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Hydroxyzine- and Cetirizine-Loaded Liposomes: Effect of Duration of Thin Film Hydration, Freeze-Thawing, and Changing Buffer pH on Encapsulation and Stability

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ABSTRACT *Purpose.* To assess the effect of the duration of film hydration, freeze-thawing, and changing buffer pH on the extent of entrapment of hydroxyzine and cetirizine, H_1-antihistamines with different polarity, into liposomes, and the stability of these liposomes. *Methods.* Multilamellar vesicles (MLV) were prepared by thin-lipid film hydration using L-α-phosphatidylcholine (PC) and buffer containing 80 mg hydroxyzine at pH 7. For MLV containing hydroxyzine, the liposomes were subjected to 1) hydration for 1 h, 24 h, or 48 h for the control batch, batch B, or batch D respectively; and 2) hydration for 1 h, 24 h, or 48 h with freeze-thawing for 5-cycles for batch A, batch C, or batch E, respectively. These formulations were stored at 10±2°C and 37±0.1°C. Small unilamellar vesicles (SUV) and MLV were prepared using L-α-phosphatidylcholine (PC), and buffer at pH 5.0, 5.5, 6.0, 6.5, and 7.0, containing 80 mg hydroxyzine or 82 mg cetirizine by the ethanol injection and thin-lipid film hydration methods, respectively. These formulations were stored at 10±2°C. Liposomes were evaluated immediately after preparation and after storage by determining percent entrapment of hydroxyzine (PETH) or of cetirizine (PEC) and by observing changes in the physical appearance (PA). Particle size (PSA) of the liposomes freshly prepared at pH=6.5 was measured from transmission electron micrographs (TEM). *Results.* Increasing thin-film hydration time or repeated freeze-thawing did not affect the initial PETH or long-term stability of control, A, B, C, D, and E batches of MLV containing hydroxyzine stored at 10±2°C. At 37±0.1°C, PETH of all MLV batches decreased considerably after 1 month. This was more evident in batches B, C, and E exposed to freeze-thawing. The PETH of SUV increased markedly from 53.0% to 84.0% when the pH of the buffer was increased from 5.0 to 5.5. As pH increased from 6.0 to 7.0, PETH continued to increase from 84% to 94%. The initial PETH of MLV increased slightly from

82.0% to 94.0% as the buffer pH values increased from 5.0 to 7.0. There was no effect of pH on initial PEC, and stability of SUV or initial PEC of MLV, which ranged from 92% to 94%, as buffer pH values increased from 5.0 to 6.5. After storage at 10±2°C PEC in MLV decreased from 94% to 74%. *Conclusions.* The freeze-thawing processes had some effect on the stability of liposomes stored at temperatures higher than ambient temperature, 37±0.1°C. The effect of changing the buffer pH from 5.5 to 7.0, and from 5.0 to 6.5 on initial PETH and PEC, respectively, was minimal. After 24 months at10±2°C, pH had no effects on PETH; however, PEC of MLV decreased.

KEYWORDS Cetirizine, Hydroxyzine, L-α-phosphatidylcholine, Liposomes, pH, Freeze-thawing, Stability

INTRODUCTION

Liposomes, closed vesicles comprised of phospholipid bilayers or lamellas with an entrapped aqueous core, are able to encapsulate medications (Idiart & Levin, 2004; Patel & Misra, 1999). Liposomes are divided into two major classes based on the number of lamellas in their structure. Multilamellar vesicles (MLV) consist of five or more lamellas, and their size ranges from 100 nm to 20 μm. Unilamellar vesicles, containing a single bilayer, are subdivided into small unilamellar vesicles (SUV,<100 nm) and large unilamellar vesicles (LUV, 100–1000 nm) (Betageri & Kulkarni, 1999; Kulkarni et al., 1995; Ostro & Cullis, 1989). These liposomes can encapsulate water-soluble drugs in their aqueous spaces and lipid-soluble drugs within the lamellas themselves (Betageri & Kulkarni, 1999; Kulkarni et al., 1995; Ostro & Cullis, 1989).

