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Research paper

Effect of pH, ionic strength and oxygen burden on the chemical stability of EPC/cholesterol liposomes under accelerated conditions Part 1: Lipid hydrolysis

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Abstract

The accelerated stability of purified egg phosphatidylcholine (EPC)/cholesterol liposomes was studied under various formulation conditions using a 2^3 factorial experimental design. The three factors included in the study were pH, ionic strength of the buffer and the headspace oxygen content in the container. The results showed that lipid hydrolysis followed pseudo first-order kinetics. Data analysis using factorial design revealed that pH of the buffer was the predominant factor influencing the rate of lipid hydrolysis. Neither the ionic strength of the buffer, nor the presence of oxygen in the headspace of the container significantly affected the EPC hydrolysis. The hydrolysis rate of EPC at pH 4.0 buffer was at least 1.75 times greater than that at pH 4.8. A prediction based on the Arrhenius equation suggests that the EPC/cholesterol liposomes should be formulated in a buffer with pH equal to or greater than 4.2 in order to have a shelf-life longer than 1 year at 5°C.

Keywords: Purified egg phosphatidylcholine; Lipid hydrolysis; Stability; Experimental design; Liposomes; Shelf-life

1. Introduction

Liposomes are proven to be an efficient drug delivery system for a number of drugs, ranging from water-soluble molecules (doxorubicin, Doxil®, Daunoxome®) to water-insoluble compounds (amphotericine B, Abelcet®, AmBisome®, Amphotec®). However, the number of liposomal products available as marketed pharmaceuticals is still limited. One of the major obstacles that prevent many promising liposomal drug candidates moving from experimental scale to the pharmaceutical market is their chemical and physical instability during manufacturing and storage. Chemical and physical stability of liposomes are closely related to the mechanically strong and well-packed bilayers which reduce the access of oxidizing and hydrolyzing agents, and therefore decrease the changes of size distribution, fusion and mechanical properties of the lipid bilayer due to the reduction of the concentration of parent lipids. Control of pH, temperature, ionic strength and addition of antioxidants [1,2] and chelators may warrant prolonged chemical stability of the liposomal product. It is shown that the stability of liposomes is sensitive to several formulation parameters, which include pH [3–6], ionic strength of the buffer [7], dissolved oxygen content within the formulation and the oxygen level in the headspace of the package. Liposomes in an aqueous dispersion can be hydrolyzed to form lysophospholipids and fatty acids. The lysophospholipid can be further hydrolyzed to glycerophospho compounds and fatty acids [3–6]. It is indicated that hydrolysis of lipids was catalyzed by hydroxyl and hydrogen ions [4], and an excess amount of oxygen present either in the formulation in the form of dissolved oxygen or in the headspace of the container could increase the chance of peroxidation of unsaturated lipids. The ionic strength of the buffer was also reported to affect the stability of liposomes [7].

In the present study, the egg phorphatidylcholine (EPC)/cholesterol liposomal dispersion was used as a model to study the effects of the pH, the ionic strength of the buffer and the oxygen content in the headspace of the container on lipid hydrolysis at three different temperatures. The EPC/cholesterol liposomes studied were intended to be used as the drug delivery system to deliver a labile drug by active loading [8–10] prior to dosing. A 2^3 factorial experimental design was constructed to determine physical and chemical

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stability of the liposomes as a function of pH, ionic strength, and headspace oxygen content. The objectives of the study were to identify the predominant formulation parameters that were associated with the instability of the EPC/cholesterol liposomes and to predict the stability behaviors of the liposomes under other conditions not included in the experiments. The stability under the stress conditions was monitored to elucidate intrinsic stability characteristics of the liposomes, and consequently provide the information as to whether a minor modification of the existing formulation could lead to optimal stability and prolonged shelf life.

2. Materials and methods

2.1. Materials

Purified EPC was obtained from Avanti (Avanti Polar Lipids, Inc., Alabaster, AL). Cholesterol was purchased from Croda (Croda, Inc., Parsippany, NJ). Methylene chloride, citric acid, monohydrate, USP, sodium chloride, USP, Sodium hydroxide, NF and methanol (UV grade) were purchased from Baker (JT Baker, Phillpsburg, PA). Chloroform (Ominsolv) was obtained from EM Science (EM Science, Gibbstown, NJ). Finally, nitrogen, NF and oxygen, USP were obtained from JWS Technologies (JWS Technologies, Inc., Hamburg, NJ). All chemicals were used as received.

