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Preparation and stability of liposomes containing 5-fluorouracil

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Summary

The aim of this study is to increase the stability of liposomes compared to their stability at room and refrigerator (4–6 °C) temperatures in aqueous media or as pellets. Additionally, the effects of freeze-drying (FD) on the stability of liposomes were observed. Both size and leakage from different types of bilayers were parameters under investigation. 5-Fluorouracil (5-FU) was used as an anticancer drug non-interacting with a bilayer. The effect of cryoprotectants (glycerol, mannitol) on the leakage of 5-FU liposomes after FD was studied.

Introduction

An acceptable shelf-life is a prerequisite for the successful introduction of liposomes into therapy. From the pharmaceutical point of view, the chemical and physical properties of liposome particles are critical parameters affecting the performance of drug-loaded liposomes in vitro and in vivo. Size, charge and composition are main parameters controlling the in vitro and in vivo behaviour. Furthermore, leakage should not occur in the period between preparation and actual administration. Particle size, chemical stability of the bilayer components and drug leakage have presented serious problems during long-term storage (Frokjear et al., 1982). Final stability strongly depends on the composition of the aqueous medium and the bilayer, bilayer–drug interaction and storage conditions. The amount of external water is important as it determines the leakage of drugs which do not interact with the bilayer, like 5-FU (Tsukada et al., 1984). Reducing the volume of external water by the liposomes and storing these pellets might be an alternative for this purpose.

The aim of this study was to increase the stability of liposomes compared to their stability at room and refrigerator (4–6 °C) temperatures in aqueous media and as pellets; additionally, to observe the effects of freeze-drying on the stability of liposomes. Both size, leakage from the bilayer and physical state of it were investigated.

5-FU is one of the most effective drugs for the treatment of solid tumors in many organs (colon, liver, breast, ovaries) and is used extensively in

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combination drug regimens (Meyers et al., 1976). It has been used in clinical practice for more than 20 years. The most common routes and doses for 5-FU administration are (a) i.v. bolus of 10–15 mg/kg with a half-life of 10–20 min; (b) constant i.v. infusion of 20–30 mg/kg/day over a 5-day period (Speyer et al., 1980). However, its clinical use is largely restricted to parenteral administration. Following oral administration it shows an incomplete and highly variable bioavailability largely due to a marked first-pass effect (Cohen et al., 1974; Chiristophidis et al., 1978; Finch et al., 1979; Fraile et al., 1980; Phillips et al., 1980; Almersjo et al., 1980) which makes oral administration an unsuitable and unreliable mode of therapy. Also, the various regimens used for i.v. treatment produce different response rates (Hillcoat et al., 1978). One of the problems encountered in 5-FU therapy is its host toxicity to the bone marrow and the gastrointestinal tract (Chabner, 1982). The drug is quite toxic, about 2/3 of patients showing signs of toxicity (Osol et al., 1982). Specific delivery of 5-FU to desired target site (liver macrophages, i.e. the Kupffer cells) was our aim in drug targetting. Liposomes with diameters of more than 100 nm are taken up by these cells as far as hepatic uptake is concerned. By incorporation into liposomes, a drug may be delivered specifically to these macrophages and as a result, this will prevent its fast excretion from the body and undesired accumulation in other cell types (Scherpof et al., 1987).

The film technique (Bangham et al., 1965) has a high efficiency of aqueous phase encapsulation. Therefore, a film technique was used for 5-FU encapsulation into liposomes.

Freeze-drying of liposomes has been investigated by Kirby and Gregoriadis (1984), Shulkin et al. (1984) and Ohsawa et al. (1984). During the freezing step in the FD process of liposomes, the rupture of liposomes and leakage from them are considerably reduced in the presence of glycerol-mannitol (Fransen et al., 1985). We tried to investigate the effect of these cryoprotectants on the leakage of 5-FU liposomes after a FD/rehydration cycle.

In this study, we report on the preparation of 5-FU liposomes as a function of their size and composition. Additionally, we tried to increase the shelf-life stability of them under different storage conditions.

Materials and Methods

Materials

5-FU (Sigma, U.S.A.), DPPC (Nattermann Chemie, F.R.G.), Cholesteryl hemisuccinate (CHEMS) (Sigma, U.S.A.), cholesterol (Sigma, U.S.A.), soy-bean lecitin (PL 100) (parenteral quality) (Nattermann Chemie, F.R.G.), mannitol (OPG, Holland), glycerol (OPG, Holland), Tris (Jansen Chimica, Belgium), methanol (Merck, F.R.G.). All other chemicals were of analytical grade.

