Stability of doxorubicin-liposomes on storage: as an aqueous dispersion, frozen or freeze-dried

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Summary

Shelf-life of doxorubicin (DXR) containing negatively charged liposomes was studied under various conditions. The following parameters were monitored: DXR-latency, stability against aggregation or fusion, and chemical stability of bilayer components and associated DXR. An ion exchange resin was used to remove free DXR from liposome-associated DXR. As an alternative for storing aqueous dispersions, the liposomes were frozen and freeze-dried. Variables under investigation were: type of cryoprotectant (saccharides, polyvinylpyrrolidone and mannitol), particle size (about 0.1, 0.25 and 0.65 μm), physical state of the bilayer ("gel"- or "fluid"-like, inclusion of α-tocopherolacetate). A comparison was made between the behaviour of a bilayer interacting compound (DXR) and a non-interacting compound (carboxyfluorescein, CF).

When stored as aqueous dispersions extruded multilamellar structures (0.25 μm) showed lower DXR leakage rates than sonicated vesicles (0.1 μm). In the presence of various saccharides freezing and freeze-drying resulted in similar DXR latencies on thawing or rehydration; they provided adequate protection against aggregation or fusion. Other cryoprotectants failed. Latency of a bilayer-interacting drug like DXR, was hardly sensitive to variation of the bilayer structure; in contrast to this observation, latency of CF strongly depended on the bilayer composition. Under the experimental conditions no effects of ageing could be observed in terms of changing leakage profiles, aggregation behaviour or chemical decomposition of DXR.

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Introduction

In the treatment of parasitic diseases and cancer liposomes have been shown to increase the therapeutic index of a number of drugs considerably (e.g. Gregoriadis, 1979, 1984). Drug latency, i.e. the fraction of the drug actually associated with the liposomes, and particle size are critical parameters for the performance of drug laden liposomes in vivo. In particular, the stability against aggregation or fusion and drug latency on long-term storage have been reported to raise problems (Frøkjaer et al., 1982). Therefore, new techniques have to be developed to improve the shelf-life of liposomes. Recently freeze-drying has been introduced as a potential technique to increase the shelf-life of liposomes designed for application in drug therapy (Evans et al., 1978; Vanderberghe and Handjani, 1979; Gordon et al., 1982; Henry-Michelland et al., 1983; Crommelin and Van Bommel, 1984).

In a previous study Crommelin and Van Bommel (1984) compared the physical stability of liposomes in aqueous media with frozen or freeze-dried dispersions. Carboxyfluorescein (CF) was encapsulated in reverse-phase evaporation vesicles (REV) as a model compound for drugs that do not interact with the bilayer. Based on their experimental results it was suggested that this interaction between drug and bilayer is essential to achieve an acceptable drug latency after a freezing/thawing or freeze-drying/rehydration cycle. Doxorubicin (DXR) associates with negatively charged bilayers (Goldman et al., 1978; Goormaghtigh et al., 1980a and b; Crommelin et al., 1983; Crommelin and Van Bloois, 1983). Besides, the results with DXR liposomes in animal studies look very promising. The therapeutic index of this cytostatic increased in all reported studies by encapsulation in liposomes (Rahman et al., 1980, 1982; Forssen and Tökes, 1981, 1983; Olson et al., 1982; Gabizon et al., 1982, 1983; Herman et al., 1983; Mayhew et al., 1983; Van Hoesel et al., 1984). DXR liposomes might be used therapeutically in the near future. This calls for studies focussing on the improvement of their shelf-life.

For these two reasons, DXR was chosen to investigate the physical stability of liposomes containing this drug under different conditions: as an aqueous dispersion, frozen or lyophilized. The chemical stability of DXR and bilayer components was determined as well. This study focusses on the stability of the negatively charged DXR-containing liposomes as used by Van Hoesel et al. (1984). Mannose and lactose were successfully applied as cryoprotectants as they improved the physical stability of liposomes (Henry-Michelland et al., 1983; Crommelin and Van Bommel, 1984). Now the effects of other saccharides and non-saccharides on DXR-latency and on particle size after a freezing/thawing cycle or after rehydration of lyophilized liposomes were investigated. Besides, the influence of the inclusion of α-tocopherolacetate (TA) on the stability of DXR liposomes was studied. Inclusion of α-tocopherol was recommended as it might reduce DXR induced cardiotoxicity (Mayhew et al., 1983). An ion exchange resin was used to remove free DXR from liposome-associated DXR.

