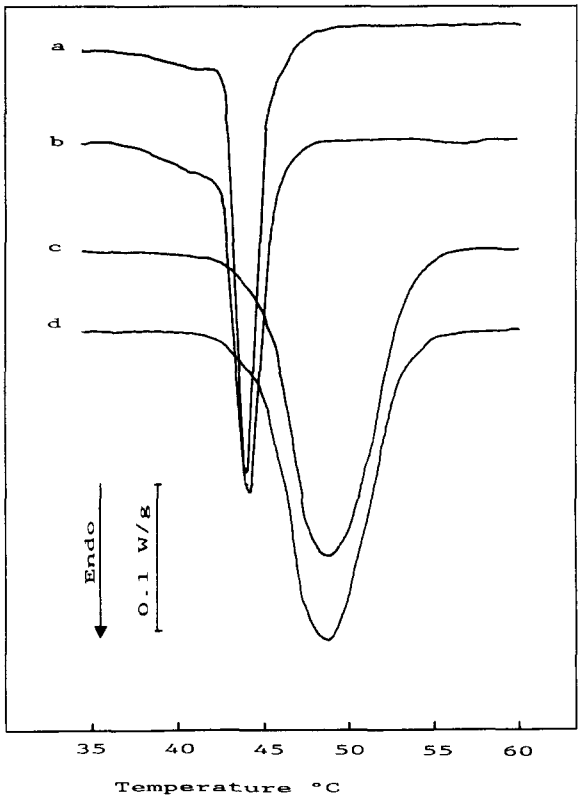

Fig. 1. Calorimetric scans in pH 7.4 isotonic phospate buffer of MLVs with different lipid composition in the absence (a, PC-PS 3:1; c, PC-PA 3:1) and presence (b, PC-PS 3:1; d, PC-PA 3:1) of 5-FU.

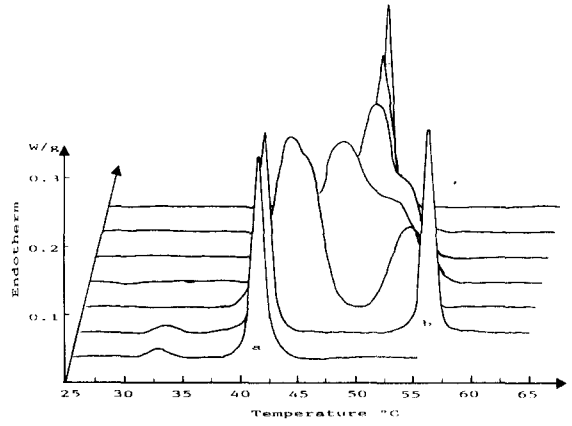

Fig. 2. DSC runs in heating mode of PC/PS (2:1 molar ratio) liposomes entrapping 5-FU (b). The samples for the fusogenic experiments were prepared in heterogeneous mode, using PC (a) as target vesicles. The measurements were carried out in pH 7.4 isotonic phosphate buffer.

The same trend was shown by the MC/PS (2:1 molar ratio) system. The only difference was the final transition peak, which was lower than that of the PC system, since MC increased the fluidity of the vesicles. For the systems containing PA no fusion process was detected.

For biological application, an important factor was the presence of CH in the phospholipid mixture; in fact, CH ensured the colloidal stability of liposome systems in biological fluids. For this reason, maintaining the molar ratio between charged and neutral lipids constant in order to avoid variation in the phospholipid fusogenic

TABLE 2

Encapsulation efficiency parameters of liposomes constituted of different lipid systems and prepared via various procedures [a]

Lipid systems	MLVs (method a)			MLVS (method b)			SPLVs			LUVs		
	EC	MF	±SD	EC	MF	±SD	EC	MF	±SD	EC	MF	±SD
PC/PS/CH 7:4:5	2.3	1.7	0.2	4.2	3.2	0.1	6.9	5.3	0.1	9.1	7.0	0.2
PC/PA/CH 7:4:5	1.0	0.8	0.2	2.5	1.9	0.2	5.6	4.3	0.1	8.9	6.8	0.1
MC/PS/CH 7:4:5	1.9	1.5	0.1	3.7	2.8	0.2	6.5	5.0	0.1	8.7	6.7	0.3
MC/PA/CH 7:4:5	0.8	0.6	0.3	2.3	1.8	0.3	5.1	3.9	0.2	8.8	6.8	0.2

[a] Each value was the average of nine experiments ± standard deviation (SD).

properties, we added CH to the liposome composition (Rand, 1981; Bonté and Juliano, 1986).