Preparation and characterization of liposomes

Liposome preparation. Two types of negatively charged liposomes were prepared, a liquid state (PL 100 : CHEMS : Chol) (10 : 1 : 4) and a gel state (DPPC : CHEMS : Chol) (10 : 1 : 4). The initial phospholipid concentration was 40 μmol/ml. All liposome dispersions were prepared at a certain initial 5-FU/PL ratio (950 mmol 5-FU/mol PL). For both types of vesicles the film method was used as described by Bangham et al. (1965) due to the high efficiency of aqueous phase encapsulation. An aqueous solution 5-FU was prepared in 10 mM Tris, pH 9. NaOH was added to pH 9 to increase the solubility to 50 mg/ml (USP XXI). This 5-FU solution was incorporated into DPPC : CHEMS : Chol bilayers at 55 °C and PL100 : CHEMS : Chol at room temperature. The liposome dispersion was left 3 h for the swelling of phospholipids at room temperature. An extrusion technique was used to reduce the size of vesicles (Szoka and Papahadjopoulos, 1980; Olson et al, 1979). For extrusion, polycarbonate filters (Biorad Richmond, CA) with pore diameters of 0.6, 0.4 and 0.2 μm were used under nitrogen pressures up to 0.6 MPa. DPPC : CHEMS : Chol vesicles were extruded at temperatures between 55 and 60 °C. For PL 100 : CHEMS : Chol, extrusion was carried out at room temperature. Free drug was removed

by ultracentrifugation (Beckman, Model L5-65, USA) by washing with iso-osmotic, 0.77 Osm 10 mM Tris, pH : 9, two times at 25 000 rpm for 45 min. Isotonic (0.9% NaCl) and iso-osmotic (0.77 Osm NaCl in 10 mM Tris at pH : 9) were used for reconstitution of pellets after long-term storage. Prepared liposomes were stored at room temperature (20 °C) and in the refrigerator (4–6 °C); and either as pellet or as aqueous dispersion. For pellets, the supernatant was completely removed after ultracentrifugation. For aqueous dispersion, pellets were left in 1 ml supernatant. In order to avoid oxidation of the bilayer structure, the vials were flushed with N_2 before sealing.

Encapsulation efficiency. To determine the amount of drug entrapped in liposomes, 0.25 ml liposome dispersion was diluted 10^3 times with chloroform : methanol (2 : 1 v/v) mixture and read spectrophotometrically at 266 nm (Hitachi 220A, Japan). Lipid phosphorus was determined according to the procedure of Fiske and Subbarow (1925).

Liposome characterization. Particle size was measured by electron microscope (Jeol 100C, U.S.A.) with the method of Kay (1967) and Jousma et al. (1987).

Stability

Leakage of 5-FU out of vesicles pelleted or dispersed in aqueous media and kept at room or refrigerator temperatures was monitored at the first, second and third day after preparation (PL 100 liposomes) and first, third and fifth day (DPPC liposomes), then weekly for both through one and 1.5 month for PL 100 and DPPC, respectively. For determination of 5-FU leakage from the pelleted vesicles the pellets were vortexed first and isotonic/iso-osmotic solution was added for reconstitution secondly and ultracentrifugation was applied at 25,000 rpm for 45 min. Then, the supernatant was removed to measure leaked 5-FU at 269 nm.

In order to evaluate the chemical stability of phospholipids, lyso-phospholipid content was determined by HPLC (Perkin Elmer Model 440, Waters Assoc., U.S.A.) (column: Lichrosorb SI 60). The lyso content was 5.5% for PL 100 and 2.8% for DPPC.

Freeze-drying of liposomes

As an alternative for removal of external water FD was applied. For this purpose 0.5 ml of liposome dispersion was filled into the vials. In some cases a solution of a cryoprotectant mixture (glycerol, 10% v/v : mannitol, 10% w/v) in the ratio of 1 : 1) was added to the dispersion in 1 : 1 v/v ratio. Freeze-drying of the vesicles was performed in a Leybould-Heraeus freeze-drier type GT-4 at −40 °C and 0.1 mbar (F.R.G.). Primary and secondary drying lasted 64 h. All aqueous dispersions and freeze-dried materials were stored at room temperature and protected from light.