Materials and Methods

Reagents

Phosphatidylcholine from egg yolk type V-E (PC), phosphatidylserine (PS), distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylglycerol (DPPG), cholesterol (chol) and α-tocopherolacetate type III (TA) were supplied by Sigma chemicals (St. Louis, MO).

Lactose, glucose and saccharose met the requirements of the Pharm. Eur., mannitol of the B.P., polyvinylpyrrolidone (PVP) of the U.S.P. XX (Plasdone K29/32), and galactose of the Ph. Ned. VII. Doxorubicin (DXR) was supplied by lab. Roger Bellon (Neuilly sur Seine, Paris) as a lyophilized powder with lactose in a ratio 1 : 5. Dowex 50W-X4 was obtained from Baker Chemicals (Phillipsburg, NJ). All other reagents were of analytical grade.

Preparation of the liposomes

During preparation of the liposomes conditions were created to keep the degree of microbiological contamination at a minimum level (LAF cabinet, sterile equipment).

DXR was mixed with phospholipids (PL) and cholesterol and if mentioned with TA (molar ratio PC–PS–chol–(TA) 10 : 1 : 4 : (0.1) or DSPC–DPPG–chol 10 : 1 : 10 in a chloroform/methanol mixture (1 : 1 v/v) in a pear-shaped flask. The mixture was rotary evaporated under low pressure at 40–45°C to yield a film. The flask was left under vacuum for at least 2 h. Then the hydration medium and glass beads were added. The medium for hydration (pH 4) consisted of 0.8% sodium chloride and 0.01 mol/l Tris-HCl in water. Nitrogen was passed through the buffer for at least 15 min. The film was hydrated under shaking at about 45°C, or in case of DSPC-containing liposomes at 60–70°C, and left after complete dispersion in a refrigerator. At this stage of preparation 1 ml of dispersion contained as a rule about 30 μmol PL and 2.8 μmol DXR. Sizing of the PC/PS/chol-liposomes was done by sequentially extruding the liposomes through membrane filters with pore diameters of 0.6 and 0.2 μm (Uni-pore, Bio-Rad, Richmond, CA) under nitrogen pressures up to 0.8 MPa. The final product was sterilized in the last extrusion step using sterile filtration equipment (0.2 μm pores). Even at pressures as high as 0.8 MPa and temperatures of 60–70°C it was not possible to extrude DSPC/DPPG/chol-liposomes through 0.2 μm pores. Therefore these liposomes only passed the 0.6 μm pores. Another way of sizing the PC/PS/chol-vesicles was by ultrasonication with a probe type sonicator (Bransonic B12, Branson Sonic Power, Danbury, CN) under nitrogen and cooling in ice-water. Every two min sonication was interrupted for one minute to allow the dispersion to cool down.

Free DXR was removed from the liposomes by mixing the dispersion with a sterile Dowex 50W-X4 suspension (in sodium form) for 1–2 min. This cation exchange resin binds free DXR and leaves the negatively charged liposomes in the aqueous medium; it removed the free DXR efficiently and quickly, with only limited dilution of the aqueous phase. Less than 5% DXR was in the free form after separating the resin from the liposome-containing supernatant. No pH change

occurred. The liposome dispersion was diluted by about 25% and the process took a few minutes. These characteristics compare favourably with alternative separation techniques like dialysis and gel chromatography.

Finally, 0.5 or 1 ml of the liposome dispersion was filled into vials under aseptic conditions. In some cases a solution of a cryoprotectant in Tris-HCl (0.01 mol/l) was added to the dispersions in a 1:1 volume ratio. The liposomes were stored in a refrigerator at 4–6°C. Freezing was carried out in a freezer at −30°C immediately after removal of free DXR from liposome-associated DXR. The vials were kept at this temperature for at least 24 h before thawing. Freeze-drying of the vesicles was performed in a Leybold Heraeus freeze-drier type GT-2. The lyophilization process was carried out immediately after removal of unbound DXR. The whole drying process took 24 h. The lyophilized dispersions were stored at 4–6°C, protected from light for at least 24 h.