By evaluating the encapsulation efficiency of the various liposome formulations (Table 2), we observed differences in EC and MF values among the various lipid mixtures, especially in the multilamellar systems.

The presence of negatively charged phospholipids tended to increase the interbilayer distance owing to electrostatic repulsive forces, which were predominant in phospholipid mixtures containing PA, whereas for systems with PS a noticeable contribution in the interlamellar space also derived from the steric repulsive forces among their polar heads, which were larger than those of PA. This explained the differences in entrapped aqueous volume, which was an important factor in the loading capacity of hydrophilic drugs such as 5-FU. No appreciable difference in encapsulation efficiency was observed among lipid mixtures of LUVs, since this system was unilamellar and the slight variation was due to a small difference in liposome size. The different entrapped aqueous volumes could explain the various encapsulation parameters among liposomes obtained by the different preparation processes.

As shown in Table 2, LUVs were the liposome formulation that was able to entrap the greatest amount of aqueous phase and of 5-FU. Attention should be focused on the loading capacity of MLVs prepared using the two different procedures. In this case, it was our opinion that a major factor affecting drug entrappment was the stage of lipid hydration during liposome preparation. The main factor governing the rate of lipid hydration was the surface available for lipid-water contact. Therefore, the larger the surface area and the thinner the lipid film, the more exposed were the lipids to the aqueous phase, and, consequently, the greater the uniformity of lipid hydration. The thickness of the film can result in markedly different preparations of MLVs in spite of identical lipid concentrations, compositions and volumes of the suspending aqueous phase. Slow hydration was beneficial due to the slow annealing of MLVs which allowed a longer period of contact between all the liposome bilayers and the drug aqueous solution.

The presence of CH did not appreciably influence the loading capacity of the various phospholipid mixtures with regard to those prepared without it (data not reported).

As reported in Table 3, the partition coefficient of 5-FU was evaluated in octanol or chloroform at different pH. This parameter had no influence on 5-FU encapsulation efficiency, whereas it strongly affected the drug release from liposomes. The permeability constant of the membrane (k) is given by:

$$k = \frac{D \cdot A \cdot P_c}{V_0 \cdot h}$$

TABLE 3

Partition coefficient of 5-FU with octanol or chloroform in the presence of 0.1 M buffer at different pH

Buffer pH	Log P_{oct}	Log P_{chlor}
4	−1.8	−2.7
5	−1.5	−2.3
6	−0.9	−1.9
7.4	−0.7	−1.7

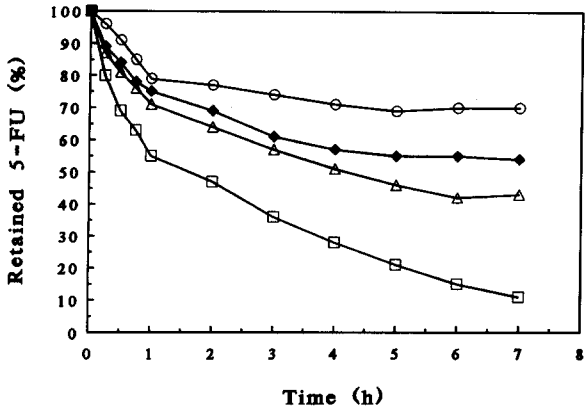

Fig. 3. 5-FU retained by liposomes in serum. The experiment was carried out at a constant temperature of 37°C. A small amount of sodium azide was added to prevent bacterial growth. (♦) LUVs (PC/PS/CH 7:4:5), (○) SPLVs (PC/PS/CH 7:4:5), (△) SPLVs (MC/PS/CH 7:4:5), (□) SPLVs (PC/PS 3:1).

where D is the diffusion coefficient of the drug in the lipid membrane, A denotes the membrane area, V_0 is the inner aqueous volume, h represents the membrane thickness and P_c is the partition coefficient of the drug.