2.2. Liposome preparation and stability study

The liposomes were prepared by a stable plurilamellar vesicles (SPLV) process [11]. Briefly, EPC and cholesterol (7:1 molar ratio) were dissolved in methylene chloride and the lipid solution was added to the citrate buffers with different pH and ionic strength in a formulation vessel. The lipid and the buffer solution were vigorously agitated together at approximately 1500–2000 rev./min. After agitation, the methylene chloride was removed by evaporation to form an aqueous dispersion of EPC/cholesterol liposomes. The size of liposomes was reduced to about 200 nm ±10% by high-pressure extrusion using a 0.1-μm organic aluminum oxide membrane (Anopore™) filter (Millipore Corporation, Bedford, MA). The entire process was carried out under nitrogen protection to minimize the oxygen exposure of the lipid mixture. Finally, the liposomal formulation was filled into 2-ml serum vials and the headspace of the vials was blanketed either with pure nitrogen or with pure oxygen. The stability of the prepared liposomal formulations was evaluated after storage at 30, 40, and 50°C for an extended period of time. The pH, EPC content, and the particle size distribution of the liposomal samples were determined as a function of the storage period. The EPC content in each formulation was analyzed by high performance liquid chromatography (HPLC), and the particle size distribution of the liposome was monitored using a light scattering method with a sub-micron particle sizer (Nicomp Model 270/370, Pacific Scientific, Menlo Park, CA).

2.3. HPLC method for EPC content analysis

The HPLC system consisted of a Waters model 300 solvent delivery system (Water Corporation, Milford, MA), a Waters model 712 WISP auto-injector, a column heater, Perkin–Elmer Nelson data acquisition software (PE Nelson System Inc., San Jose, CA), an Astec diol bonded phase column 250 × 4.6 mm (Advanced Separations) and an evaporative light scattering detector (ELSD, SEDEX, SEDERE, Alfortville, France). The mobile phase consisted of chloroform, methanol and water at a volume ratio of 65:25:4. An aliquot of 50 μl of the sample was diluted to 10 ml with chloroform:methanol (85:15, v/v) and injected into the HPLC system. The sample was prepared in duplicate and was analyzed at a flow rate of 1.3–1.5 ml/min. The amount of EPC in the formulation was quantitatively determined using external standards.

2.4. Experimental design

A two-level non-repeating factorial design was employed in this study. This design method is also known as 2^k factorial design, in which k is the number of the factors used in the study (Table 1).

In this study, the factors were defined as follows:

- Factor A (X_1) = pH of the citrate buffer used for preparing the liposomes.
  High level (+): 4.8
  Low level (−): 4.0
- Factor B (X_2) = oxygen level in the headspace of the serum vials containing the liposomal formulation.
  High level (+): pure oxygen blanketing. The average oxygen content in the headspace determined from the oxygen-blanketed samples ranged from 70 to 90%.
  Low level (−): pure nitrogen blanketing. The average oxygen content in the headspace determined from the nitrogen blanketed samples was less than 4%.

Table 1
Factorial design for short term EPC/cholesterol liposome stability study

Factor				
Batch	Run order[a]	pH	Oxygen (%)	Ionic[b] (m)
1027–164	4	4.0	N_2	0.2
1095–025	6	4.8	N_2	0.2
1027–064	2	4.0	O_2	0.2
1027–200	5	4.8	O_2	0.2
1095–055	7	4.0	N_2	0.8
1027–043	1	4.8	N_2	0.8
1095–080	8	4.0	O_2	0.8
1027–100	3	4.8	O_2	0.8

[a] Random order for preparing each formulation.
[b] Additional NaCl was added to adjust ionic strength.