Results and Discussion

The objective of this study was to prepare 5-FU liposomes with optimum stability during long-term storage. Different storage conditions (room and refrigerator temperatures for 4/6 weeks) and freeze-drying were applied in order to improve shelf-life stability under isotonic and iso-osmotic conditions as aqueous dispersion or pellets.

Preparation and characterization of liposomes

Liposome preparation. DPPC and PL 100 liposomes with cholesterol have proven to be relatively successful in animal studies concerning the improvement of therapeutic index of cytostatics (Heath-Timoty et al. 1985; Mazumder, 1981). CHEMS was employed as a negative charge inducer. As there is a tendency to choose negatively charged liposomes for in vivo experiments this study also focused on negatively charged liposomes (Heath-Timoty et al., 1985; Crommelin 1984; Crommelin and Van Bommel, 1984).

As 5-FU does not associate with lipid bilayers (Tsukada et al., 1984), the encapsulated volume is of major importance to achieve a high loading capacity. Therefore, MLV dispersions seem convenient for studies using liposomes as carriers of 5-FU, MLV are suitable for the encapsulation of a variety of lipid compositions (Szoka and Papahadjopoulos, 1980).

We prepared only negatively charged liposomes, because positively charged or neutral liposomes are less efficient for drug delivery and have

less in vivo antitumor activity (Heath-Timoty et al., 1985).

The solubility of 5-FU in water (pK_a 8–13) increases when it is converted to the ionized form by adding NaOH. It has been reported that the decomposition of 5-FU occurs when it is subjected to strongly basic conditions (Rudy and Senkowski, 1972). The official monograph of USP XXI (1981) for 5-FU injection allows pH 8.6–9. Therefore, pH 9 was selected for our study.

The final step in the preparation of 5-FU liposomes deals also with the removal of "free" nonencapsulated 5-FU from the liposome dispersion. Although dialysis and chromatography are the common procedures, the former one is rather time-consuming and gel chromatography causes dilution of the dispersion. In this study, several adsorbents were studied for their potential to remove free 5-FU from liposome dispersion. These adsorbents were different resins such as Dowex 50W-X4, Amberlite XAD-2, SP-Sephadex C-50 and in addition A1203 and Silicagel 60H. Dowex 50W-X4 and SP-Sephadex C-50 chemically interacted with 5-FU. A typical odor was smelled. Amberlite XAD-2, however, adsorbed the phospholipids and the integrity of liposomes was attacked. A1203 and Silicagel 60H could not remove the free drug efficiently. As an alternative, ultracentrifugation and washing were used. Two times ultracentrifugation at 25,000 rpm for 45 min and washing with 10 mM Tris 0.77 Osm (at pH : 9) two times reduced the external 5-FU to 2% in the centrifuged dispersion or pellet.

Encapsulation efficiency. With fluid type of liposomes encapsulation efficiency was much lower (2.11%) than gel type (5.99%). Inclusion of cholesterol and the presence of fully saturated acyl chains resulted in higher encapsulation efficiency of gel type liposomes. These values were quite satisfactory when compared to the previous studies with dipalmitoyl lecitin : cholesterol : phosphatidic acid (7 : 2 : 1)/5-FU liposomes (Mazumder, 1981) and DSPC/5FU liposomes (Tsukada et al., 1984).

In the first preparation step, the initial 5-FU concentration was high (47.24 mg/ml for DPPC, 45.16 mg/ml for PL 100) by the enhanced solubility of 5-FU with high pH up to 9. But, extrusion for uniform size distribution through 0.2 μm filters (less than 10%) and two times ultracentrifugation for removal of free 5-FU decreased the amount of entrapped drug (31.88 μmol/ml for DPPC; 29.80 μmol/ml for PL 100, although the initial concentration was 40 μmol/ml). This agrees with literature (Jousma et al., 1987).

Liposome characterization. The diameter of the gel type liposomes (DPPC : CHEMS : Chol; 10 : 1 : 4) was about 375 nm; this value was less for PL 100 : CHEMS : Chol liposomes, nearly 340 nm. These findings indicate that the liposomes above 100 nm can prevent their rapid excretion from the body and/or Kupffer cells (targetting) can be achieved; i.e. the size will affect the intrahepatic destination of the liposomes.