Taking into consideration the fact that the diffusion coefficient D is not markedly affected by a change in drug and A, V_0 and h are dependent on the type of liposome, the partition coefficient P_c strongly influences the permeability coefficient k. Hence, the partitioning of the drug could be the rate-determining step in the process of drug leakage. Therefore, the lower the value of log P for 5-FU, the slower will be the rate of release (Tsukada et al., 1984). For this reason, pH 6 phosphate buffer was chosen for the liposome formulation; this buffer provides a compromise between appropriate values of log P for 5-FU and high biocompatibility (Table 3).

By examining the serum leakage of 5-FU from liposomes, it was noted (Fig. 3) that the presence of CH in the lipid composition greatly influenced the release behaviour of the liposome formulations; in fact, phospholipid mixtures without CH became very permeable in the presence of serum. When the experiments were carried out in pH 7.4 buffer (Fig. 4), the release profile was almost equal for all phospholipid systems. A difference was also observed between multilamellar systems and LUVs, which were more permeable through the leakage of a greater amount of drug than the others. This was due to the more rapid and easier passage of 5-FU through the only liposomal membrane characterizing the system (Figs 3 and 4).

The limiting step in the clinical application of liposomes for drug delivery was the technical feasibility of using the system in practice. In particular, this was due to the following critical factors: (i) the preparation of sterile and pyrogen free liposomes on an industrial scale; (ii) high and reproducible levels of drug entrapment; (iii) storage possibility, both in terms of chemical stability of the lipid components and drugs, and the physical stability concerning liposome size and drug leakage.

To obtain sterile liposome suspensions, the lipid mixtures, both solubilized in organic solvents and aqueous buffer solution, were filtered through 0.2 μm filters. In addition, the liposome formulations were submitted to sterility assay as described in FUI 1985, to verify whether sterile conditions were maintained during the preparation procedures. As shown in Table 2, all preparation procedures showed reproducible loading capacity values.

Previous studies (Ozer and Talsma, 1989) have reported the possibility of long-term storage for 5-FU loaded liposomes, submitting them to a

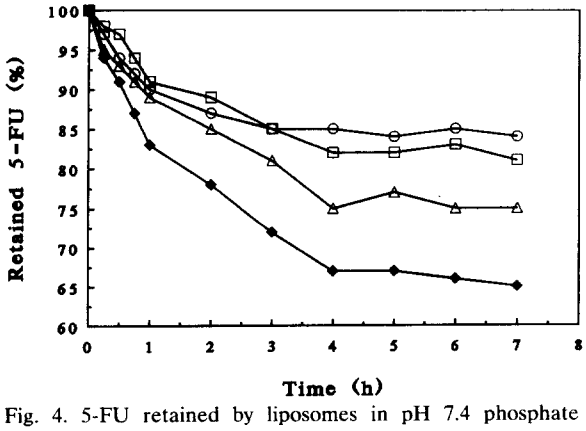

Fig. 4. 5-FU retained by liposomes in pH 7.4 phosphate buffer. The experiment was carried out at a constant temperature of 37 ± 0.2°C. Symbols as in Fig. 4.

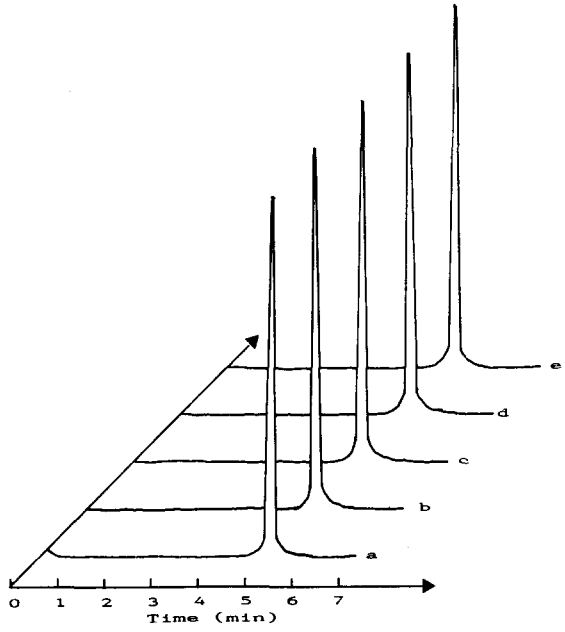

Fig. 5. 5-FU chromatograms recorded for different storage times: (a) before storage, (b) 1 month storage, (c) 2 months storage, (d) 3 months storage, (e) 4 months storage.

storage temperature of 4–6°C. To assess the storage stability, some liposome suspensions were submitted to a storage period of 4 months as a pellet at a temperature of 4°C under sterile conditions. 5-FU stability was monitored by HPLC, which showed no alteration (peak splitting or broadening) of the drug (Fig. 5). Chemical stability of the lipid component (Stewart, 1980; Ozer and Talsma, 1989) was determined by evaluating the lysophospholipid content of the various liposome formulations. The lysophospholipid content was very low for all systems, particularly for those comprising PC (Table 4).

