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Formulation and characterisation of primaquine loaded liposomes prepared by a pH gradient using experimental design

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Abstract

The effect of different formulation factors (lipid type, cholesterol, charge, internal buffer capacity, drug-to-lipid incubation ratio) on the encapsulation efficiency and size of primaquine liposomes (SUV's) in response to a pH gradient was investigated by a fractional factorial screening design. Three of the factors (charge, internal buffer capacity, drug-to-lipid incubation ratio) were further studied in a Box–Behnken optimisation design. The lipid type was the most important parameter followed by the drug-to-lipid incubation ratio, buffer capacity, cholesterol and charge. Several of the interactions were important. In the optimisation design a robust region with high encapsulation efficiency ($>95\%$) was obtained for DSPC: 33.33 mol% cholesterol-liposomes at high internal citrate concentration (200 mM) by maintaining the drug-to-lipid incubation ratio below 0.15:1 (mol:mol) and varying the charge incorporation between 2 and 10%. In order to achieve long-term stability and sterility, the liposomes were lyophilised followed by gamma irradiation. The pH gradient was maintained during this treatment with little chemical degradation of the substances. The final preparation consisted of three separate vials with lyophilised liposomes, solid state primaquine and hydration medium.

Keywords: Liposomes; pH gradient; Primaquine; Lyophilisation; γ-Irradiation; Experimental design

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DSC, differential scanning calorimetry; DSPC, distearoylphosphatidylcholine; DSPG, distearoylphosphatidylglycerol; HPLC, high performance liquid chromatography; MLR, multi linear regression; MLV, multi lamellar vesicles; PCA, principal component analysis; PCS, photon correlation spectroscopy; PEG, polyetylenglycol; PQ, primaquine; SUV, small unilamellar vesicle; T_c, main phase transition temperature; TGA, thermogravimetric analysis; UV, ultra violet.

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1. Introduction

Malaria has still a major impact on world health. Malaria is caused by four different species of the genus *Plasmodium* with a complex life cycle. Briefly; sporozoites are injected from an anopheline mosquito into the blood and are taken up by the liver. In the hepatocytes, the parasite divides (exoerythrocytic shizogony) releasing merozoite forms which invade the erythrocytes and divide again (erythrocytic schizogony) leading to further cycles in other erythrocytes (Ridley and Hudson, 1998). Primaquine is mainly used curatively to eliminate persistent liver forms of the malaria parasites *Plastnodium vivax* and *Plasmodium ovale*. The drug is not used in general prophylaxis due to severe dose-limiting side effects (haemolysis and methaemoglobinacia), which are especially prominent in patients with glucose-6-phosphate dehydrogenase deficiency.

Sufficiently small liposomes are able to pass through the approximately 100 nm pores of the fenestrated endothelium lining the hepatic sinusoid followed by interaction and internalisation by the hepatocytes (Wisse, et al., 1985; Yao et al., 1995; Scherphof et al., 1997). A further increased hepatocytic uptake can be achieved by inclusion of lipids with galactosyl residues (Yao et al., 1995). By incorporation of primaquine in small liposomes, the drug can be targeted directly to the site of action, thus enhancing its therapeutic efficacy. At the same time, primaquine entrapped in the aqueous interior is protected from any destabilising components present in the blood and, conversely the toxicity of primaquine is markedly reduced. Primaquine is photochemically unstable and in vitro phototoxic (Kristensen, 1997). Earlier studies have shown that liposome encapsulated primaquine results in reduced acute drug toxicity. This result was most probably due to a change in the biodistribution pattern of the drug that resulted in a high concentration in the target organ, the liver, whilst limiting drug uptake and, hence, the toxicity elsewhere (Trouet et al., 1981). However the therapeutic effect was not enhanced compared to the free drug because the multilamellar liposomes (MLVs) were taken up by the kupffer cells and not by the hepatocytes in the liver (Smith et al., 1983).

The low encapsulation volume and poor chemical stability of primaquine in aqueous medium readily explains the poor encapsulation efficiency of primaquine into small unilamellar liposomes (SUV's) employing passive loading procedures. However, active or remote loading strategies have been developed with encapsulation efficiencies up to 100% (Madden et al., 1990). The driving force is a pH gradient (inside acidic) established by changing the pH of the extraliposomal phase or by using an ammonium sulphate gradient (induction of a pH gradient) (Haran et al., 1993). These strategies allow empty liposomes to be loaded with drug immediately before use, which is preferable for labile drugs such as primaquine.

Liposomes can successfully be lyophilised in order to achieve long term stability if a suitable lyoprotectant is included and proper lyophilisation conditions are met (van Winden et al., 1997). γ-Irradiation has become an alternative sterilisation method for some pharmaceuticals (Woods and Pikaev, 1994; Jacobs, 1995; Reid, 1995) and has turned out to be an interesting and promising technique also for the sterilisation of liposomes (Zuidam et al., 1996). Recent work has shown that γ-irradiation of lyophilised liposomes is possible due to limited chemical and physical changes (Zuidam et al., 1995; Stensrud et al., 1999).

In this study, the effect of liposome composition (lipid type, cholesterol, charge), internal buffer capacity (citrate concentration) and primaquine concentration on the encapsulation efficiency and liposome size in response to a pH gradient was evaluated in a fractional factorial screening design. The combined influence of liposome charge, citrate concentration and primaquine concentration was further studied by means of response surface methodology using a rotatable Box–Behnken design. This latter design has earlier been applied to optimise liposome formulations (Loukas, 1996; de la Maza et al., 1996). In order to achieve long-term stability and sterility of the preparation lyophilisation followed by γ-irradiation was employed.

2. Materials and methods

2.1. Materials

Distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylglycerol (DSPG) and dimyristoylphosphatidylglycerol (DMPG) were kindly provided by Nattermann Phospholipids, Köln, Germany. PEG 5000-L-α-distearoylphosphatidylethanolamine and cholesterol (99%, from porcine liver) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Primaquine diphosphate (99% pure) was obtained from Aldrich Chem. Co. (Milwaukee, WI, USA). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Liposome preparation and sterilisation

Liposomes were prepared by the film-method as follows; the phospholipids and cholesterol were co-dissolved in chloroform:methanol (2:1) and evaporated to dryness under reduced pressure. The thin film obtained was hydrated and gently shaken above the phase transition temperature (T_c) for 2 h with citrate buffer pH 4.0. The liposomes (25.3 μmol/ml) were subjected to three freeze-thaw cycles employing liquid nitrogen and thawing above T_c, followed by extrusion (Lipex extruder, Biomembranes Inc., Vancouver, Canada) using 50 nm polycarbonate membranes (Nucleopore®, Costar Corp., Cambridge, USA) in the last ten extrusion steps.