Analysis

The total DXR content of liposome dispersions was determined spectrophotometrically (Crommelin et al., 1983). Liposome-bound DXR was separated from free DXR by gel chromatography (Sephadex G50 fine, Pharmacia Fine Chem., Uppsala). The chemical stability of DXR was monitored with a HPLC method (Janssen et al., 1984). The purity of the phospholipids in PC/PS/chol-liposomes was assessed by two-dimensional TLC on precoated Silicagel 60, F_{254} (Merck) plates. The liposomes were chromatographed with a chloroform–methanol–water–ammonia (90:54:5.5:5.5) mixture and consecutively with a chloroform–methanol–water–acetic acid (90:40:2:12) mixture. After visualization of the spots by iodine vapour the phospholipids (PC and PS) and the corresponding lyso-compounds were scraped off and determined by phosphate analysis (Fiske and Subbarow, 1925).

Mean particle size was determined by dynamic light scattering (Nanosizer, Coulter Electronics, Luton, U.K.). Particle size is given in combination with a polydispersity index (p.i.) ranging from 0 (monodisperse) to 9 (polydisperse). An indication for the interpretation of this scale is given by Crommelin et al. (1983).

Results

Stability of DXR-containing liposomes on storage: as aqueous dispersions

DXR latency of liposomes consisting of PC–PS–chol–(TA) 10:1:4:(0.1) as a function of time is given in Fig. 1. After an initial drop drug latency stabilized around 80–90%. Inclusion of TA did not affect DXR latency. Sonicated vesicles showed a substantial loss of DXR. The first data point shown was taken about 4 h after removal of free DXR.

For the extruded liposomes with or without TA the mean particle size was 0.25 μm (p.i. = 3). No change was observed over the 2 month period of time. Sonication of liposomes reduced the average diameter to about 0.10–0.12 μm but increased the polydispersity index (6–7). The mean diameter of sonicated vesicles tended to increase on storage.

Freezing and freeze-drying of DXR-containing liposomes

Two potential techniques to increase the long-term stability of liposomes on storage are freezing and freeze-drying of the dispersions. As described previously (Crommelin and Van Bommel, 1984) cryoprotectants were needed to provide a stable physical structure in case of encapsulated compounds that do not interact with the bilayer. In Table 1 DXR latency and particle diameter are given of 0.25 μm liposomes after a freezing/thawing and a freeze-drying/rehydration cycle in the presence of different types of additives. Crommelin and Van Bommel (1984) found that saccharides were effective protectants in terms of preserving the original particle size and polydispersity index of the liposomes. Regularly used additives like mannitol or PVP did not give satisfactory results. Different types of mono- and disaccharides were equally effective. DXR latency improved in the presence of mono- and disaccharides, but never exceeded 72% for the frozen and 66% for the freeze-dried dispersions. It was investigated whether manipulation of experimental conditions could increase DXR latency up to acceptable levels. As glucose is widely used in parenteral i.v. injections it was selected as cryoprotectant for these experiments. Variables under investigation were: (a) the concentration of the cryoprotectant (glucose); (b) the rehydration volume after freeze-drying; (c) the mean particle size of the dispersions; and (d) the physical state of the bilayer (gel- or fluid-like).

In Table 1 the influence of the glucose concentration on drug latency and the particle size (p.i.) is shown. Changing the glucose concentration from 2.5% to 10% did not improve DXR latency. The particle size tended to decrease in the presence of 5 or 10% glucose, possibly because of osmotic effects; no increase in polydispersity index was observed.