- Factor C (X_3) = ionic strength of the citrate buffer in the liposomal formulation.
  High level (+): 0.8 M
  Low level (−): 0.2 M

2.5. Data analysis

The hydrolysis rate constant of each liposomal formulation was calculated by linear regression of the logarithmic percentage of the lipid remaining (ln %EPC) vs. storage time using a Microsoft Excel program. Other data treatment was performed by Design-Ease® Software (State-Ease Corporation, Minneapolis, MN). A P value of less than 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Lipid hydrolysis

3.1.1. Order of reaction

The EPC content in the liposomal dispersion was determined as a function of the storage time for each pH condition, ionic strength, headspace oxygen content and storage temperature studied. The EPC percentage remaining was plotted against the storage time on a semi-logrithmic scale and the typical graphs are presented in Figs. 1 and 2. It was found that the degradation of EPC followed pseudo first-order kinetics regardless of the pH of the liposomal dispersion, ionic strength of the buffer, headspace oxygen levels or the storage temperature. A close examination of the plot revealed that the slope of the plot remained consistent up to 75% EPC degradation (25% EPC remaining). This suggests that the formation of the degradation products exerted no significant impact on the degradation kinetics of the lipid. The slope of the linear plot presented in Figs. 1 and 2 represented the observed rate constant (k_{obs}) for each corresponding formulation condition under different storage temperatures. The k_{obs} and the correlation coefficient for each formulation and temperature condition were calculated by a least-squares linear regression method. Statistical analysis indicated that the linear relationship between the natural log percentage of EPC remaining (ln %EPC) and the storage time of each plot was statistically significant ($P < 0.05$), which confirmed the validity of the conclusion that the degradation of EPC followed a pseudo first-order reaction. The observed hydrolysis rate constant of EPC under different conditions are presented in Fig. 3.

The shelf life, $t_{80,obs}$ of the formulation was defined as the time required to reach 20% degradation (80% EPC remaining) and it was calculated based on the first-order kinetics

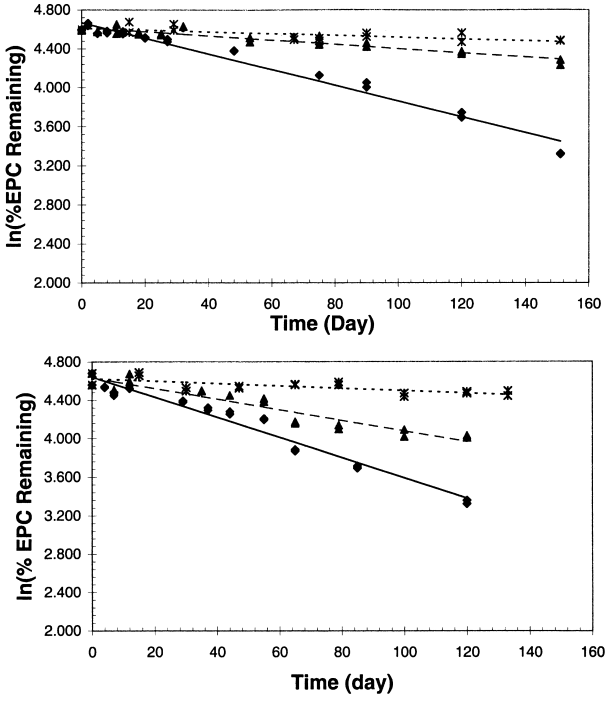

Fig. 1. Degradation profiles of EPC in liposomes at different temperatures. Top: formulation conditions: pH 4.8, nitrogen blanketing, ionic strength of buffer: 0.8. Bottom: formulation conditions: pH 4.8, oxygen blanketing, ionic strength of buffer: 0.8. ◆, 50°C samples; ▲, 40°C samples; * 30°C, samples. Each line presents the data that are fitted with a pseudo first-order kinetic model.