Egg phosphatidylcholine (PC) is the phospholipid (PL) most widely used to prepare liposomes. It is a naturally occurring, neutral PL with a low transition temperature of −15°C (Personal communication, Information Center, Avanti Polar Lipids, Inc., Alabaster, AL, USA. Jan. 2000.), composed of a mixture of the diglycerides of stearic, palmitic, and mainly unsaturated oleic fatty acids with the phosphate esterified with choline (Avanti Polar Lipids, INC.; http://www.lipidat.chemistry.ohio-state.edu/, 2003; Fiume, 2001). Cholesterol (CH), used as an additive material in the preparation of liposomes, is comprised of amphipathic molecules that orientate themselves at the exterior portion of the lipid region of the bilayer membrane of the liposomes with the polar hydroxyl group located at the level of the bridge region, where hydrogen bonding can take place (New, 1995). This arrangement reduces leakage of medications through the bilayer membranes of the liposomes and increases the stability of the liposomes (Coderch et al., 2000; Fang et al., 2001; Roger, 1995; Veatch & Keller, 2003; Yarosh, 2001).

The three most important evaluation parameters for medication-loaded liposomes are entrapment efficiency, medication/lipid ratio, and medication retention (Betageri & Kulkarni, 1999; Kulkarni et al., 1995). Various factors that influence medication encapsulation within MLV liposomes include film thickness after evaporation of the solvent during preparation of the liposomes, the rate of hydration of the thin film, and the temperature, speed, and duration of shaking during the hydration process. Repeated freeze-thawing of MLV without using organic solvents or detergents may increase the entrapped aqueous volume of the liposomes considerably, and theoretically increase the total entrapment of medications within them. During freezing, the slow rate of cooling leads to the formation of ice crystals that exert pressure on the bilayers of the liposomes, widening the spaces between the bilayers, and resulting in the formation of liposomes with larger internal aqueous volumes and potentially higher entrapment capacity for medications (Betageri & Kulkarni, 1999; Percot et al., 2004; Sriwongsitanont & Ueno, 2004).

Retention of medications entrapped in liposomes is considered to be the most important measurement of their stability. The physico-chemical properties of the entrapped medications and the pH of the aqueous phase within the liposomes (Betageri, 1993) determine

the rate at which they leak out. Some preparation methods depend on a pH gradient or adjustment of the pH of the aqueous phase. Therefore, adjustment and maintenance of a pH balance by using buffers may lead to enhanced formation and stability of the liposomes and increased entrapment and retention of medications within them (Patel & Misra, 1999).

Hydroxyzine and cetirizine are piperazine-class H_1-antihistamines effective in the treatment of urticaria and other allergic skin disorders in which histamine plays a role. After conventional oral administration, adverse effects of the first generation H_1-antihistamine hydroxyzine include central nervous system sedation and impairment of cognitive and psychomotor function, and anticholinergic effects including dry mouth and urinary retention (Simons et al., 1984; Watson et al., 1989).

Cetirizine, the active carboxylic acid metabolite of hydroxyzine, is a potent second-generation H_1-antihistamine that has antiinflammatory properties and high specific affinity for histamine H_1-receptors. It is widely used to treat symptoms of allergic disease in patients of all ages. It is effective in relieving pruritis, whealing, and erythema in urticaria, and it reduces the pruritus of atopic dermatitis. Although it is relatively nonsedating compared to hydroxyzine, dose dependent somnolence may occur after oral administration (Simons et al., 1984, 1995; Watson et al., 1989).

Topical application of first-generation H_1-antihistamines in ointments and creams for treatment of symptoms of allergic skin disorders has been in use for many years; however, considerable systemic absorption may occur, potentially leading to adverse effects (Huston et al., 1990). In a rabbit model, hydroxyzine or cetirizine liposomes, prepared using phosphatidylcholine, when applied to the skin provided enhanced cutaneous antihistaminic effects with reduced systemic absorption (Elzainy et al., 2003, 2004).

Hydroxyzine and cetirizine are similar in chemical structure but differ in polarity (Simons, 2002). Their different ionic forms at different pH values may affect their entrapment and retention within liposomes and consequently the stability of the liposomes (Balen et al., 2001).

We hypothesized that by varying the duration times of hydration of the thin lipid film, freeze-thawing treatments during the formation of MLV, and the use of hydrating buffers at various pH values might affect the extent of hydroxyzine or cetirizine entrapment within the liposomes and/or the long-term stability of these liposome formulations after storage at various temperatures.

The objectives of this research were to study the effect of duration of film hydration time, of freeze-thawing exposure during formation of the liposomes, and of changing the pH of the hydrating buffer on the extent of entrapment of hydroxyzine and cetirizine and on the stability of these liposome formulations (Kommanaboyina & Rhodes, 1999; Manosroi et al., 2004; Matthews, 1999).