Rehydration of the freeze-dried liposomes (5% glucose) in half the original volume – thus concentrating the vesicles by a factor of 2 – resulted in slightly lower
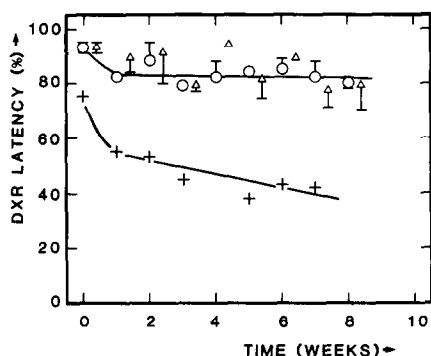

Fig. 1. DXR latency in extruded (0.2 μm pores) or sonicated dispersions stored at 4–6°C. Lines are drawn. O = extruded, composition PC–PS–chol 10:1:4; △ = extruded, composition PC–PS–chol–TA 10:1:4:0.1. As a rule: number of experiments (n) = 4, for n > 2: vertical bars indicate standard deviations. PL concentration is about 12 μmol/ml; DXR concentration is about 0.4 μmol/ml. + = sonicated, composition PC–PS–chol 10:1:4. Mean of two experiments. PL concentration is about 12 μmol/ml; DXR concentration is about 0.1 μmol/ml.

TABLE 1

DXR LATENCY (%), MEAN PARTICLE SIZE (μm) AND – IN PARENTHESES – POLYDISPERS-
ITY INDEX (RANGING FROM 0 TO 9) AFTER A FREEZING/THAWING OR A FREEZE-DRY-
ING/REHYDRATION CYCLE IN THE PRESENCE OF VARIOUS ADDITIVES

Experiments were carried out at least in duplicate; for n > 2 the standard deviation is given. Composition
of the liposomes: PC–PS–chol 10:1:4.

Additive [a]	Freezing		Freeze-drying	
	Latency	Part. size	Latency	Part. size
galactose 2.5%	72	0.26 (3)	62	0.27 (3)
glucose 2.5%	68	0.31 (3)	65	0.28 (3)
glucose 5%	63 ± 4	0.25 (3)	60 ± 6	0.24 (3)
glucose 10%	65	0.25 (2)	66	0.22 (3)
lactose 5%	70 ± 5	0.29 (2)	55 ± 10	0.29 (3)
lactose[b]	65	0.29 (2)	61	0.26 (2)
saccharose 5%	63	0.27 (3)	66	0.27 (3)
mannitol 5%	n.d.	n.d.	28	0.36 (6)
PVP 5%	n.d.	n.d.	3	2.7 (7)
TA [c]	n.d.	n.d.	55	n.d.
no additive	37	0.36 (5–7)	30	> 3 (9)
glucose 5% [d]	n.d.	n.d.	55	n.d.

[a] The solution of the protectant is added to an isotonic (0.8% sodium chloride, 0.01 mol/l Tris-HCl
buffer) liposome dispersion in a 1:1 ratio. The percentages of protectants mentioned in the table refer
to the final concentrations in the dispersion. PL concentrations were about 12 μmol/ml, DXR
concentration about 0.4 μmol/ml
[b] The PL/DXR film in the pear-shaped flask was dispersed in a 10% lactose and 0.01 mol/l Tris-HCl-
containing medium.
[c] Composition of the bilayer PC–PS–chol–TA 10:1:4:0.1.
[d] Rehydration in half the original volume.
n.d. = not determined

TABLE 2

DXR LATENCY (%) IN LIPOSOMES WITH VARIOUS DIAMETERS AFTER A
FREEZING/THAWING OR A FREEZE-DRYING/REHYDRATION CYCLE

Mean diameter (μm)	Latency	
	Freezing (%)	Freeze-drying (%)
0.1 (a)	68	58
0.25 (b)	63 ± 4	60 ± 6
0.65 (c)	40	46

Composition PC–PS–chol 10:1:4. (a) Sonicated vesicles; (b) extruded through 0.2 μm pores, (c)
extruded through 0.6 μm pores. 5% glucose solutions were mixed in a 1:1 (v/v) ratio with isotonic (0.8%
sodium chloride/0.01 mol/l Tris·HCl buffer) liposome dispersions. PL concentration is about 12
μmol/ml; DXR concentration is about 0.1 (a); 0.4 (b); 0.6 (c) μmol/ml. Mean of at least duplicate
experiments, standard deviations are given for n > 2.

latencies than rehydration in the original volume (Table 1).