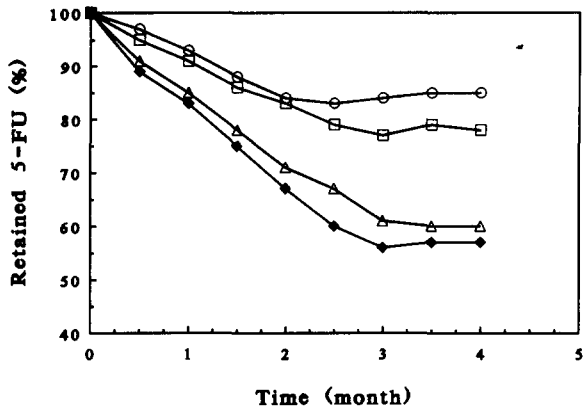

Fig. 6. Retention of 5-FU in SPLVs stored at 4°C as a pellet. Each point was the average of three experiments. (○) PC/PS/CH (7:4:5), (△) MC/PS/CH (7:4:5), (□) PC/PA/CH (7:4:5), (◆) MC/PA/CH (7:4:5).

By maintaining structural integrity, the liposome systems demonstrated no change in membrane permeability thereby allowing elevated retention of the entrapped drug. In fact, after a period of drug leakage, a plateau level was reached by fixing the amount of retained 5-FU at values of about 80% for liposomes with PC and 60% for those with MC (Fig. 6). This was due to the greater membrane fluidity of the MC systems (Table 1), which were consequently more permeable to 5-FU than liposomes containing PC. In fact, the drug loss in PC systems was probably due to a deabsorption process of 5-FU from the external liposome layers rather than to permeation through the liposome membranes.

This trend was similar for all the multilamellar liposomal systems. Only LUVs showed (Fig. 7) considerable differences in leakage of the various phospholipid mixtures, total drug leakage being

TABLE 4

Lysophospholipid content of the various phospholipid mixtures as a function of storage time [a]

Phospholipid mixtures	Storage period		
	0	2 months	4 months
PC/PS/CH 7:4:5	0.2 ± 0.1	1.1 ± 0.5	1.7 ± 0.4
MC/PS/CH 7:4:5	0.5 ± 0.2	1.0 ± 0.4	1.8 ± 0.6
PC/PA/CH 7:4:5	0.3 ± 0.1	0.9 ± 0.2	1.5 ± 0.6
MC/PA/CH 7:4:5	0.5 ± 0.3	1.3 ± 0.6	1.9 ± 0.3

[a] Lysophospholipid content is expressed as percent ± SD. Each value was the average of six experiments ± standard deviation (SD).

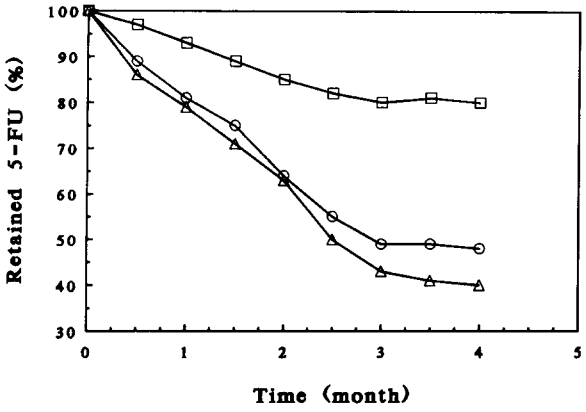

Fig. 7. Retention of 5-FU in different liposome systems stored at 4°C as a pellet. Each point was the average of three experiments. (○) LUVs (PC/PS/CH 7:4:5), (△) LUVs (MC/PS/CH 7:4:5), (□) MLVs (PC/PS/CH).