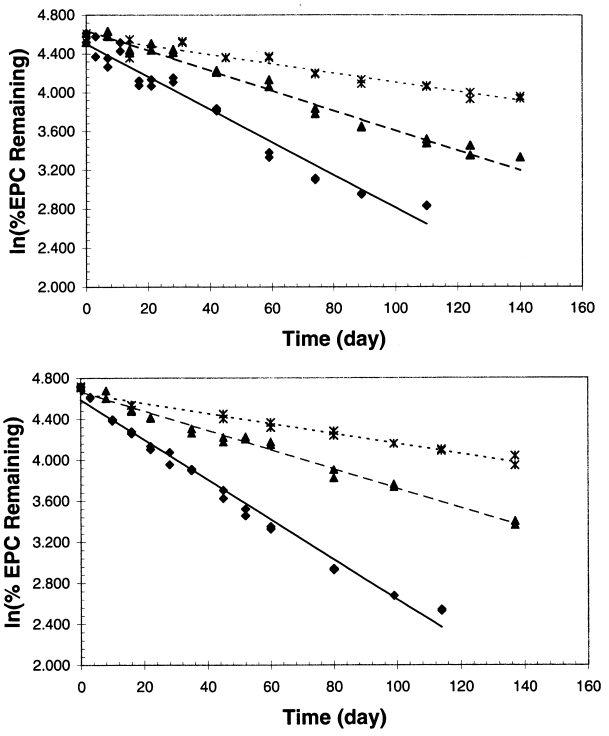

Fig. 2. Degradation profiles of EPC in liposomes at different temperatures. Top: formulation conditions: pH 4.0, nitrogen blanketing, ionic strength of buffer: 0.2. Bottom: formulation conditions: pH 4.0, oxygen blanketing, ionic strength of buffer: 0.2. ◆, 50°C samples; ▲, 40°C samples; *, 30°C samples. Each line presents the data that are fitted with a pseudo first-order kinetic model.

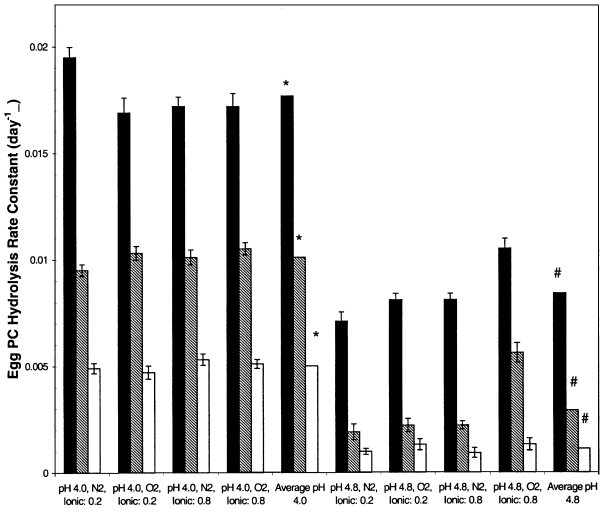

Fig. 3. Hydrolysis rate constants of egg phosphatidylcholine/cholesterol liposomes under different formulation conditions. ■, 50°C samples; ▨, 40°C samples; □, 30°C samples. The * represents the average values obtained from all batches made with pH 4.0 buffer. The # represents the average values obtained from all bathes made with pH 4.8 buffer.

using k_{obs} obtained (Fig. 3) and Eq. (1)

$$t_{80,obs} = \frac{0.2231}{k_{obs}} \quad (1)$$

The results of the calculated $t_{80,\,obs}$ are presented in Fig. 4.

3.1.2. Effect of pH, ionic strength and headspace oxygen content

A first-order polynomial model was designed to determine the significance of the factors included in the factorial experimental design

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_1X_2 + a_5X_1X_3 + a_6X_2X_3 + a_7X_1X_2X_3 \quad (2)$$

where Y is the lipid hydrolysis rate, X_1 (pH), X_2 (headspace oxygen content), and X_3 (ionic strength) are the three factors investigated, and X_1X_2, X_1X_3, X_2X_3, and $X_1X_2X_3$ represent the factor-to-factor interactions. From a typical normal probability plot, the factors that contributed significantly to the rate of hydrolysis would appear as outliners. If the factors did not contribute to the rate of lipid hydrolysis significantly, they would be expected to fit onto a straight line.

The normal probability plots at 30, 40, and 50°C (Fig. 5) revealed that the pH (factor X_1) departed greatly to the left of the line. Therefore, the pH of the liposomal dispersion had contributed significantly to the hydrolysis of EPC at all storage conditions studied. In contrast, all other factors including the headspace oxygen content, the ionic strength and the interactions among the three factors investigated (X_1X_2, X_1X_3, X_2X_3 and $X_1X_2X_3$) were in proximity to the straight line, indicating that their effects on the lipid hydrolysis were not statistically significant. As a result of the statistical analysis, the headspace oxygen content, the ionic strength and the interactions among the three factors were eliminated from the model (Eq. (2)). Therefore, Eq. (2) was reduced to

$$Y=a_0+a_1X_1 \quad (3)$$

Analysis of variance (ANOVA) conducted by the Design-Ease® software has further confirmed that the hydrolysis rates of EPC were significantly influenced by pH of the buffer at the storage conditions of 30, 40 and 50°C ($P < 0.05$).