For PC–PS–chol-liposomes the effect of liposome diameter on DXR latency after freezing or freeze-drying (5% glucose) is given in Table 2. Reducing the size of the vesicles down to 0.1 μm by sonication yielded results comparable to the liposomes with a diameter of 0.25 μm. However, when the liposomes were extruded only through 0.6 μm pores the latency tended to be lower. In all cases the mean particle size remained unaffected by the freezing or freeze-drying process. The influence of the physical state of the bilayer and the type of encapsulated compound on latency and particle size after the freeze-drying process is given in Table 3. CF latencies were taken – partly – from a previous paper (Crommelin and Van Bommel, 1984). Although the experimental conditions were not completely similar (bilayer composition, liposome type, particle diameter) there is a clear trend in the data. For the DXR-containing liposomes the physical state of the bilayer (gel- or fluid-like) is relatively unimportant. However, for the compound without bilayer interaction (CF) the latency after a freeze-drying/rehydration cycle is much more sensitive to the physical state of the bilayer. Under the conditions studied a gel-type structure without chol showed a maximum latency.

The liposomes with a multilamellar gel state bilayer structure (see Table 3 (c)) could not be extruded through 0.2 μm pores at pressures as high as 0.8 MPa even at elevated temperatures. Therefore only extrusion through 0.6 μm pores was performed. The relatively high value for the polydispersity index might be ascribed to the presence of liposomes smaller than 0.6 μm in freshly prepared, non-extruded dispersions. These liposomes are not affected by the extrusion process, while the

TABLE 3

EFFECT OF TYPE OF ENCAPSULATED COMPOUND (DXR OR CF) AND BILAYER STRUCTURE ON LATENCY (%), PARTICLE SIZE (μm) AND p.i. (IN PARENTHESES) AFTER A FREEZE-DRYING/REHYDRATION CYCLE

	Latency	Diameter (p.i.)
PC–PS–chol (DXR) 10:1:4 + lactose 5% (a)	55 ± 10	0.29 (3)
PC–PS–chol (CF) 10:1:4 + lactose 5% (b)	20 ± 4	0.20 (3)
PC–PS–chol (DXR) 10:1:4 + glucose 5% (a)	60 ± 6	0.24 (3)
DSPC–DPPG–chol (DXR) 10:1:10 + glucose 5% (c)	60 ± 8	0.78 (6)
DSPC–DPPG–chol (CF) 10:1:0 + lactose 5% (d)	66 ± 10	0.20 (3)
DSPC–DPPG–chol(CF) 10:1:5 + lactose 5% (d)	21 ± 8	0.21 (3)

Standard deviations are given for n > 2.
(a) See Table 1; particle diameter of non-freeze dried liposomes: 0.25 μm (3).
(b) Liposomes were REV, extruded through 0.2 μm pores. A 10% lactose solution was added to the iso-osmotic liposome dispersion in a 1:1 dilution. The liposome preparation procedure and experimental conditions were described before (Crommelin and Van Bommel, 1984). The freezing temperature was −30°C; particle diameter of non-freeze-dried liposomes: 0.20 μm (3).
(c) Extruded through 0.6 μm pores; particle diameter of non-freeze-dried liposomes: 0.72 μm (6).
(d) Taken from Crommelin and Van Bommel (1984). A 10% lactose solution was added to the liposome dispersion in a 1:1 dilution. These liposomes were REV extruded through 0.2 μm; particle diameter of non-freeze dried liposomes: 0.21–0.26 μm (3).

dimensions of the oversize liposomes are adjusted to approximately the size of the pores. Therefore, the particle size distributions of these dispersions will be skewed and the polydispersity index relatively large. Abra et al. (1984) presented a dialysis technique to remove undersize particles.