Transmembrane pH gradients were established by passing the liposomes over a Sephadex® G-25 M column (Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with isotonic phosphate buffered saline pH 7.4. Primaquine was then added from a 10 mM stock solution in phosphate buffered saline pH 7.4 and incubated for 15 min at room temperature (22°C). In the temperature experiments, liposomes were incubated up to 4 h at 22, 37 and 60°C with primaquine encapsulated during or after the heat treatment.

For lyophilisation, liposomes were formulated with 9.2% (w/w) sucrose as a lyoprotectant in the internal aqueous phase whereas an iso-osmotic sucrose solution pH 7.4 was used instead of phosphate buffered saline in the outer phase. The vials with liposomes in aliquots of 2.0 ml were quickly frozen in liquid nitrogen for 10 min and placed on the shelf of a Leybold GT 4 pilot-production freeze-dryer at a temperature of −30°C for 2 h. Slowly frozen samples were achieved by positioning the samples directly on the shelf at −30°C for 2 h or alternatively by decreasing the shelf temperature from 20 to −30°C stepwise for 3 h. The freeze-drying process was analogous for all samples. In the primary drying, the shelf temperature was maintained at −30°C for 68 h at a chamber pressure of 5 Pa followed by a ramp time to secondary drying for 16 h. The secondary drying was performed at 30°C for 5 h followed by 2 h at 20°C at a pressure of 2 Pa.

Some of the lyophilised liposome samples were sterilised by γ-irradiation. These samples were exposed to a dose of 25 kGy (15 kGy/h, ^{60}Co source) at ambient temperature. The lyophilised liposomes were reconstituted with water at room temperature.

2.2.2. Liposome characterisation

Untrapped primaquine was removed by passing the liposomes over a Sephadex® G-25 M column equilibrated in unbuffered isotonic saline. After a Bligh and Dyer extraction (pH 2.0) (Bligh and Dyer, 1959), the primaquine concentration was determined in the upper aqueous phase using a Shimadzu UV-2101PC UV-VIS scanning spectrophotometer at 340 nm. The phospholipid concentration was determined by phosphorus analysis according to Rouser et al. (1970). Trapping efficiencies (%) were calculated by comparing the amount of primaquine present before and after removal of the free drug.

The osmolarity of the buffers and liposomes was measured with a Knauer Cryoscopic unit (Dr Ing. Knauer GmbH, Berlin, Germany). If necessary, sodium chloride was added to the buffers to adjust the osmolarity.

The mean diameter of the liposomes was measured at 90° (25°C) by photon correlation spectroscopy (PCS) using a Coulter N4 MD. Liposome morphology and size were characterised by means of cryo-transmission electron mi-

croscopy (Dubochet et al., 1988). Briefly; the liposomes (5 mg/ml) were vitrified into thin films on 200 mesh copper grids coated with a perforated carbon film. Liquid ethane was used as cryogen. The preparation of the samples was carried out in a flow of humid air in order to reduce the evaporation and the drying effects (Cyrklaff et al., 1990). A Gatan 626 cryo-transfer system was used and the samples were observed at −170°C in a Philips CM 200 transmission electronmicroscope operating at 120 kV.

The ζ-potential of the liposome suspension diluted with phosphate buffered saline pH 7.4 was measured at 25°C using a Doppler-Elecrophoretic Light Scattering Analyser (Coulter DELSA 440).

The phase transition behaviour of the liposomes was analysed with a Perkin Elmer DSC 7 equipped with Intracooler 1 (scanning rate of 2.5°C/min). Fifty milligrams of the pellets, obtained after concentration of the liposome suspensions by ultracentrifugation (100 000 × *g* for 2 h, Sorvall® Ultra Pro 80), was used. The lipid content of the pellets was measured by phosphorous analysis after a Bligh and Dyer extraction. The melting behaviour of the lyophilised liposomes and solid state primaquine was analysed with a scanning rate of 10°C/min.

The residual moisture content of the lyophilised liposomes was measured with a Perkin Elmer TGA 7. The samples were investigated at 20–200°C with a heating rate of 20°C/min.

Morphological examination of the surfaces and cross–sections of the lyophilisates was carried out using a Scanning Electron Microscope (JEOL-6400, Jeol Ltd., Japan) at 5 kV after coating the lyophilisates with gold-palladium for 6 min at 24 μA (E5000 SEM coating unit, Polaron, UK).

The stability of cholesterol following γ-irradiation was analysed by GC. Direct gas chromatographic injection of underivatised cholesterol was performed on a Fison 8060 capillary GC equipped with a flame ionisation detector. 6-Ketocholestanol was used as internal standard. Data were collected and integrated with a Class-VP (version 4.2) data system. The column was a 032 mm lD × 3o m bonded phase 5% phenylsilicone column with 0.25 μm film thickness (SPB-5, Supelco). Helium was the carrier gas at 1 ml/min and the injector split was 1:10. The column temperature was programmed from 280 to 300°C at 10°C/min with a total analysis time of 20 min. The injector and detector temperatures were 280 and 300°C, respectively.

2.2.3. Primaquine analysis

The stability of primaquine towards heat and γ-irradiation was determined spectrophotometrically at 352 rim and by HPLC (Kristensen et al., 1998). The solubility of primaquine in 200 mM citrate buffer as a function of pH was determined by preparing saturated solutions of the drug followed by 30 min stirring in the dark at room temperature. Undissolved drug was removed by centrifugation and filtration prior to spectrophotometric concentration determination at the upper absorption maxima.

The partition coefficient (log *P*) of primaquine in 200 mM citrate as a function of pH was determined by the shake-flask method (Danielsson and Zhang, 1996). Primaquine was introduced into the aqueous phase and shaken together with n-octanol for 60 min. After centrifugation, the concentration of primaquine in the aqueous phase was determined spectrophotometrically while the concentration in the octanol phase was determined by HPLC after sample dilution with methanol and mobile phase.

2.3. Experimental design

The design of the statistical experiments and the evaluation were performed using the computer program Modde 4.0 (Umetri AB, Umeå, Sweden). A principal component analysis (PCA) was performed on the data set of the fractional factorial design using unscrambler software (Camo, Trondheim, Norway).