Based on the statistical data analysis by Design-Ease Software, a_0 and a_1 in Eq. (3) were calculated, and the following equations were obtained

$$Y\{\text{Rate, ln (\%EPC remaining)} \times \text{day}^{-1}\} = 0.0640 - 0.01157\ X_1\ (\text{pH}) \text{ at } 50°\text{C} \quad (4)$$

$$Y\{\text{Rate, ln (\%EPC remaining)} \times \text{day}^{-1}\} = 0.0458 - 0.00893\ X_1\ (\text{pH}) \text{ at } 40°\text{C} \quad (5)$$

$$Y\{\text{Rate, ln (\%EPC remaining)} \times \text{day}^{-1}\} = 0.0245 - 0.00486\ X_1\ (\text{pH}) \text{ at } 30°\text{C} \quad (6)$$

Evidently, the rate of EPC hydrolysis in EPC/cholesterol liposomes is inversely proportional to pH of the bulk formulation at all the storage temperatures studied. Since the pH-stability profiles of phosphadylcholine (PC) liposomes was found to be linear within the pH range of 3–6.5 (Grit et al. [3,4,6,12]), the degradation rate (Y) at any pH condition within this pH region can be predicted using the Eqs. (4)–

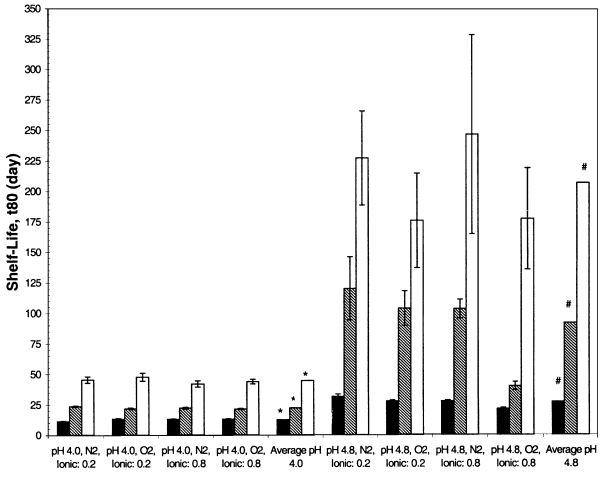

Fig. 4. Shelf-life, t_{80} of egg phosphatidylcholine/cholesterol liposomes under different formulation conditions. ■, 50°C samples; ▨, 40°C samples; □, 30°C samples. The * represents the average values obtained from all batches made with pH 4.0 buffer. The # represents the average values obtained from all bathes made with pH 4.8 buffer.