Freezing and freeze-drying of DXR-containing liposomes: long-term storage

DXR latency and mean particle diameter of liposomes thawed or rehydrated after a certain period of storage in the presence of 5% glucose are given in Tables 4 and 5. Inclusion of TA did not affect the results (not shown). DXR latency, particle size and polydispersity index did not change during the experiment. Table 6 shows that no loss of DXR from the liposomes occurred when free DXR was not removed from liposome-associated DXR prior to freezing. On thawing or rehydration the mean particle size of these dispersions was equal to the diameters of corresponding dispersions without free DXR after a similar treatment (Table 5). Because latency is defined as the fraction of liposome-associated DXR relative to the total amount of DXR, the latencies in Table 6 are lower than in Table 4. As is shown in the bottom row of Table 6, DXR latency in the original dispersions – before freezing or freeze-drying – equalled the DXR latency after these manipulations. Thus, under these conditions no DXR loss from the liposomes occurred during freezing or freeze-drying.

It was investigated whether storage of frozen or freeze-dried liposomes changed their DXR leakage profile. After thawing and rehydration of the liposomes that were stored for 8 weeks in frozen or freeze-dried state without prior removal of associated DXR, these vesicles were treated with a Dowex suspension as described in Methods and Materials and kept at 4–6°C. Latency and particle size were monitored during one month. The results are given in Fig. 2.

Chemical stability of the phospholipids and DXR on storage

The chemical stability of the phospholipids was determined by TLC. Liposomes (about 2 μmol PL), which were stored for 8 weeks at 4–6°C or in freeze-dried state (4–6°C) were chromatographed. The content of lysocompounds was less than 10%

TABLE 4

DXR LATENCY (%) AFTER A FREEZING/THAWING OR A FREEZE-DRYING REHYDRATION CYCLE ON LONG-TERM STORAGE IN THE PRESENCE OF 5% GLUCOSE[a].

	Period of storage (weeks)		
	1	5	8
freezing	64 ± 4	68 ± 5	66 ± 5
freeze-drying	61 ± 5	57 ± 2	58 ± 3

Mean of duplicate experiments; for storage conditions: see Materials and Methods. Composition: PC–PS–chol 10:1:4.
[a] See Table 1(a)

TABLE 5

PARTICLE SIZE (μm) AND – IN PARENTHESES – POLYDISPERSITY INDEX AFTER A FREEZING/THAWING AND A FREEZE-DRYING/REHYDRATION CYCLE ON LONG-TERM STORAGE IN THE PRESENCE OF 5% GLUCOSE [a]

	Period of storage (weeks)		
	1	5	8
freezing	0.25 (2)	0.25 (3)	0.28 (3)
freeze-drying	0.23 (4)	0.23 (3)	0.26 (3)

Mean of duplicate experiments; mean diameter (μm) and p.i. of the original aqueous dispersion: 0.25 μm (3).

[a] See Table 1. Composition: PC–PS–chol 10:1:4. For storage conditions, see Materials and Methods.

TABLE 6

DXR LATENCY (%) AFTER A FREEZING/THAWING AND A FREEZE-DRYING/REHYDRA-TION CYCLE ON LONG-TERM STORAGE WITHOUT PRIOR REMOVAL OF FREE DXR FROM LIPOSOME ASSOCIATED DXR IN THE PRESENCE OF 5% GLUCOSE [a]

	Period of storage (weeks)	
	1	8
freezing	33 $\pm$ 3	31 $\pm$ 6
freeze-drying	38 $\pm$ 2	37 $\pm$ 3
aqueous dispersion	34	36

Mean of duplicate experiments; for storage conditions: see Materials and Methods. Composition PC–PS–chol 10:1:4.
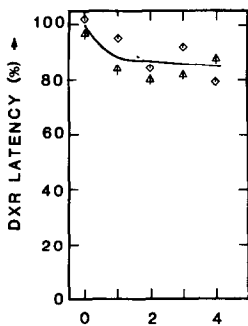
[a] See Table 1(a).

Fig. 2. DXR latency in extruded (0.2 μm pores) dispersions after being frozen or freeze-dried for 8 weeks without prior removal of free DXR. After thawing and rehydration free DXR was removed and the dispersions were stored at 4–6°C. Line is drawn. $\Diamond$ = after a freezing period of 8 weeks; $\blacktriangle$ = after a freeze-drying period of 8 weeks. Mean of duplicate experiments.

for the two samples. After 5 weeks no detectable loss of DXR was found in any of the dispersions (stored at 4–6°C, frozen or freeze-dried). The chromatograms did not show degradation products such as doxorubicinone or doxorubicinol in detectable quantities (detection limit: about 2% of the amount of DXR).