2.3.1. Screening

The effects of the phospholipid type, cholesterol. charge, citrate and drug concentrations on the encapsulation efficiency (%) and size increase (nm) after loading were studied in a 2^{5-1} fractional factorial design, comprising 16 runs. In order to estimate the experimental error and check the linearity triplicates were added at two

centre points (one for each phospholipid type) giving a total of 22 runs. The design resolution was V+, i.e. main effects and two-factor interactions are unconfounded. The levels of the factors are shown in Table 1. The batches were produced in a random order and triplicate measurements were run on each batch.

2.3.2. Optimisation

A Box–Behnken optimisation design with three variables was applied to find the optimum condition and to analyse how sensitive the responses were to variations in the settings of the experimental variables (Box and Behnken, 1960). This design is a factorial design with three levels, using middle points instead of corner points and is useful for estimating the coefficients in a second-degree polynomial. A total of 15 experiments were performed including triplicates of the centre point. The centre points improve the assessment of the response surface curvature and simplify estimation of the model error. The charge, citrate and drug concentrations were varied and the encapsulation efficiency (%) and liposome size-increase (nm) after loading were assessed. Phospholipid type and cholesterol concentrations were both on their +1 level based on results from the factorial screening design. The levels of the three factors are shown in Table 1. The batches were produced in random order and triplicate measurements were run on each batch.

Table 1
Levels of the factors studied in the 2^{5-1} fractional factorial design

Factor	Level		
	−1	0	+1
A: phospholipid type	DMPC		DSPC
B: cholesterol concentration (mol%)	0	16.67	33.33
C: charge concentration (mol%)[a]	0	5	10
D: citrate concentration (mM)[a]	50	125	200
E: drug concentration (mol drug:mol lipid)[a]	0.05	0.125	0.2

[a] Factor C, D and E were further studied in a Box–Behnken optimising design. In that design, the levels of factor A and B were both kept on their high levels (+1).

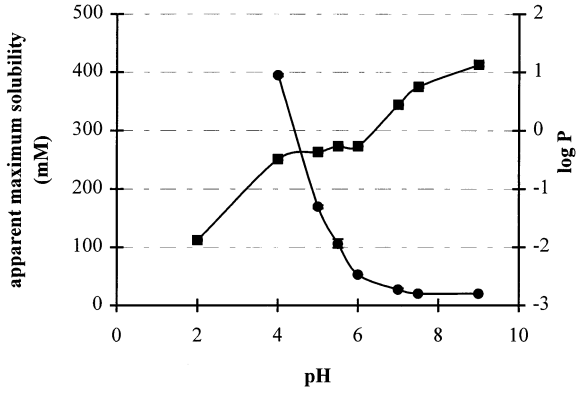

Fig. 1. The apparent maximum solubility (●) (200 mM citrate) and log P (n-octanol: 200 mM citrate) (■) of primaquine as a function of pH. Error bars are the maximum and minimum values ($n \geq 3$).

2.3.3. Evaluation

A principal component analysis (PCA) was first applied on the data set of the fractional factorial design (Esbensen et al., 1994). The data were autoscaled before any statistical operations were performed. Full cross validation was performed. Multiple linear regression (MLR) was further used to fit the encapsulation efficiency (%) to the variables. The significance of the estimated effects was tested by analysis of variance. The accuracy of the statistical model used is described by the R^2 and Q^2 parameters. R^2 is the fraction of the data explained by the model, whereas Q^2 is the predictive ability of the model. Values close to 1 indicate a good model. For the evaluation of the optimisation design only an MLR-model was used.

3. Results

3.1. Solubility and log P of primaquine

Primaquine (8-((4-amino-1-methylbutyl) amino)-6-methoxyquinoline diphosphate) is an amphiphatic drug (pK_a 3.2 and 10.4) with pH dependent solubility and log P values as shown in Fig. 1. In isotonic phosphate buffer pH 7.4, the solubility and log P values were 11 mM and 0.74 mM, respectively (the outer phase).

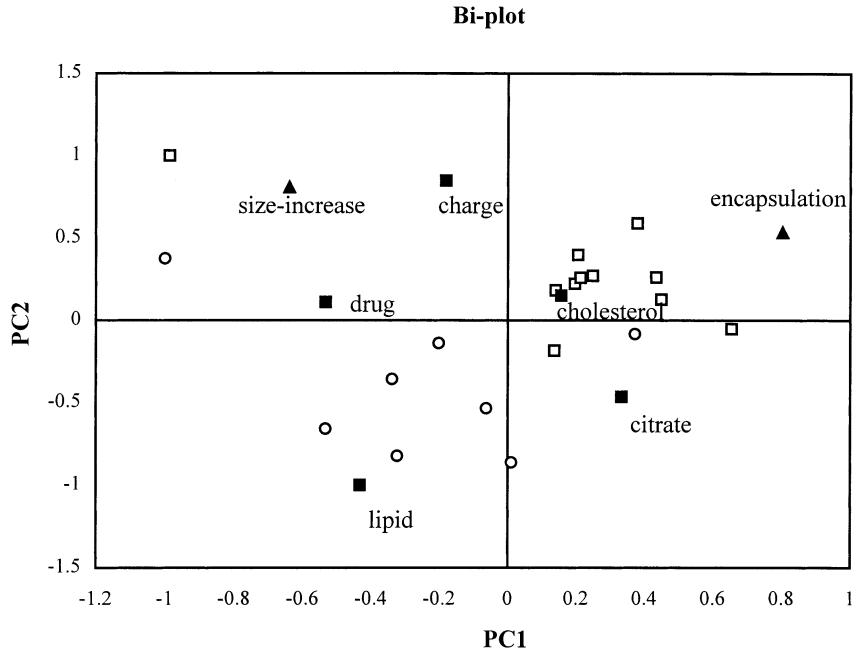

Fig. 2. The first two principal components (PC1 and PC2) from the principal component analysis (PCA). Loadings (factors (■) and responses (▲)) are shown together with the scores (open symbols). Liposomes containing DMPC (○) or DSPC (□).

3.2. Screening design

Liposomes were prepared and loaded according to the fractional factorial plan. It was not possible to prepare liposomes at the centre point (phospholipid type at low level) that were stable with regard to size before loading. Some important interactions may be responsible for this behaviour. These three experiments were therefore omitted in the analysis.