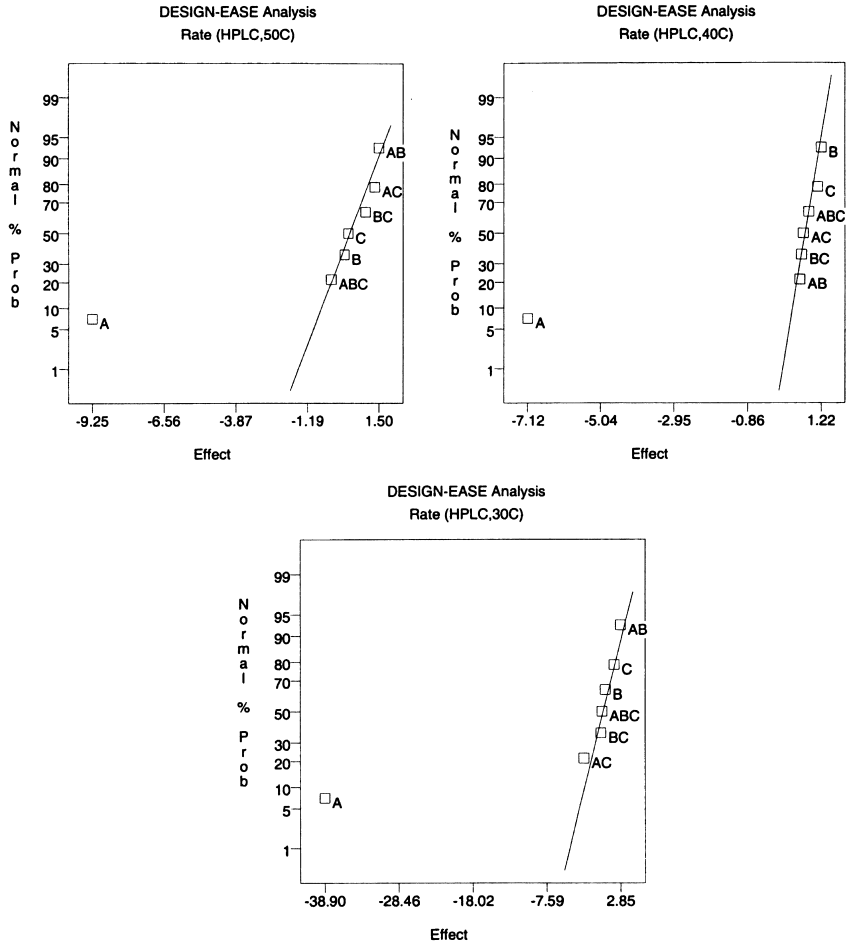

Fig. 5. 2^3 Experimental Design. Normal probability plot of the effect. Top right: EPC hydrolysis at 50°C. Top left: EPC hydrolysis at 40°C. Bottom: EPC hydrolysis at 30°C. A: X_1, pH effect; B: X_2, oxygen effect; C: X_3, ionic strength effect; AB, AC, BC and ABC are the factor-to-factor interaction.

(6). Table 2 summarized the hydrolysis rate constants and shelf-life ($t_{80,\ Y}$) predicted using Eqs. (4)–(6) and the first-order kinetics.

Since the ionic strength of the formulation and the headspace oxygen content did not contribute to the hydrolysis rate of the lipids, the observed rate constants (k_{obs}) and the shelf life ($t_{80,\ obs}$) determined using the previously discussed log-linear model were grouped together according to the pH condition of the formulation. The average value was calculated for each pH condition at each storage temperature studied. The results were presented in Figs. 3 and 4. As compared with the hydrolysis rate constant (Y) and the shelf life ($t_{80,\ Y}$) predicted using Eqs. (4)–(6) (presented in Table 2), a good agreement was found between the two sets of the values, confirming the validity of both approaches used in this study.

It was also found that the hydrolysis rate of EPC liposomes at pH 4.0 buffer was at least 1.75 times faster than

Table 2
Predicted hydrolysis rate constants[a] and $t_{80,\ Y}$[b]

Temperature (°C)	pH 4.0		pH 4.8	
	Rate constant (Y)[c]	$t_{80,\ Y}$ (day)	Rate constant (Y)[c]	$t_{80,\ Y}$ (day)
30	5.06×10^{-3}	44.1	1.17×10^{-3}	190.4
40	1.01×10^{-2}	22.1	2.94×10^{-3}	75.9
50	1.77×10^{-2}	12.6	8.46×10^{-3}	26.4

[a] Predicted from Eqs. (4–6).
[b] $t_{80,\ Y}$: the time to reach 20% degradation (80% EPC remaining). It was determined based on first-order kinetics, i.e. $t_{80,\ Y} = 0.2231/Y$.
[c] Unit in ln(%EPC remaining) × day^{-1}.

those at pH 4.8 regardless of the headspace oxygen content or the ionic strength of the bulk formulation. Grit et al. [3,12] have found that the natural soybean phosphatidylcholine and partially saturated egg phosphatidylcholine both showed V-shape pH stability profiles with a minimum degradation rate occurring at pH of 6.5. They also reported that both protons and hydroxyl ions catalyzed the hydrolysis of liposomal phospholipids. As indicated in their study, the rate of lipid hydrolysis decreased significantly as pH of the buffer was increased from 3 to 6.5. The present study confirmed their findings in that the hydrolysis rate of EPC decreased dramatically as the pH of the buffer was increased from 4.0 to 4.8.