Stability

The following aspects concerning the stability of the liposomes were examined: (i) the occurrence of changes in vesicle size during storage (4/6 weeks for solid/liquid bilayers, respectively; (ii) the amount of encapsulated drug; (iii) chemical stability of the encapsulated drug.

Mean particle size did not change for any of the negatively charged liposomes during storage at 4–6 °C and room temperature for over a period of 4–6 weeks. This is in accordance with previous findings for doxorubicin liposomes captured in the same molar phospholipid (Storm, 1987). It was expected that "fluid" liposome types would be sensitive to drug loss on long-term storage. "Solid" type liposomes were predicted to be relatively stable. Figs. 1 and 2 show the results with respect to the 5-FU leakage on long-term storage (4/6 weeks) at room and refrigerator temperatures. 5-FU started to leak from the solid liposomes stored as pellets and aqueous dispersions after storage and continued until a plateau level of about 30%. It reached a plateau after a 4-week storage. We did not observe any difference between liposomes stored as pellets and aqueous dispersion although they had different volumes of external water into which the encapsulated 5-FU can leak. However, liposomes stored in the refigerator as pellets under iso-osmotic conditions showed relatively low leakage rates when compared with other pellets. For PL 100 : CHEMS : Chol lipo-

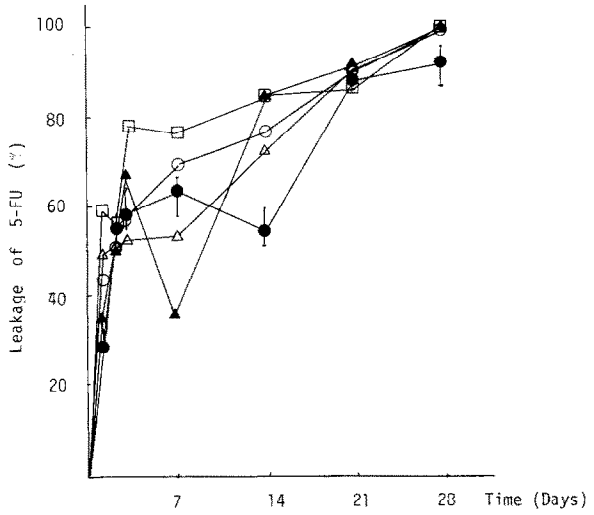

Fig. 1. The leakage of 5-FU from PL-100:CHEMS:Chol (10:1:4) liposomes. Average of 2 determinations. All dispersions are under iso-osmic conditions except the last. ○, Pellet, room temp.; □, aqueous dispersion, room temp.; △, pellet, ref. temp.; ▲, aqueous dispersion, ref. temp.; ●, pellet, ref. temp.; PL 100, soy-bean lecitin; CHEMS, cholesteryl hemissuccinate. Values show means, and some indicate S.D. bars.

somes 5-FU leakage was much higher and reached a plateau level of about 84% within two weeks. The sensitivity of fluid types of liposomes to the marker loss has been shown with previous studies (Frokjear et al., 1982; Gregoriadis, 1974). Simmons and Kramer (1984), also, the gel type liposomes mentioned (sphingomyelin : cholesterol : DCP) were much more successful for 5-FU entrapment than the fluid types (lecitin : cholesterol : DCP) (3.6% > 1.0%). They emphasized that the retention of materials within the aqueous interstices of liposomes was directly related to the ability of the material to hydrogen bond. Temperature and pellet/aqueous dispersion storage form were not effective for leaked amount of 5-FU except liposomes stored in the refrigerator as pellets under isotonic conditions. A relatively low leakage was observed under these conditions. Additionally, we did not observe a significant difference between isotonic and iso-osmotic conditions used for rehydration of liposomes. This may be due to the presence of lysolecitin, because according to Kitagawa et al. (1976), liposomes containing lysolecitin are stable in an isotonic solution. They were osmotically more fragile than liposomes containing no lysolecitin. The extent of osmotic fragility increased proportionally with the increase of the lysolecitin content in liposomes. The shelf-life could be prolonged by weeks (nearly 4 weeks for DPPC/5-FU liposomes and 2 weeks for PL 100/5-FU ones). Simmons and Kramer (1977) could achieve to keep 5-FU in sphingomyelin : cholesterol : DCP (4.8 : 2.8 : 1.0) liposomes only 40 h at room temperature.