MATERIAL AND METHODS

Preparation of the Liposomes

Effect of Duration of Hydration and Freeze-Thawing on the MLV Liposomes Containing Hydroxyzine

Multilamellar vesicles (MLV) of hydroxyzine were prepared using 406 mg egg L-α-phosphatidylcholine 95% (PC) (Avanti Polar Lipids Inc., Alabaster, AL) (PC) and 61.5 mg of cholesterol (CH) (Fisher Scientific Co., Fair Lawn, NJ) and the lipid film hydration method of Bangham et al., (1965). A molar ratio of PC:CH 3.3:1 was found to be optimal, resulting in the highest percent entrapment, and was therefore selected for use in this study (Bawati, 1999). Chloroform solutions, 5 mL volumes, were prepared containing 406 mg of PC and 61.5 mg of CH and designated as the lipid phases. Phosphate buffer, 0.02 M, 100 mL volumes, at pH 7 containing 80 mg hydroxyzine dihydrochloride (Sigma Chemical, St Louis, MO) was used as the aqueous phases. In 250-mL round-bottom flasks, the solvent was removed from the lipid phases at 40°C under vacuum using a rotary evaporation apparatus resulting in thin lipid films formed on the wall of the flasks. The lipid films of PC were hydrated with the aqueous phase by continuous wrist-action shaking (Wrist-Action Shaker: Burrell Corporation, Pittsburgh, PA) for 1 h. The formulations of MLV containing hydroxyzine were evaluated using various hydration times and 5-cycles of freeze-thawing. The liposomes were subjected to 1) hydration for 1 h, 24 h, or 48 h for the control batch, batch B, or batch D respectively; and 2) hydration for 1 h, 24 h, or 48 h with freeze-thawing for 5-cycles for batch A, batch C, or batch E, respectively.

The hydration of the lipid films of PC formed on the walls of the flasks, with the aqueous phase containing 80 mg hydroxyzine, was carried out using wrist-action shaking at varying lengths of time: 1 h, 24 h, or 48 h at ambient temperature (25±3°C). One freeze-thawing cycle of the liposomes was performed by freezing the liposome suspensions for 24 h in a freezer at −20°C. Then the liposome suspensions were thawed at room temperature for approximately 3 h. After thawing, the liposome suspensions were frozen again at −20°C for 24 h. This freeze-thawing cycle was repeated five times.

Effect of Using Buffer of Different pH Values

Small unilamellar vesicles (SUV) were prepared using PC and the ethanol injection method of Batzri and Korn (1973) as modified in our laboratory. Ethanol solutions, 7 mL volumes, prepared containing 406 mg of PC and 61.5 mg of CH were designated as the lipid phases. Exactly 80 mg of hydroxyzine dihydrochloride or 82 mg of cetirizine dihydrochloride (UCB-Pharmaceutical Sector R&D, Braine-L' Alleud, Belgium) were dissolved in 100 mL of a phosphate buffer of 0.2 M K_2HPO_4 at pH values of 5.0, 5.5, 6.0, 6.5, and 7.0 and designated as the aqueous phases. The lipid phases were injected into the rapidly stirred aqueous phases at a rate of 0.5 mL/min using a 22-G needle and a calibrated syringe pump (Pump 22-Ealing Scientific Ltd, St. Laurent, Quebec, Canada).

Multilamellar vesicles (MLV) were prepared using PC and the lipid film hydration method described above. Exactly 80 mg of hydroxyzine or 82 mg of cetirizine were dissolved in 100 mL of a phosphate buffer of 0.2 M K_2HPO_4 at pH values of 5.0, 5.5, 6.0, 6.5, and 7.0 and designated as the aqueous phases to hydrate the lipid films by continuous wrist-action shaking for 1 h.

Stability Studies of Hydroxyzine and Cetirizine Liposomes

To evaluate the stability of hydroxyzine or cetirizine liposomes, all batches were placed in opaque containers, purged with nitrogen to remove atmospheric oxygen, sealed air-tight, and stored in desiccators. For each parameter to be evaluated three liposome batches were prepared (one batch per day). Assessment of the stability of these formulations was not carried out at all temperatures due to the number and volumes of samples available for testing. The stability studies of MLV containing hydroxyzine prepared using various hydration times and 5-cycles of freeze-thawing were conducted every month for up to 24 months at 10±2°C (refrigeration temperature) and 37±0.1°C using an incubator (THELCO® General Purpose Incubator (2DG): Precision Scientific Corporation, Chicago, IL). The stability studies of hydroxyzine or cetirizine liposome batches prepared using buffer of different pH values were conducted every month for up to 24 months or at 12 and 24 months, respectively, at 10±2°C (refrigeration temperature).