Fig. 2 shows a PCA bi-plot (loadings and scores) with the five factors, the two responses and all the samples. Variables close to each other and along the same straight line through the origin covary. They are positively correlated if they, are situated on the same side of origin and negatively correlated if they lie on the opposite side. Variables of little importance lie near the origin. The PCA explained 51% of the variance in the original matrix by the two first components. The explained calibration variances for encapsulation efficiency and size-increase were 95 and 84%, respectively. The two responses (encapsulation and size-increase) seem to be negatively correlated. Three groups of scores may be distinguished in the plot. In the upper right comer a group is situated in direction of the response encapsulation and hence shows high encapsulation efficiencies (65–100%). These liposomes were prepared from DSPC. In the lower left corner DMPC-liposornes with low encapsulation efficiencies (7–41%) are situated. However, one DSPC-based liposome preparation was different from the others (high cholesterol content, charge, citrate concentration and low drug concentration). This preparation showed high encapsulation efficiency (96%) and is situated near the DSPC-liposomes. Two liposome-preparations are located in the upper left comer. They exhibit low encapsulation efficiencies (7 and 14%) together with a pronounced size-increase after loading (66 and 90 nm). These samples are prepared with high levels of charge and drug concentration and low level of citrate concentration thereby complying with the overall pattern. Same results were obtained with replicate productions. In Fig. 3, a cryo-electron micrograph of one of these samples (DSPC: 10% DSPG in 50 mM citrate buffer and with a drug:

lipid ratio of 0.2:1 (mol:mol)) is shown where aggregation is evident. For the other liposome-preparations the size-increase after loading was small (0–9 nm).

MLR was further used to fit the encapsulation efficiency (%) to the variables. The effects of increasing the factors from a low to a high level in the 2^{5-1} fractional factorial design are shown in Fig. 4. The optimal model was determined by a step down procedure leaving only the significant terms. One insignificant main effect was included in the final model due to limitations of the software. The R^2 and Q^2 were 0.9879 and 0.9092, respectively. The linear regression of the model was significant (< 0.01) and no significant model error was seen ($P > 0.05$).

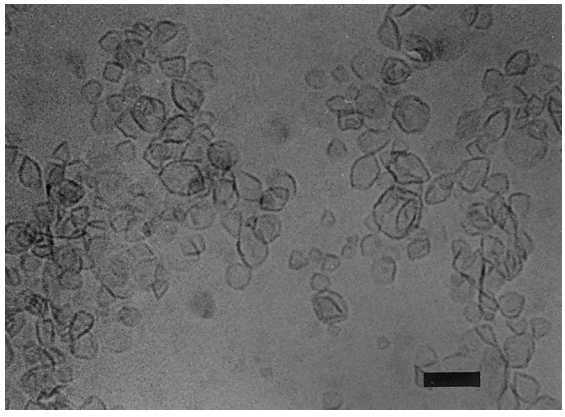

Fig. 3. Cryo-electron micrographs of DSPC: 10% DSPG in 50 mM citrate buffer (inner phase) and with a drug:lipid ratio of 0.2:1 (mol:mol). Bar represents 100 nm.

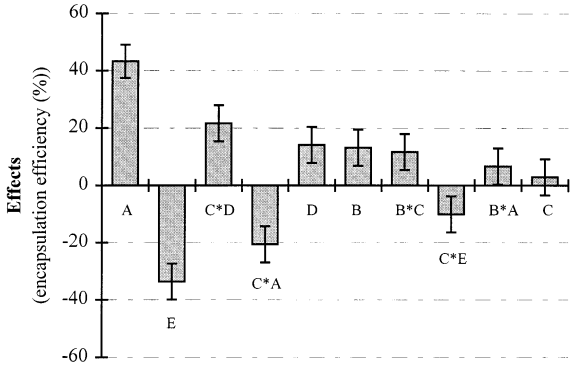

Fig. 4. The estimated effects of increasing the factors from a low to a high level on the encapsulation efficiency (%) in the 2^{5-1} fractional factorial design. Error bars are the confidence intervals. (A) Phospholipid type; (B) cholesterol concentration; (C) charge concentration; (D) citrate concentration and (E) drug concentration.

3.3. Optimisation design

Based on the results from the fractional factorial screening design, a Box–Behriken optimisation design was constructed. Phospholipid type and cholesterol were both at their high level due to their favourable effect on the encapsulation efficiency, while the other three factors were varied (Table 1). The response surfaces obtained from the results obtained in the Box–Behriken design with encapsulation efficiency (%) as the response are shown in Fig. 5 A–C. A second order regression model was developed. The fitted equation based on the scaled and centred significant regression coefficients was:

$$Y = 81 + 4.75C + 22.875D - 16.125E + 4.5C^*E + 9.25D^*E - 5.375C^2 - 7.125D^2 - 7.125\ E^2,$$

where Y is the encapsulation (%) and C, D and E are the coded levels of the independent variables. The model was significant (< 0.01) and no significant model error was seen ($P > 0.05$). The R^2 and Q^2 were 0.9890 and 0.9052, respectively. Only, a small increase in the liposome-size was observed after loading (up to 8 nm).

A robust region with high encapsulation efficiencies ($> 95\%$) was achieved with 200 mM citrate and drug:lipid incubation ratio below 0.15:1 (mol:mol) with charge varying between 2–10 mol% (Fig. 5c). It was therefore decided to perform further experiments with: DSPC, 10 mol%; DSPG, 33.33 mol%; cholesterol; and 200 mM citrate buffer pH 4.0 as the inner phase. In order to obtain a high encapsulation efficiency a drug:lipid ratio of 0.125:1 (mol:mol) was chosen.

3.4. Liposome-primaquine interaction

In Fig. 6 the phase transition behaviour of liposomes before and after loading with primaquine at a drug:lipid ratio of 0.125:1 (mol:mol) is shown. The ζ-potential (-14 mV) was not influenced by the encapsulation of primaquine.