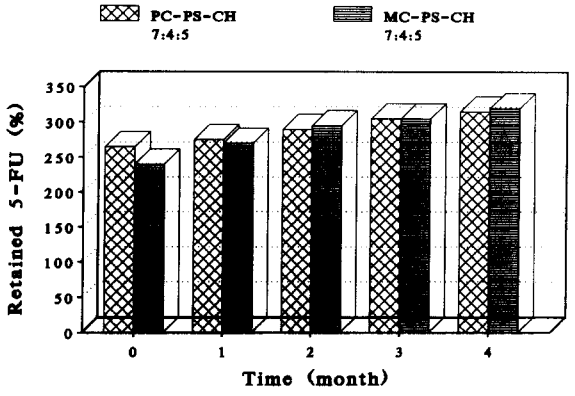

Fig. 8. Size variation of LUVs during storage at 4°C as a pellet. Each point was the average of five experiments.

higher than those of MLVs. Furthermore, in this case, the cause was due to the fact that the drug could pass through only one phospholipid bilayer.

As reported in Table 5, the liposome size during storage underwent no apreciable change in multilamellar systems. Only LUVs showed changes in vesicle size (Fig. 8). Evidently, this system was more sensitive to storage procedure.

Conclusions

We conclude from our present data that phospholipid mixtures containing PS could provide the best biological 5-FU delivery devices. In fact, owing to their fusogenic properties, these systems should be able to interact with cells, not only through fusion or endocytotic processess but also through the juxtaposition of drug transfer when, upon binding of liposomes to the cell surface, transient permeabilization of the cellular membrane occurs (Poste, 1980). On consideration of the pharmaceutical formulation parameters of the various systems, the SPLVs appeared to be the most appropriate for preparing liposomes. In fact, LUVs showed the best 5-FU loading capacity but involved drawbacks in serum and storage stability, whereas SPLVs demonstrated not only suitable 5-FU encapsulation values and vesicle size but also serum and stotage stability.

Acknowledgements

The present work was supported by a grant of the Italian C.N.R. The authors wish to thank Istituto di Tecniche Spettroscopiche C.N.R. Messina and particularly Dr Norberto Micali for help with light scattering measurements. We are

TABLE 5

Variation of SPLV size during storage as a pellet at 4°C [a]

Phospholipid mixture	Storage period				
	0	1 month	2 months	3 months	4 months
PC/PS/CH 7:4:5	610	605	615	610	620
MC/PS/CH 7:4:5	590	605	615	625	620
PC/PA/CH 7:4:5	575	575	570	580	585
MC/PA/CH 7:4:5	560	575	580	575	585

[a] Size is expressed as nm. Each value was the average of five experiments.

especially grateful to Professor Gioacchino Mazzone for stimulating discussions.

References

Ackerman, N., Experimental studies on the role of the portal circulation in hepatic tumourvascularity. *Cancer,* 58 (1986) 1653–1657.

Amselem, S., Gabizon, A. and Barenholz, Y., Optimization and upscaling of doxorubicin-containing liposomes for clinical use. *J. Pharm. Sci.,* 79 (1990) 1045–1052.

Benita, S., Poly, P.A., Puisieux, F. and Delatre, J., Radiopaque liposomes: effect of formulation conditions on encapsulation efficiency. *J. Pharm. Sci.,* 73 (1984) 1751–1754.

Bangham, A.D., Standish, M.M. and Watkins J.C., Diffusion of univalent ions across the lamellar of swollen phospholipids. *J. Mol. Biol.,* 13 (1965) 238–252.

Bonté, F. and Juliano, R.L., Interaction of liposomes with serum proteins. *Chem. Phys. Lipids,* 40 (1986) 359–372.

Buur, A. and Bundgaard, H., Prodrugs of 5-FU: I. Hydrolysis kinetics and physicochemical properties of various *N*-acyl derivatives of 5-FU. *Int. J. Pharm.,* 21 (1984) 349–364.

Castelli, F., Puglisi, G., Pignatello., R. and Gurrieri, S., Calorimetric studies of the interaction of 4-biphenylacetic acid and its βcyclodextrin inclusion compound with lipid model membrane. *Int. J. Pharm.,* 52 (1989) 115–121.

Chabner, B.A., Pyrimidine antagonist. In Chabner, B.A. (Ed.), *Pharmacologic Principles of Cancer Treatment,* Saunders, Philadelphia, 1982, pp. 132–142.