Evaluation of Hydroxyzine and Cetirizine Liposomes

The integrity of the liposome formulations both immediately after their preparation and after storage for various periods of time was assessed by: determining the percent entrapment of hydroxyzine (PETH) or the percent entrapment of cetirizine (PEC) and by observing any change in the physical appearance (PA) of the liposome suspensions. Particle size analysis (PSA) of the liposomes freshly prepared at pH = 6.5 was performed using transmission electron micrographs (TEM).

Percent Entrapment of Hydroxyzine and Cetirizine

The liposome suspensions in each batch, immediately after preparation, and after storage for various periods of time at several temperatures, were filtered using an Amicon Ultrafiltration Apparatus (ultrafiltration Apparatus: Amicon, Beverly, MA) and an ultrafiltration membrane with a greater than 100,000 M.W. cut-off. The liposomes were then washed twice with 10 mL of phosphate buffer (0.2 M K_2HPO_4) of the same pH used during the preparation, using rapid stirring under a nitrogen pressure of less than 10 psi. The initial filtrate and the two 10 mL-wash volumes were combined. The amount of hydroxyzine or cetirizine that was not entrapped into the liposomes (F) was determined by measuring the hydroxyzine or cetirizine content in the combined clear filtrate and

wash volumes, obtained from concentrating and washing the liposome formulations using the Amicon Ultrafiltration Apparatus.

Hydroxyzine or cetirizine concentrations were measured using validated HPLC methods developed in our laboratory (Simons et al., 1984, 1995; Watson et al., 1989). Each experiment was repeated three times. The mean percent entrapment of hydroxyzine (PETH) and cetirizine (PEC) were calculated using Eq. 1:

$$\text{Percent Entrapment} = (I - F/I) \times 100 \qquad (1)$$

where I=Initial amount of hydroxyzine or cetirizine added during preparation; F=mean amount (n=3) of hydroxyzine or cetirizine in the filtrate, not entrapped into the liposomes.

The molar ratios of the PC: CH: hydroxyzine per 1 mL of the concentrated final SUV and MLV formulations were 3.5:0.86:1 and 3.2:1:1, respectively. The molar ratios of the PC: CH: cetirizine per 1 mL of each of the concentrated final SUV or MLV formulations were 3.2: 1: 1. These ratios were calculated using the percentage entrapment values of hydroxyzine or cetirizine in the liposomes.

At various times the samples stored at 10±2°C and 37±0.1°C were subjected to additional filtration and washing to assess PETH and PEC.

Validation of the Percent Entrapment of Hydroxyzine and Cetirizine

The method used to determine PETH or PEC was validated. The extent of loss of hydroxyzine or cetirizine by adsorption to the filtration membranes used to concentrate the liposomes was evaluated using separate filtration of aqueous solutions of hydroxyzine or cetirizine at various concentrations. The amount of hydroxyzine or cetirizine in the aqueous solutions was determined before and after filtration, then any loss by adsorption to the filtration membrane was calculated.

Physical Appearance

The visual subjective evaluation of the physical appearance of the liposomes was performed periodically to monitor any discoloration or change in appearance of the liposome suspensions.

Particle Size Analysis by Transmission Electron Micrograph

The particle sizes of the freshly prepared SUV and MLV prepared using buffer pH 6.5 were determined from transmission electron micrographs (TEM). To examine SUV and MLV of hydroxyzine or cetirizine, liposome samples were negatively stained and evaluated using a transmission electron microscope (Hitachi H-7000: Hitachi Scientific Instruments, Tokyo, Japan) (Hayat, 1970). Three fields of view were chosen at random and photographed. The final magnification was calculated to be 112,200 X. From each TEM, the largest diameters of at least 10 liposomes, or the maximum number of liposomes visible in the micrograph, were measured. The mean (±SEM) diameters of the liposomes were calculated.

RESULTS AND DISCUSSION

The method of concentrating liposome suspensions to determine the extent of hydroxyzine or cetirizine entrapment by ultrafiltration was validated. There was no detectable hydroxyzine loss by adsorption onto the filtration membrane. Only 0.6% of the cetirizine content was adsorbed from aqueous solutions onto the filtration membrane, and this loss was included in the calculation of PEC results.