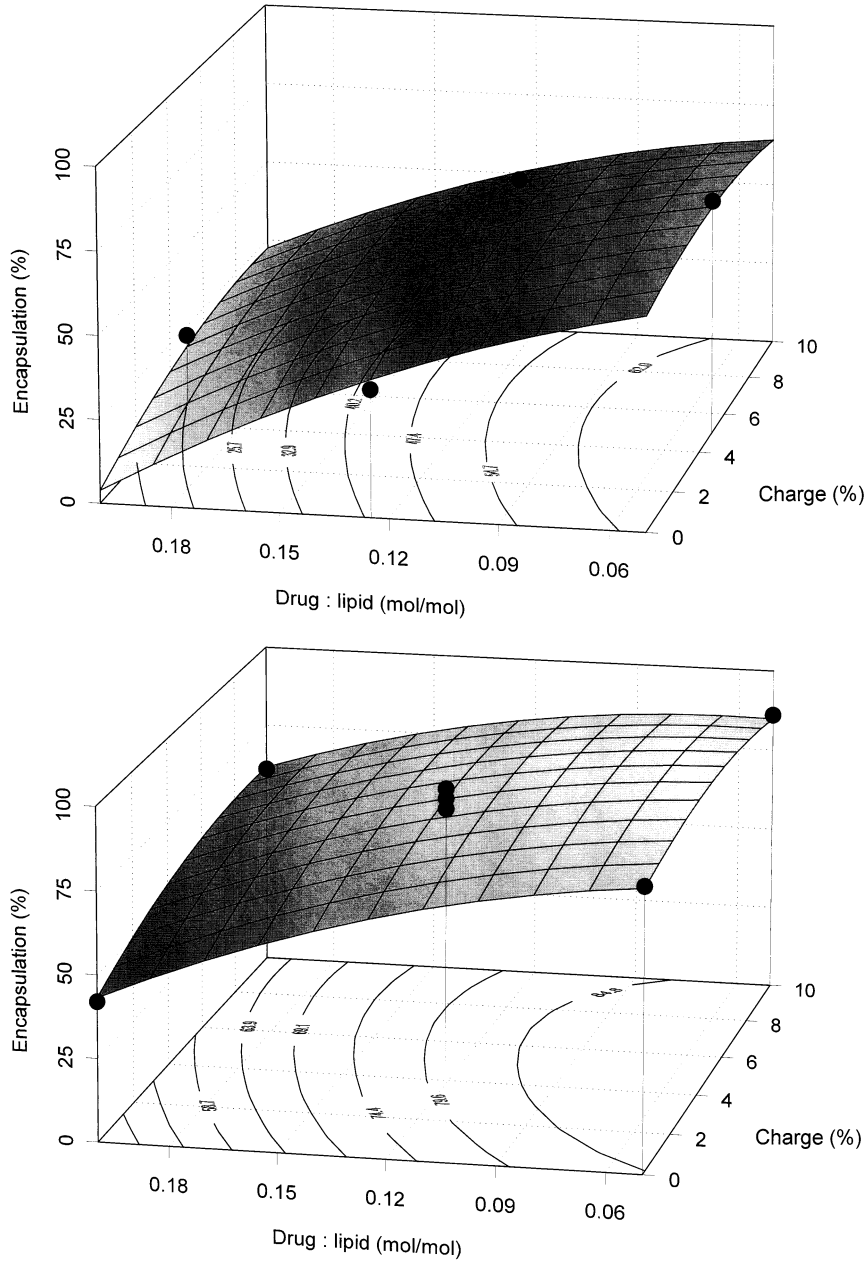

Fig. 5. Response surface obtained from the Box–Behnken design showing the encapsulation (%) of primaquine in DSPC: 33.33 mol% cholesterol-liposomes as a function of primaquine concentration (drug:lipid mol:mol) and charge concentration (mol %). (A) 50 mM; (B) 125 mM; and (C) 200 mM citrate buffer pH 4.0 as the inner phase. The points are the measured values.

3.5. Temperature effects

The efficiency of primaquine encapsulation in response to the pH gradient proved to be temperature dependent (Fig. 7). The encapsulation of primaquine was stable even after 4 h at 22°C (98% encapsulated). Incubation of the liposomes at 60°C for 1 h before loading (15 min at room

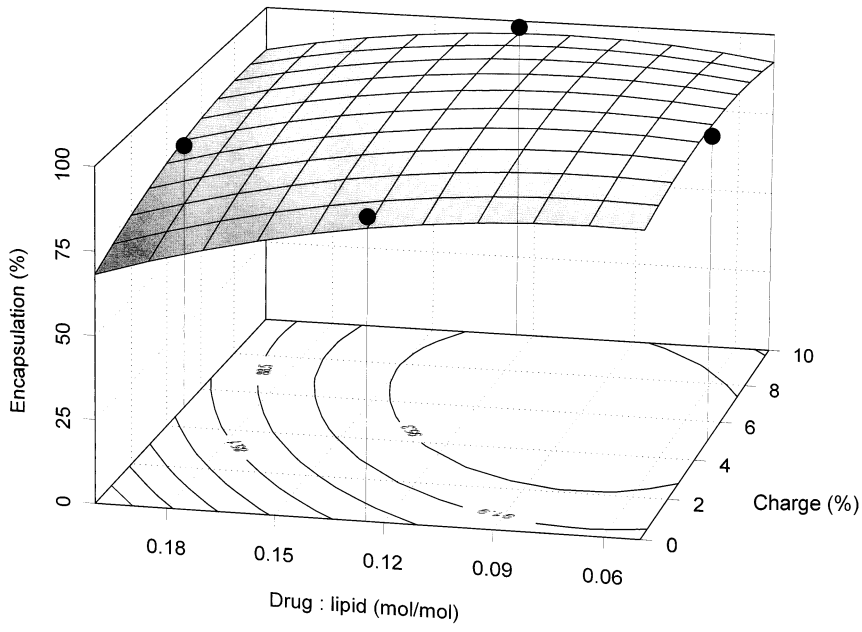

Fig. 5. (*Continued*)

temperature) gave the same low encapsulation efficiency as direct loading at 60°C. The liposome-size was not considerably affected by the loading procedure, and varied between 73 and 79 nm for all preparations. The stability of primaquine was not affected by the heat treatment (HPLC, UV).

3.6. Other factors

An encapsulation efficiency of only 4% was obtained by passive loading of primaquine into liposomes (drug:lipid ratio 0.125:1 mol:mol) in isotonic phosphate buffer pH 7.4 (primaquine added in the hydration buffer). The size of the liposomes achieved after extrusion was 92 nm in average and increased considerably during storage.

The inclusion of 5 mol% DSPE-PEG (5000) did not affect the liposome size or the encapsulation efficiency of primaquine by use of the remote loading technique.