During the early stage of the hydrolysis, nitrogen blanketed EPC/cholesterol liposomal formulations remained white and opalescent in appearance with no sedimentation, whereas the formulations that were blanketed with oxygen turned yellow during the storage period. The color change may be due to oxidation of EPC lipid at a high oxygen content environment. Nevertheless, the changes in the composition of EPC/cholesterol liposomes due to discoloration was insignificant to cause any effects on lipid hydrolysis as verified by statistical analysis. At the later stage of the hydrolysis, the turbidity of the formulations increased regardless of the bulk pH or the headspace oxygen content. Furthermore, when the EPC degradation reached up to 60% (40% of lipid remaining), a phase separation was observed. This phenomenon may be due to the formation of large amount fatty acids when the EPC was hydrolyzed. It is known that most of the fatty acids are less dense than the citrate buffer, therefore, as the ratio of fatty acids to EPC increases, a phase separation may result. The phase separation caused by the re-distribution of EPC and fatty acids induced liposome fusion to form larger size of vesicles (confirmed by light scattering particle size analysis results and visual observation using microscope). At the storage temperature conditions studied, the mean particle size of the liposomes remained unchanged at 200 nm until about 60% EPC was hydrolyzed. The size of the liposomal vesicles increased dramatically thereafter.

Results of this study also revealed that hydrolysis was not affected by the ionic strength of the buffer (the kinetic salt effect). This may be due to insufficient net charge on EPC before and after degradation. Carstensen [13] theorized from Debye–Hückel and Brønsted–Bjerrum equations that if a decomposition does not exhibit a kinetic salt effect, the reaction of two charged species can be eliminated from consideration. He suggested that hydrolysis which is not affected by salt should only happen between charged and non-charged species (EPC). The findings in this study were in agreement with the theory in that the hydrolysis of EPC involved no net charge changes. Therefore, it is concluded from the study that sodium chloride can be safely added to the formulation to adjust the tonicity as needed without jeopardizing the product stability.

Table 3
Activation energies (E_a) and frequency factor (A) for the hydrolysis of EPC/cholesterol liposomes under various conditions[a]

Lot #	Condition	E_a (kJ/mol)	A (day^{-1})
1027–164	pH 4.0, N_2, ionic: 0.2	56.20	2.32×10^{7}
1095–025	pH 4.8, N_2, ionic: 0.2	80.09	5.50×10^{10}
1027–064	pH 4.0, O_2, ionic: 0.2	67.66	1.63×10^{9}
1027–200	pH 4.8, O_2, ionic: 0.2	73.98	6.28×10^{9}
1095–055	pH 4.0, N_2, ionic: 0.8	55.55	1.74×10^{7}
1027–043	pH 4.8, N_2, ionic: 0.8	89.28	2.51×10^{11}
1095–080	pH 4.0, O_2, ionic: 0.8	49.44	1.74×10^{6}
1027–100	pH 4.8, O_2, ionic: 0.8	85.41	7.63×10^{11}

[a] E_a and A were estimated using only three temperatures.

3.1.3. Effect of temperature

The effect of temperature on the hydrolysis of EPC/cholesterol liposomes was investigated at 30, 40 and 50°C at two pH levels. Figs. 1 and 2 present the temperature dependence of the lipid hydrolysis at various conditions. Clearly, the rate of EPC hydrolysis increased as the storage temperature was increased.

Additionally, the temperature dependence of the overall degradation process at various formulation conditions was characterized using the Arrhenius equation as follows

$$k = A\mathrm{e}^{-\frac{E_a}{RT}} \tag{7}$$

where k is the reaction rate constant of any order, A is the frequency factor or pre-exponential factor, and E_a is the activation energy of the chemical reaction, R is the gas constant, and T is the absolute temperature. This equation can be re-arranged as follows

$$\ln(k) = \ln A - \frac{E_a}{RT} \tag{8}$$

Eq. (8) indicates that a graph of ln$k$ against $1/T$ will be linear with a slope of $-E_a/R$ and a intercept of ln$A$. The estimated E_a values for the EPC hydrolysis reaction are presented in Table 3. The higher E_a value is, the greater the effect of temperature on the rate of reaction is. The E_a of hydrolysis of liposomes at pH 4.0 ranged from 56 to 68 kJ/mol, whereas the formulations at pH 4.8 ranged from 74 to 89 kJ/mol (Table 4). These values were similar to the results of partially saturated egg PC hydrolysis reaction reported by Grit [12]. The greater value of E_a for the formu-