All entrapment results were the mean data of three experiments and were very reproducible, yielding coefficient of variation values of 0.58% to 5.30%. In stability study samples resulting in no quantifiable loss of percent entrapment of hydroxyzine (PETH) over 24 months, intermediate time PETH values that were >±5% of initial PETH values were considered as outliers and were not included in the data analysis.

The SUV and MLV liposome formulations, using phosphate buffer, pH 6.5, resulted in a mean PETH of 86.0%±0.5% and 94.3%±0.4%, respectively.

Effect of Duration of Hydration and Freeze-thawing on MLV Liposomes Containing Hydroxyzine

Some investigators (Percot et al., 2004; Sriwongsitanont & Ueno, 2004) have used freeze-thawing processes to increase the amount of encapsulated

DNA within liposomes (Percot et al., 2004), and to influence the size of the liposomes prepared using PC after using more than 10 freeze-thawing cycles (Sriwongsitanont & Ueno, 2004). However, there are no reported studies to date on the long-term stability of the liposomes after freeze-thawing treatment. In this study, the effect of freeze-thawing processes on the initial and long-term stability of the liposomes prepared using PC was evaluated.

Multilamellar vesicles (MLV) of hydroxyzine were prepared using PC, CH, phosphate buffer, 0.02 M, at pH 7.0, and the lipid film hydration method. The initial PETH was not affected either by an increase in hydration time of the lipid films for up to 48 h, nor by five cycles of freeze-thawing (Fig. 1). Overall, PETH averaged 95%. Neither the length of time of hydration of the lipid film nor the freeze-thawing cycle processes appear to affect the initial PETH.

The extensive entrapment of hydroxyzine into the lipid vesicles at pH 7 was probably due to hydroxyzine existing mainly in non-ionic form in this liposome system (log P = 3.4) (Balen et al., 2001). As the lipophilic non-ionic form of hydroxyzine was entrapped into the liposomes, a shift in the equilibrium between the ionic and non-ionic form of hydroxyzine in the aqueous medium at pH 7.0 yielded more of the non-ionic form of hydroxyzine. This resulted in further entrapment of the non-ionic form from the external aqueous medium into the liposomes.

The long-term stability of the liposomes stored at 10±2°C monitored by PETH was not affected either by the increase in hydration time or by the freeze-thawing cycles. Overall, PETH was similar to the initial values even after 24 months of storage at 10±2°C.

There was no change in the physical appearance of the liposomes in batch B and the control batch. Some slight discoloration was observed in the liposomes in batches A, C, D, and E after storing the liposomes for 4 months but the long-term stability of the liposomes evaluated by monitoring PETH was not affected. The

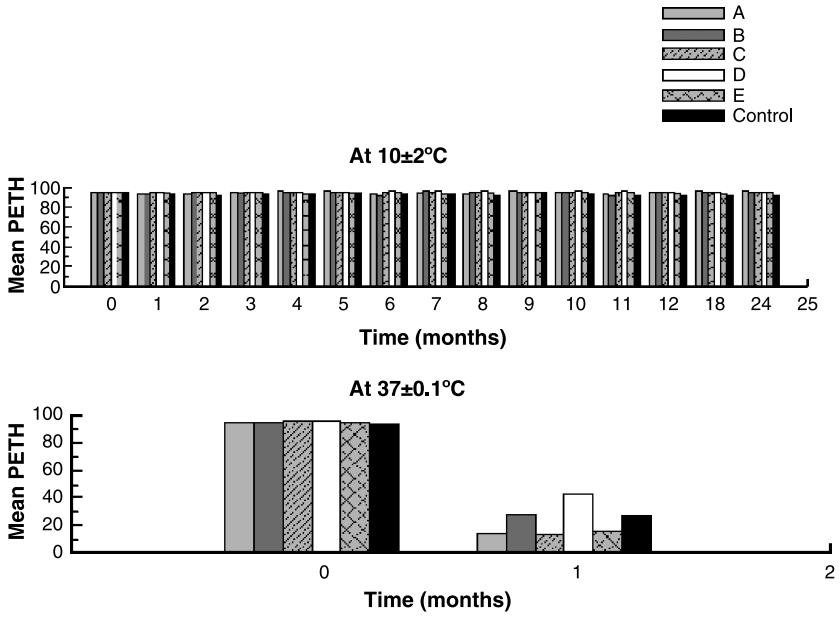

FIGURE 1 **Effect of Duration of Hydration Period and Freeze-Thawing Processes on the Initial and Long Term Stability of PC-MLV Containing Hydroxyzine, pH=7, Stored at 10±2°C and 37±0.1°C, Measuring Percent Entrapment of Hydroxyzine (PETH). n=3, PC-MLV=Multilamellar Vesicles (MLV) Prepared Using L-α-Phosphatidylcholine (PC).**