Only 35% encapsulation was obtained using a smaller pH gradient (200 mM internal citrate (pH 5) concentration and isotonic phosphate buffer pH 7.4 externally) and the liposome size also increased during storage.

3.7. Lyophilisation and sterilisation

The effects of lyophilisation and γ-irradiation of the DSPC, 10%; DSPG, 33.33%; cholesterol-liposomes on the liposome-size and encapsulation efficiency are shown in Table 2. The liposomes

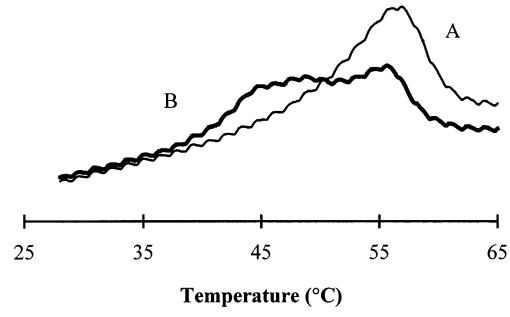

Fig. 6. Typical examples of the phase transition behaviour of DSPC: 10% DSPG: 33.33%. Cholesterol-liposomes before (A) and after (B) loading with primaquine at a drug:lipid ratio of 0.125:1 (mol:mol). The pellets obtained after ultracentrifugation was analysed. Endothermic peaks go up.

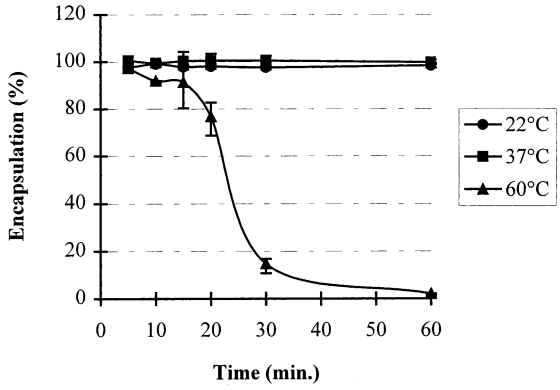

Fig. 7. Effect of incubation temperature on the primaquine uptake into DSPC:10% DSPG:33.33% cholesterol-liposomes with a drug:lipid ratio of 0.125:1 (mol:mol) in response to a pH gradient. Primaquine and liposomes were mixed at room temperature and incubated at the indicated temperatures. Error bars are the minimum and maximum values ($n = 3$).

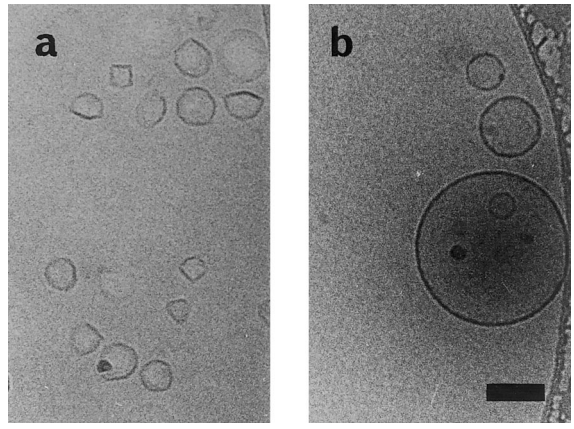

Fig. 8. Cryo-electron micrographs of DSPC:10% DSPG:33.33% cholesterol-liposomes after lyophilisation followed by rehydration and loading (a). Liposomes loaded with primaquine before lyophilisation (b). Bar represents 100 nm.

were 70 nm in average and showed an encapsulation efficiency of 98% before lyophilisation. The pH gradient proved to be stable against lyophilisation and rehydration as only a small decrease in the encapsulation efficiency was observed. This was associated with the increased liposome size.

The pH gradient was also stable against γ-irradiation. Solid state primaquine proved to be stable against γ-irradiation as no changes were observed by HPLC, UV-spectroscopy, pH measurements or DSC. The melting temperature of the lyophilised liposomes was lowered with 1.9°C, the pH decreased (Table 2) and cholesterol showed 5% degradation following γ-irradiation.

In Fig. 8a a cryo-electron micrograph of the liposomes after lyophilisation (slow freezing) followed by re-hydration and loading at a drug:lipid ratio of 0.125:1 (mol:mol) is shown. The liposomes are mainly unilamellar and faceted and appear as single units. On the other hand, liposomes loaded with primaquine before lyophilisation showed a considerably size-increase followed

Table 2
The effect of freezing rate during lyophilisation and gamma irradiation on the size (nm) and encapsulation efficiency (%) upon rehydration and loading of DSPC:10% DSPG:33.33% cholesterol-liposomes prepared with a pH gradient ($n = 3$)[a]

	After rehydration		After loading	
	Size (nm)	pH	Size (nm)	Encapsulation (%)
Quick freezing[b]	87	7.3	92	91
Quick freezing followed by γ-irradiation	82	6.5	85	93
Quick freezing with primaquine	608	7.1	–	56
Slow freezing[c]	90	7.3	98	94
Slow freezing followed by γ-irradiation	88	6.6	93	95
Slow freezing with primaquine	568	7.2		62
Slow freezing[d]	94	7.3	96	96

[a] The liposomes were 70 nm and showed an encapsulation efficiency of 98% before lyophilisation (pH 7.4 in the outer phase).
[b] Liposomes were frozen in liquid nitrogen.
[c] Liposomes were frozen by placing the vials directly on the pre-cooled plate (−30°C).
[d] Liposomes were frozen by placing the vials on the plate at 20°C followed by a gradual decreasing of the temperature.

by low encapsulation efficiencies after rehydration (Table 2). The cryo-electron micrograph in Fig. 8b shows that in this case the small liposomes have fused to larger vesicles.

The residual water content (w/w) in the lyophilisate was dependent upon the freezing protocol. The quickly frozen samples had a residual water content of 1.7%. the samples which were placed on the shelf at −30°C had a content of 1.4% and the samples which underwent a controlled freezing procedure had a water content of 0.7% ($n = 3$).

In Fig. 9a–d scanning electron micrographs of the lyophilisates are shown. Most noteworthy is the smooth and continuous surface of the quickly frozen liposomes (Fig. 9a) compared to the creaky and ruptured surface of the slowly frozen liposomes (Fig. 9b). No changes were seen with the slow freezing protocol when liposomes were loaded with primaquine before lyophilisation. With the quick freezing protocol a rougher surface with larger holes was observed (Fig. 9c). The structure of the 1yophilised cakes was similar independent of the freezing procedure (9d).