Table 4
Predicted shelf-life for EPC/cholesterol liposomes at different pH

pH	Y at 5°C (Day^{-1})[a]	$t_{80,\,Y}$ at 5°C (Day)
4.0	8.30×10^{-4}	269
4.2	5.99×10^{-4}	372
4.3	4.90×10^{-4}	455
4.4	3.87×10^{-4}	577
4.5	2.91×10^{-4}	768
4.8	6.44×10^{-5}	3463

[a] Data were estimated using Eqs. (4)–(6) and Arrenhius equation.

lations at pH 4.8 resulted in a more profound effect of temperature on the hydrolysis rates of the phospholipids (rate constant increased 664% going from 30 to 50°C), as compared with the formulations at pH 4.0 (rate constant increased 254% going from 30 to 50°C).

Based on the rate constants predicted using Eqs. (4–6) and the Arrhenius equation for the formulations at 4.0, 4.2, 4.3, 4.4, 4.5 and 4.8, the shelf life for EPC/cholesterol liposomes at 5°C were calculated as 269, 372, 455, 577, 768 and 3463 days, respectively (Table 4). It is shown that the predicted shelf life of the liposomes at 5°C was less than 1 year when the liposomes were formulated in pH 4.0 buffer, however when the liposomes were formulated at a higher pH range, the shelf life increased dramatically. It is evident that the liposomes should be formulated at pH equal to or greater than 4.2 to maintain a shelf life longer than 1 year.

Finally, no discontinuity in the Arrhenius plots were found for the formulations investigated (Fig. 6), indicating that there was no change in reaction mechanism. Therefore, gel to liquid crystalline phase transition, which was the probable cause for changes in hydrolysis mechanism of the phospholipids, was not a concern at the storage temperature conditions investigated in this study. This finding suggests that the stability studies conducted at 30, 40 and 50°C as the accelerated conditions for EPC/cholesterol liposomes are appropriate to predict liposomal behaviors at 5°C. However, this approach would not be suitable for some of the liposomal products in which the phase transition temperature (T_m) of the lipids is within the storage temperature range studied. If this is the case, the stability behavior of the liposomes in one physical state (e.g. at higher temperature) should not be used to predict that in another physical state (e.g. at lower temperature), as the physicochemical behaviors of the liposomes are different in gel or liquid crystalline state [14].

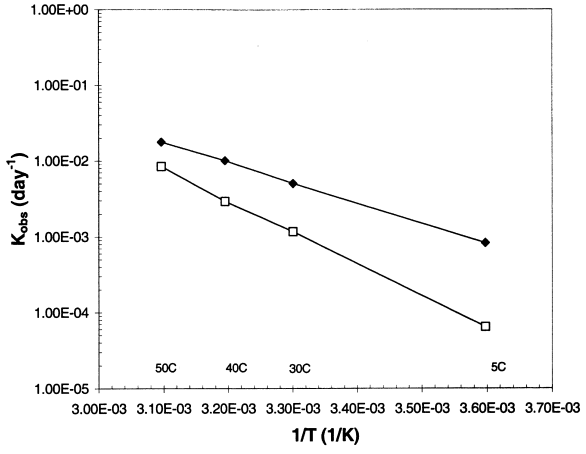

Fig. 6. Arrhenius plot of the effect of the temperature on the hydrolysis of egg phosphatidylcholine liposomes. ◆, pH 4.8; □, pH 4.0. Each data point represents the mean of four separate results determined in the experimental batches.

4. Conclusions

In conclusion, hydrolysis of egg phosphatidylcholine in the liposomal formulation investigated followed pseudo first-order kinetics. The lipid hydrolysis was greatly dependent upon pH of the buffer in which the EPC was formulated, but was independent on the ionic strength of the buffer and the headspace oxygen content. The study suggests that the liposomes formulated at higher pH level tend to have longer shelf life as compared with those formulated at lower pH levels. Furthermore, since the phase transition temperature (T_m) of the EPC/cholesterol liposomes was not found in the temperature range of 5–55°C, the storage conditions of 30, 40 and 50°C were appropriate in serving as the accelerated conditions to predict the shelf life of this type of liposomes at 5°C. Finally, the study demonstrated that the factorial experimental design employed in this study successfully identified the predominant factor which influenced the stability of the EPC liposomal formulation. Thus, the use of a factorial experimental design is very valuable in screening and optimizing a formulation during pharmaceutical development.

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