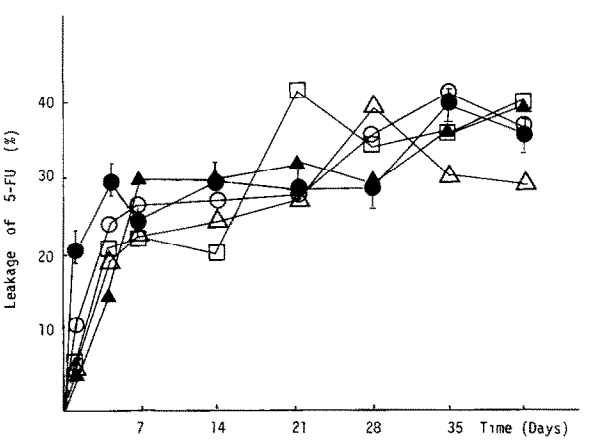

Fig. 2. The leakage of 5-FU from DPPC:CHEMS:Chol (10:1:4) liposomes. Average of 2 determinations. All dispersions are under iso-osmic conditions except the last. Symbols as in Fig. 1.

At the end of long-term storage we observed no change in HPLC peaks for 5-FU. Chemical stability of 5-FU could be kept under experimental conditions. Lysophospholipids are known as the compounds increasing the fragility of bilayers (Kitagawa et al, 1976). We did not observe any significant increase in lyso content.

Freeze-drying of liposomes

FD did not affect the particle size of PL 100 and DPPC liposome dispersion containing no cryoprotectants (Table I). On the contrary, FD caused an increase in particle size for both DPPC (630 nm) and PL 100 (990 nm) liposomes. FD was harmful for liposome integrity, as freezing caused a pronounced increase in the release of the drug. Abu-Zaid et al. (1985) also observed significant increases in both ionic and non-ionic markers trapped from the liposomes.

TABLE 1

The effect of FD on particle size and retention of 5-FU in the liquid and gel state liposomes

Composition	Particle size (nm)		Retention of 5-FU (%)	
	Before FD	After FD	Before FD	After FD
PL-100 : CHEMS : Chol with 5-FU	252	990	3,63	59,36
PL-100 : CHEMS : Chol with 5-FU and mannitol : glycerol	256	209	3,63	36,45
DPPC : CHEMS : Chol with 5-FU	313	660	6,07	58,62
DPPPC : CHEMS : Chol with 5-FU and mannitol : glycerol	296	257	6,07	52,32

PL-100, Phospholipon 100 = soy-bean lecitin; DPPC, dipalmitoylphosphatidyl cholin; FD, freeze drying/rehydration.

The amount of 5-FU retained in the liposomes was 9% for "solid" liposomes and 7% for the "fluid" type. Even in the presence of cryoprotectants, these values appeared to be rather low when compared with the results obtained for a period of 6 weeks for DPPC (about 20%) and 4 weeks for PL 100 (about 10%) liposomes. Briefly, freeze-drying tended to destroy the membrane barrier function in both fluid and gel-like bilayers. Crommelin and Van Bommel (1984) observed that FD tends to destroy the integrity of liposomes containing non-interacting substance with bilayers. Particle size increase after FD was in good agreement with leakage.

Conclusions

A prerequisite for any reproducible clinical work with liposomes is the use of well-standardized and stable preparations with reproducible physicochemical properties (Fildes, 1965). In order to assess critical parameters controlling the quality of 5-FU containing liposome formulations the influence of liposomal composition and characteristics in vitro behavior of extrusion-MLV was investigated. This study clearly demonstrates that the preparation of 5-FU liposomes in the composition of DPPC : CHEMS : Chol (10 : 1 : 4) is an important factor in manufacturing stable liposome dispersions with optimum pharmaceutical and therapeutic characteristics. The enhancement of the solubility by pH and the use of gel type liposomes are advanteous over the fluid ones for higher encapsulation capacity of 5-FU. In general, both the storage at 4–6 °C and the presence of either isotonic or iso-osmotic medium are convenient conditions. Long-term stability strongly depends on liposome composition. Loss of encapsulated 5-FU was observed during long-term storage of fluid PL 100 : CHEMS : Chol (10 : 1 : 4) liposomes. Freeze-drying conditions in the presence of other cryoprotectants especially tretralose will be detected in detail in order to increase physical stability of 5-FU liposomes.

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