Conditions of the Experiments:

Hydration time	Freeze-thawing for 5-cycles after hydration	
	No	Yes
1 h	Control Batch	Batch A
24 h	Batch B	Batch C
48 h	Batch D	Batch E

slight discoloration might be due to some oxidation or hydrolysis of the PC since antioxidants were not added to these liposome formulations. There was virtually no difference in PETH, and visual appearance between the liposomes of the control batch and batch B, indicating that the hydration time of the lipid films for 1 h or 24 h without freeze-thawing may yield the most stable formulations. These results are in agreement with those of Patel and Misra (1999) who found that a hydration time of 2 h was optimum for the preparation of clofazimine-loaded MLV liposomes prepared using PC. The percent entrapment of clofazimine was 71±0.05 using a molar ratio of 1:7.85:1.02 of drug:PC:CH. These liposomes were stable for 3 months at 2–8°C, and 25±2°C, but started to lose their drug entrapment after 20 days at 37°C.

Batches of liposomes were also stored at 37±0.1°C to evaluate their long-term stability above ambient temperatures of 25±3°C. The data in Fig. 1 reveals that the PETH of the liposomes stored at 37±0.1°C decreased considerably after just 1 month. Loss of the liposome structure probably occurred, as evidenced by the appearance of brown droplets on the surface of the liposome suspensions in all batches based on visual inspection. This was probably due to extensive hydrolysis and oxidation of the PC, leading the rupture of most of the liposomes after storage at 37±0.1°C for 1 month. Liposome formulations of the control batch, 26.5%, batch B, 27.6%, and batch D, 43.0%, appeared to retain slightly higher PETH than that of batch A, 13.2%, batch C, 12.6%, and batch E, 15.1%. The freeze-thawing of the liposomes after hydration may have caused an increase in the fragility of the liposome bilayer membrane and adversely affected the long-term stability of liposomes prepared using PC and stored at 37±0.1°C. This possible loss of liposome structure and low PETH may be due to the fact that PC changes from a gel state to a fluid state (Betageri & Kulkarni, 1999) at the transition temperature of −15°C (Tc) (Personal communication, Information Center, Avanti Polar Lipids, Inc., Alabaster, AL, USA. Jan. 2000.), which is well below the elevated storage temperature of 37±0.1°C.

Based on these results, the method used to prepare the control batch using lipid film hydration for 1 hour and no freeze-thawing, and storage at 10±2°C may result in the most stable formulations. It was therefore selected to prepare the MLV containing hydroxyzine or cetirizine using buffers at various pH values.

Effect of Using Buffer of Different pH Values

Hydroxyzine and cetirizine were reported (Balen et al., 2001) to have different ionic forms at different pH values that might affect the extent and duration of PETH and PEC and consequently the liposome stability. There are no reported studies to date on the stability of liposomes prepared using different pH. To evaluate the effect of changing the buffer pH on the entrapment of hydroxyzine or cetirizine and the stability of their liposomes, a series of buffer solutions at pH values of 5.0, 5.5, 6.0, 6.5, and 7.0 was used to prepare the liposomes. The pH range was chosen to approximate the reported pH range of human skin (pH 4.5 to 6.5) in order to avoid skin irritation, especially in patients with allergic skin disorders (Huston et al., 1990; Valenta et al., 2001).

In Fig. 2, the initial PETH and PEC of freshly prepared SUV and MLV liposomes are shown. The PETH of SUV markedly increased from 53% to 84% when the pH of the buffer used was increased from 5.0 to 5.5. As pH increased from 5.5 to 7.0, PETH continued to increase from 84% to 94%. The lower entrapment of hydroxyzine at pH 5.0 may be due to the presence of hydroxyzine mainly in the cationic