3.8. Long-term stability

No changes in the encapsulation efficiency nor liposome size were recorded after three months storage of the lyophilised product in the refrigerator (2–8°C) ($n = 3$). However, the size tended to increase for liposome suspensions exhibiting a pH gradient (101 nm after 3 months compared to 70 nm after preparation).

4. Discussions

4.1. Solubility and log P of primaquine

The pH dependent solubility and suitable log P values render primaquine an excellent candidate for active loading into pre-formed liposomes in response to a pH gradient (acidic interior).

Primaquine might permeate the membrane as PQ or PQ^+. With the pH gradient method, primaquine mainly in the PQ^+ form is added to pre-formed liposomes with an imposed pH gradient (acidic interior). Membrane permeation of

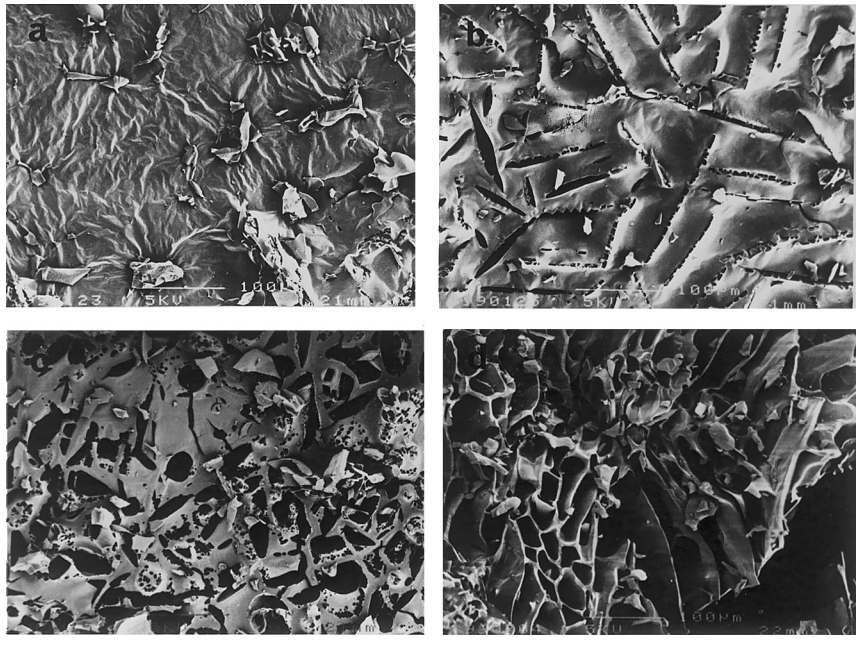

Fig. 9. Scanning electron micrographs of lyophilised DSPC:10% DSPG:33.33% cholesterol-liposomes. (a) Top view, quick freezing. (b) Top view, slow freezing. (c) Top view, loaded with primaquine before quick freezing. (d) Cross section.

PQ^+ results in a depletion of the internal proton pool as PQ^+ is further protonated upon exposure to the acidic intravesicular environment. The resulting diprotonated PQ^{2+} exhibits a low bilayer permeability (Fig. 1).

The internal liposomal primaquine concentration did not probably exceed the solubility limit in these experiments. Judging from the cryo-electron micrographs no precipitates were observed in contrast to the intraliposomal precipitation of doxorubicin, acridine and phenantridine after a remote loading-based encapsulation technique (Lasic et al., 1992; Johnsson et al., 1999).

4.2. Screening design

The phospholipid type proved to be the most important factor in order to achieve a high encapsulation efficiency as shown both in the PCA-plot (Fig. 2) and by the high levels in the effects plot (Fig. 4). At the incubation temperature (22°C), the DSPC membrane will be in the gel-phase ($T_c = 55.5$°C) and is less permeable than a fluid DMPC membrane ($T_c = 23.5$°C), thus creating a more stable transmembrane pH gradient. In addition, primaquine may have an increased partitioning into the more fluid DMPC bilayer and is thereby able to further disturb the pH gradient by, altering the bilayer permeability. However, that one of the DMPC-based liposome preparations showed a high encapsulation efficiency (Fig. 2), proves that the preparation can benefit from having the other factors (charge, cholesterol content, citrate and drug concentration) at an optimal level.

Primaquine entrapment efficiency was further dependent upon the drug:lipid ratio in the initial incubation mixture. High levels of accumulated drug deplete the interior buffering capacity and collapse the pH-gradient, thereby inhibiting further drug uptake.

The internal buffering capacity proved to be important in order to achieve high encapsulation efficiencies. This is consistent with the consumption of entrapped protons by PQ^+ during the uptake process and the shift in the equilibrium towards PQ^{2+} due to the high ionic strength. Citrate buffer is a widely used and pharmaceutically acceptable excipient for use in injectable therapeutic formulations. However, care must be taken with regards to the total amount and concentration of citrate in vivo, as citrate is able to chelate plasma calcium. Further, isotonic formulations are preferable and may restrict the use of higher citrate concentrations. An increase in the internal citrate concentration may also lead to osmotic gradients and disturbances when the liposomes are suspended in normal isotonic solutions. Lowering the pH of the intravesicular medium can also increase the internal buffering capacity, but a further decrease in pH exacerbates lipid stability problems due to hydrolysis.

The presence of cholesterol in the liposomes facilitated the permeation of primaquine as incorporation of cholesterol into a gel-like membrane renders the latter more hydrophobic and less rigid. Contrarily, for liposomes in the fluid-phase the rigidity is increased. The effect of cholesterol incorporation was most pronounced for DSPC-liposomes compared to DMPC-liposomes because the former liposomes were in the gel-state whereas DNIPC-liposornes were partly in the gel-phase and partly in the more fluid-state at the loading temperature.