Crommelin, D.J.A., Influence of lipid composition and ionic strength on the physical stability of liposomes. *J. Pharm. Sci.,* 18 (1984) 1559–1563.

El Hag, I.A., Jakobsson, B., Jönsson, P.E. and Steuram, U., The incorporation of 5-FU into rat liver tumour and normal tissues after administration by the hepatic artery during temporary portal vein clamping. *Res. Exp. Med.,* 190 (1990) 183–192.

Ericksen, C., Christensson, P.I., Eriksson, G., Yngner, I., Jönsson, P.E. and Stenram, U., Effect of administration routes on the uptake of uridine and 5-fluorouridine into an adenocarcinoma trasplanted to rat liver. *J. Surg. Oncol.,* 33 (1986) 76–80.

Foldvari, M., Mezei, C. and Mezei, M., Intracellular delivery of drugs by liposomes containing Po glycoprotein from pheriferal nerve myelin into human 4 melanoma cells. *J. Pharm. Sci.,* 80 (1991) 1020–1028.

Foldvari, M., Mezei, M. and Mezei, C., Reconstitution into liposomes of highly purified Po glycoprotein from avian peripheral nerve myelin. *Biochem. Cell Biol.,* 68 (1990) 499–511.

Fountain, M.W., Weiss, S.J., Fountain, A.G. and Shen, A., Treatment of Brucella canis and Brucella abortus in vitro and in vivo by plurilamellar vesicle-encapsulated aminoglicosides. *J. Infect. Dis.,* 152 (1985) 529–535.

Fresta, M., Puglisi, G., Panico, A.M., Di Marco, S. and Mazzone, G., CDP-choline entrapment and release from multilamellar and reverse-phase evaporation liposomes. *Drug Dev. Ind. Pharm.,* 19 (1993) 559–585.

FUI: Farmacopea Ufficiale della Republica Italiana, 9th Edn, Istituto poligrafico e Zecca dello Stato, Roma, 1985, Vol. 1, pp. 277–328.

Gabizon, A., Dagon, A., Goren, D., Barenholz, Y. and Fuks, Z., Liposomes as in vivo carriers of Andriamycin: reduced cardiac uptake and preserved antitumour activity in mice. *Cancer Res.,* 42 (1982) 4734–4739.

Gabizon, A., Meshorer, A. and Barenholz, Y., Comparative long-term study of the toxicities of free and liposome-associated doxorubicin in mice after intraveneous administration. *J. Natl. Cancer Inst.,* 77 (1986) 459–469.

Gruner, S.M., Lenk, R.P., Janoff, A.S. and Ostro, M.J., Novel multilayered lipid vesicles: comparison of physical characteristics of multilamellar liposome and stable plurilamellar vesicles. *Biochemistry,* 24 (1985) 2833–2842.

Heath-Timoty, D., Lopez, N.G. and Papahadjopoulos, D., The effect of liposome size and surface charge on liposome mediated delivery of methotrexate-asprtate to cell in vitro. *Biochim. Biophys. Acta,* 820 (1985) 74–84.

Jurima-Romet, M., Barber, R.K, Demeester, J. and Shek, P.N., Distribution studies of liposomes-encapsulated glutathione administered to the lung. *Int. J. Pharm.,* 63 (1990) 227–235.

Kreuter, J. and Hartman, H.R., Comparative study on the cytostatic effect and the tissue distribution of 5-FU in a free form and bound to polybuthylcyanoacrylate nanoparticles in sarcoma 180-bearing mice. *Oncology,* 40 (1983) 363–366.

Kundu, N.G. and Schmits, S.A., *N*-Alkylated derivatives of 5-Fluorouracil. *J. Pharm. Sci.,* 71 (1982) 935–938.

La Rosa, C., Grasso, D., Fresta, M., Ventura, C. and Puglisi, G., Phospholipid vesicles as drug delivery systems: II. A study on kinetic fusion between vesicles containing CDP-choline and dipalmitoylphosphatidylcholine vesicles. *Thermochim. Acta,* 198 (1992a) 181–190.

La Rosa, C., Grasso, D., Fresta, M., Ventura, C. and Puglisi, G., Phospholipid vesicles as a drug delivery system: I. Interaction of cytidine-5′-diphosphate choline with charged and zwitterionic phospholipids. *Thermochim. Acta,* 195 (1992b) 139–148.