Discussion and Conclusions

The objective of this study was to evaluate the stability of DXR-containing liposomes on storage and to explore new techniques to further increase their shelf-life. When stored as aqueous – extruded – dispersions the liposomes showed a 15% loss of DXR after two months. Particle size remained constant. Sonication of the liposomes decreased their stability; then the vesicles tended to aggregate and DXR leaked out relatively fast. The reason for this high leakage rate may be found in the supposedly unilamellar structure of these sonicated particles. Extruded liposomes have a multilamellar structure. In this structure the DXR molecules localized in internal bilayers will have more barriers to pass through on their way to the external aqueous phase than in the case of a unilamellar structure. Crommelin and Van Bloois (1983) found that sonicated vesicles with the same bilayer composition and initial mean diameter (0.11 μm) showed no aggregation or fusion. However, there were differences in the preparation procedure. Those dispersions were ultracentrifuged to remove multilamellar structures and dialyzed to remove free DXR. TA inclusion into the liposome bilayers consisting of PC–PS–chol (10 : 1 : 4) did not affect the stability. TA was chosen primarily for its reported benificial properties with respect to DXR-induced cardiotoxicity. It has less pronounced anti-oxidant properties than α-tocopherol itself. Under the experimental conditions in this study no signs of autoxidation as manifested by a sudden drop in drug latency were observed (Hunt and Tsang, 1981).

Freezing and freeze-drying of DXR-containing liposomes in the presence of saccharides did not induce aggregation or fusion of the vesicles. The loss of associated DXR was about 35%. In the experimental range the saccharide concentration did not influence the DXR latency after thawing or rehydration. DXR loss on freezing tended to be less than on freeze-drying. However, the differences were small. As freezing is part of the procedure in both techniques, it is hypothesized that DXR leaves the liposomes mainly during the freezing process. For further optimization of DXR latency on freezing or freeze-drying one should therefore focus on reducing the loss of liposome-associated DXR during the freezing process.

The mechanism behind DXR loss on freezing is not clear. It was reported before for fluid-type liposomes (Crommelin and Van Bommel, 1984) that latency of a non-bilayer interacting compound (CF) after a freezing/thawing cycle was extremely low. One might hypothesize that DXR encapsulated in the aqueous phase was mainly lost on freezing, while bilayer-associated DXR remained bound to the liposome structure. An attempt was made to validify the proposed model. To this end free DXR was removed from thawed liposomes. They were frozen for a second time. DXR latency was determined after consecutive thawing. The results were

comparable with those obtained after only one freezing/thawing cycle. Thus, bilayer-associated DXR that had "survived" the first cycle was lost in the second cycle. It is therefore incorrect to ascribe DXR loss on freezing to mere leakage of non-bilayer-associated DXR.

The influence of the physical state of the bilayer was investigated by freezing or freeze-drying different types of liposomes (cf. Table 3). If the drug, like DXR, interacted with the bilayer a gel state or a fluid state structure gave similar results. However, if the encapsulated drug did not interact (CF) only with certain gel-type liposomes latencies over 50% could be found after thawing or rehydration. Apparently, in the "fluid" bilayers pores existed that were large enough to allow free transport of encapsulated water and water-associated compounds. In the presence of a saccharide the liposome structure as a whole was preserved. If liposomes were frozen or freeze-dried without prior removal of free DXR, no leakage of DXR out of the liposomes was observed after thawing or rehydration. Therefore, long-term storage of liposomes as freeze-dried dispersions in the presence of a saccharide without removal of free DXR might provide a way of preserving them. Prior to use they should be rehydrated and mixed with a cation exchange resin. After complete removal of the resin the dispersions are ready for use. The DXR latency profile and the physical stability were not affected by this procedure. Over an eight week period of storage PL degradation products were still at a low level while no DXR degradation products could be detected.

Acknowledgements

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