The use of charged liposomes showed several important effects on the encapsulation efficiency. It is known that the use of negatively charged lipids increases the amount of positively charged drugs associated to the liposome membrane. In these experiments, it seemed that the positively charged primaquine (pH 7.4) interacted with the negatively charged liposomes and facilitated the encapsulation in the presence of a strong pH gradient (high citrate concentration). On the other hand, with a low citrate concentration where primaquine was not immediately accumulated in the intravesicular phase, the negative charge was not desirable. The reason is that non-entrapped primaquine most probably binds to the negatively charged lipid bilayer, which in turn interacts with membranes of other liposomes thus promoting aggregation and thereby also decreasing further loading. This behaviour was obvious from the PCA plot (and the cryo-electron micrograph) where two batches showed low encapsulation efficiencies followed by a size-increase. It has to be

mentioned that the size-measurements were performed after dilution of the liposomes, so de-aggregation might occur and this may explain, in some instances, the limited size increase as measured by PCS. It has earlier been shown that under certain conditions also non-entrapped doxorubicin induces aggregation of small negatively charged liposomes (Fonseca et al., 1997). Similarly, charge facilitated the encapsulation process at low primaquine concentration, whilst at higher concentrations excess primaquine was able to induce liposome aggregation and reduce the encapsulation efficiency. Furthermore. charge incorporation was less advantageous for DSPC-liposomes. This might be explained by the greater ability of liposomes to aggregate in the gel-state by the presence of primaquine. When cholesterol was present in the membrane, the use of charged liposomes increased the encapsulation efficiency. The negatively charged liposome membrane seemed to facilitate the interaction with primaquine and due to the presence of cholesterol the drug partitioning into the membrane occurred rapidly. In addition. cholesterol has shown to enhance the resistance against liposome aggregation (Fonseca et al., 1997).

4.3. Optimisation design

The significant interaction terms resulted in twisted response surfaces. At lower primaquine concentration. the encapsulation efficiency was already high and therefore benefits less from a higher citrate concentration or charge incorporation.

The curved response surfaces are due to the quadratic terms which all showed a negative influence on the encapsulation efficiency meaning that an optimum exists.

A robust region with high encapsulation efficiency ($> 95\%$) was achieved by proper settings of the factors (Fig. 5C). Small changes in the settings of the factors in this region cause negligible variations in the encapsulation efficiency and indicates a relatively robust encapsulation method.

4.4. Liposome-primaquine interaction

The changes in the phase transition behaviour after loading show that primaquine interacts with the membrane, causing packing default and alterations in the bilayer. The ζ-potential was not influenced and indicates that primaquine is not associated to the liposome surface by charge interactions but rather to the inner monolayer with the amino function oriented towards the aqueous phase and the hydrophobic region oriented towards the hydrocarbon (Harrigan et al., 1993).

4.5. Temperature effects

It seems that the pH gradient was depleted at temperatures above T_c due to a more permeable bilayer and, hence, faster transmembrane exchange of ions and water molecules. These observations are contrary to previously reported results where higher temperatures were employed to overcome the activation energy and, thereby, achieve a higher loading efficiency (Mayer et al., 1989; Harrigan et al., 1993; Lim et al., 1997). The complete uptake of primaquine at room temperature demonstrates that the pH gradient mediated uptake properties are clearly dependent upon the physico/chemical properties of the drug to be entrapped.

4.6. Other factors

The low trapping efficiency obtained by passive loading of primaquine is due to the use of SUV's with low encapsulation volume and, hence, poor encapsulation efficiency for hydrophilic drugs.

The polymeric chains of PEG did not affect the encapsulation efficiency or the size of the liposomes. It was therefore possible to make steric stabilised primaquine liposomes displaying a long circulating time. Also others have reported high encapsulation efficiencies with PEG-liposomes (Johnsson et al., 1999).

The internal pH proved to be a very important parameter in order to obtain high trapping efficiencies as the latter decreased dramatically by increasing the pH from 4 to 5.

4.7. Lyophilisation and sterilisation

The ability of liposomes to maintain a proton or a Na^+/K^+ gradient during lyophilisation and re-hydration has been earlier observed (Madden et al., 1985; Vemuri and Rhodes, 1994). Slow freezing compared to quick freezing markedly enhanced the stability of the pH gradient since quick freezing in liquid nitrogen induces a stress vector (van Winden et al., 1997). Unfortunately, primaquine loaded into liposomes prior to lyophilisation/rehydration leaked out and a large liposome size-increase was measured. The non-encapsulated primaquine may be responsible for the size increase/fusion of the liposomes resulting in even more drug leakage. It might be that an optimal lyophilisation procedure was not obtained for the primaquine-entrapped liposomes due to a different interior compared to the 'empty' liposomes.

The surface of the lyophilisates was sensitive to changes in the freezing-rate. The smooth surface seen for the quickly frozen samples probably reduces the sublimation rate during the primary drying process. The changes appearing on the surface for the quickly frozen samples in the presence of primaquine indicate that slow freezing allows the liposomes more time to relax and to adapt to changes induced by the freezing process.

γ-Irradiation proved to be a suitable method in order to sterilise the lyophilised product although some chemical degradation was observed. The decrease in pH and the lowering of the melting point of the lyophilised liposomes indicated chemical degradation of the phospholipids as earlier observed (Zuidam et al., 1995; Stensrud et al., 1999). However, the degradation was small ($<$ l.5% for DSPC which is the main component, Stensrud et al., 1999). The pH decrease might also result from degradation of the lyoprotectant sucrose (Triolet et al., 1992; Zuidam et al., 1995). Degradation of cholesterol following irradiation has also been reported by others (Samuni et al., 1997). Primaquine on the other hand proved to be stable against γ-irradiation in the solid state, an interested finding due to the reported low photochemical stability in aqueous solution (Kristensen, 1997). The smaller size increase of liposomes prepared from irradiated lyophilisate is due to the presence of degradation products like lysophospholipids and fatty acids since such a mixture of lipids can pack into a more curved bilayer (Stensrud et al., 1999).

4.8. Long-term stability

A lyophilised liposome product seemed to be favourable compared to a liposome-suspension. Stability problems due to hydrolysis of the phospholipids may, occur during storage of the lipo some-suspension at low internal pH (pH 4.0), with a further possibility to influence the physical stability.

5. Conclusions

Primaquine has successfully been encapsulated in liposomes in response to a pH gradient. The encapsulation efficiency depended upon the lipid composition, the internal buffer capacity, the drug-to-lipid incubation ratio and the presence of cholesterol and charge in the liposomes. The pH gradient was maintained during the lyophilisation-rehydration process and after sterilisation of the lyophilised product by γ-irradiation.

The final preparation consisted of a three-vial kit with solid state primaquine, lyophilised liposomes and hydration medium, where the encapsulation is assumed to occur immediately before use.

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