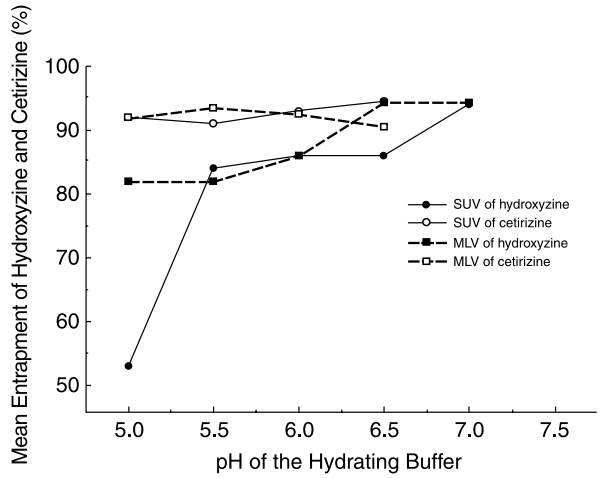

FIGURE 2 **Effect of Changing pH on the Initial Percent Entrapment of Hydroxyzine (PETH) or of Cetirizine (PEC) in SUV and MLV. SUV = Small Unilamellar Vesicles, MLV = Multilamellar Vesicles.**

form, which has lower lipophilicity of log P^{C}_{oct}=0.93, than the non-ionic form with log P^{N}_{oct}=3.5 (Balen et al., 2001). These results indicate that by increasing the pH of the buffer from 5.5 to 7.0, the fraction of hydroxyzine as the non-ionic form may have increased, consequently showing more affinity in the anisotropic liposomes/water systems, and resulting in higher PETH. This explanation is in agreement with the results of Balen et al. (2001), who determined that the values of log D, distribution coefficient of different forms of hydroxyzine, used as indicators of the lipophilicity, were 2.76, 3.13, and 3.46, at the pH range from 3.1 to 4.9, and at pH 7.4, and pH 9, respectively. These values indicate that hydroxyzine demonstrated increased lipophilicity when the pH of the buffer was increased, due to increased conversion from the cationic form to the non-ionic form.

The initial PETH of MLV increased slightly from 82% to 94% as the buffer pH values increased from 5.0 to 7.0. It is clear that the effect of buffer pH has less impact on the extent of entrapment of hydroxyzine in MLV than in SUV. To explain this finding we developed the model shown in Fig. 3, supported by the results of Balen et al. (2001).

In MLV, it is assumed that hydroxyzine gained lipophilicity by increased conversion from the cationic form to the non-ionized form after penetration and retention of the non-ionized molecules into the outer MLV bilayers. When the non-ionic molecules penetrated into the deeper multilamellar bilayers of the MLV, a concentration gradient was established across the liposome membranes that encouraged more penetration of the non-ionic form of hydroxyzine into the deeper bilayers, consequently enhancing the overall entrapment. In contrast, this wide concentration gradient across the liposome membrane did not occur in the SUV liposomes because they are unilamellar. Instead of the concentration gradient, a simple equilibrium is achieved across the single membrane of SUV between the non-ionic molecules of hydroxyzine in the aqueous core of the liposomes and those in the aqueous vehicle surrounding the liposomes, so no further entrapment occurs. This explanation is supported by our finding that only 53.0% PETH occurred at pH 5.0 in SUV. As more of the non-ionized form of hydroxyzine was present when pH increased from 5.5 to 7 the PETH increased ranging from 84.0% to 94.0% respectively, to maintain the equilibrium across the membrane of SUV between the non-ionic molecules of hydroxyzine in the aqueous core of the liposomes and those in the aqueous vehicle surrounding the liposomes.

In contrast to hydroxyzine, for cetirizine there was no effect of pH on PEC in SUV or MLV, which ranged from 92% to 94% as buffer pH values increased from 5.0 to 6.5 as shown in Fig. 2. These findings can be explained by the results of Balen et al. (2001), that cetirizine exists in three forms, cationic (log P^{C}_{oct}=