Lindell, B., Arosen, K.F., Nosslin, B. and Rothman, U., Studies in pharmacokinetics and tolerance of substances temporarily retained in the liver by microsphere embolization. *Ann. Surg.,* 1 (1978) 95–99.

Mayhew, E. and Rustum, Y.M., The use of liposomes as drug carriers of therapeutic agents. *Prog. Clin. Biol. Res.,* 172 (1985) 301–310.

Mollgard, B., Hoelgaard, A. and Bundgaard, H., Prodrugs as drug delivery system: XXIII. Improved dermal delivery of 5-Fluorouracil through human skin via *N*-acyloxymethyl prodrug derivatives. *Int. J. Pharm.,* 12 (1982) 153–162.

Nicholas, A.R. and Jones, M.N., The absorption of phospholipid vesicles by perfused rat liver depends on on vesicle

surface charge. *Biochim. Biophys. Acta,* 860 (1986) 600–607.

Notari, R., Structural effects in pharmacokinetcs and drug response. Symposium on pharmacokinectis and drug effects, Stockholm, Nov. 1974. *Acta Pharm. Suec.,* 11 (1974) 633.

Ozer, A.Y. and Talsma, H., Preparation and stability of liposomes containing 5-fluorouracil. *Int. J. Pharm.,* 55 (1989) 185–191.

Poste, G., The interaction of lipid vesicles (liposomes) with coltured cells and their use as carriers for drugs and macromolecules. In Gregoriadis, G. and Allison, A.C. (Eds), *Liposome in Biological Systems,* Wiley, New York, 1980, pp. 102–151.

Puglisi, G., Fresta, M., La Rosa, C., Ventura, C.A., Panico, A.M. and Mazzone, G., Liposome as potential drug carrier for citicoline (CDP-choline) and the effect of formulation conditions on encapsulation efficiency. *Pharmazie,* 47 (1992) 211–215.

Rand, R.P., Interacting phospholipid bilayers: measured forces and induced structural changes. *Annu. Rev. Biophys. Bioeng.,* 10 (1981) 277–314.

Sasaki, H., Matsukawa, Y., Hashida, M. and Sezaki, H., Characterization of alkylcarbamoyl derivatives of 5-fluorouracil and their application to liposome. *Int. J. Pharm.,* 36 (1987) 147–156.

Schenk, P., Nuhm, P., Fichtner, I. and Arndt, D., Studies on sucrose-palmitatestearate-containing vesicles encapsulating the cytostatic drug methylglyoxal-bis-guanyl-hydrazone. *Pharmazie,* 45 (1990) 747–749.

Sigurdson, E.R., Ridge, J.A., Kemeny, N. and Daly, J.M., Tumor and liver drug uptake following hepatic artery and portal vein infusion. *J. Clin. Oncol.,* 5 (1987) 1836–1840.

Stetson, P.L., Shukla, U.A. and Ensminger, W.D., Sensitive high-performance liquid chromatographic method for the determination of 5-fluorouracil in plasma. *J. Chromatogr.,* 344 (1985) 385–390.

Stewart, J.C., Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal. Biochem.,* 104 (1980) 10–14.

Straubinger, R.M., Duzgunes, N. and Papahadjopoulos, D., pH-sensitive liposome mediate cytoplasmic delivery of encapsulated macromolecules. *FEBS Lett.,* 179 (1985) 148–154.

Straubinger, R.M., Hong, K., Fried, D.S. and Papahadjopoulos, D., Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after interaction in coated vesicles. *Cell,* 32 (1983) 1069–1079.

Tsukada, K., Ueda, S. and Okada, R., Preparation of liposome-encapsulated anti-tumour drug; relationship between lipophilicity of drugs and in vitro drug release. *Chem. Pharm. Bull.,* 32 (1984) 1929–1935.

Vainstein, A., Herskovitz, M., Israel, S., Rabin, S. and Loyter, A., A new method for reconstitution of highly fusogenic Sendai virus envelopes. *Biochim. Biophys. Acta,* 773 (1984) 181–188.

Yamashita, J., Yamawaki, I., Ueda, S., Yasumoto, M., Unemi, N. and Hashimoto, S., Studies on antitumour agent: V. Synthesis and antitumour activity of 5-FU derivatives. *Chem. Pharm. Bull.,* 30 (1982) 4258–4267.