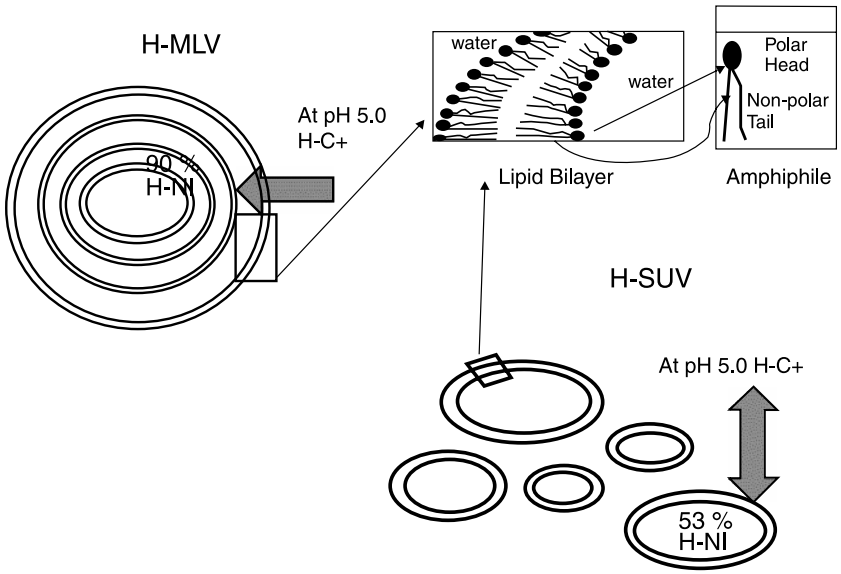

FIGURE 3 **Proposed Model to Explain Hydroxyzine Entrapment in Liposome Structure. H-C^{+}=Cation Form of Hydroxyzine, H−NI=Non-Ionic Form of Hydroxyzine, H-SUV=Small Unilamellar Vesicles Containing Hydroxyzine, H-MLV=Multilamellar Vesicles Containing Hydroxyzine.**

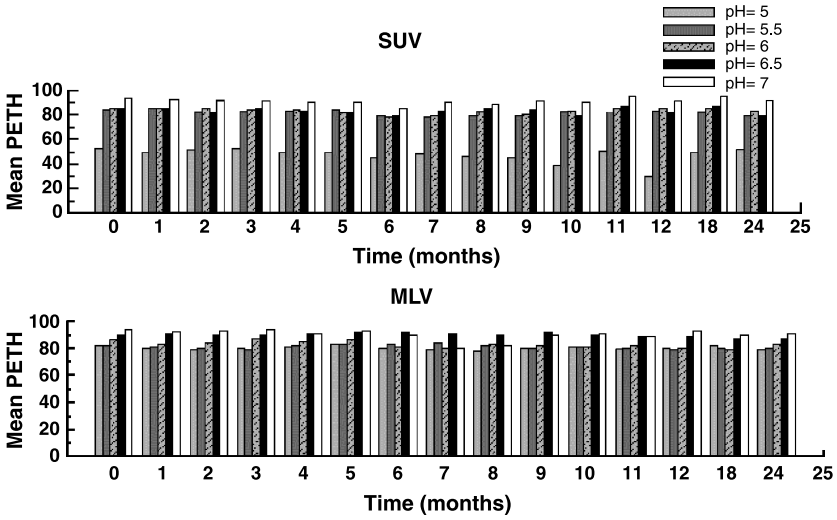

FIGURE 4 **Effect of Changing pH on the Stability of the Percent Entrapment of Hydroxyzine (PETH) in SUV and MLV Stored at 10±2°C. SUV = Small Unilamellar Vesicles, MLV = Multilamellar Vesicles.**

1.12, log P^{C}_{lip}=3.2), zwitterionic (log P^{Z}_{oct}=1.55, log P^{Z}_{lip}=2.3), and anionic (log P^{A}_{oct}=−0.19, log P^{A}_{lip}=2.3). However, all three forms have similar distribution coefficients, log D_{oct}=1.5, log D_{lip}=2.3, at pH values ranging from 3.1 to 9.0. This indicates that over the pH range from 3.1 to 9.0, which includes the pH range of 5.0 to 7.0 in the present studies, all forms of cetirizine passed equally from the isotropic system, n-octanol/water, compared to anisotropic system liposome/water. Also, all forms of cetirizine gained in lipophilicity when passing from the isotropic system to the anisotropic system, creating a driving force that encouraged drug entrapment within the liposomes. From these results, it is clear that the change in pH from 5.0 to 6.5 had minimal effect on cetirizine entrapment within PC liposomes.

The data in Fig. 4 shows the long-term stability of both SUV and MLV of hydroxyzine. There was no change in PETH in SUV and MLV prepared using buffers at pH 5.0, 5.5, 6.0, 6.5, and 7.0 when stored at 10±2°C for up to 24 months compared to initial PETH values. Also, there were no observed visual subjective changes in coloration or in the physical appearance of the liposomes, indicating that when buffers at pH range from 5.5 to 7.0 are used to prepare the liposomes, PETH values of >90% persist for up to 24 months at 10±2°C. These results are supported by those of Balen et al. (2001), who reported that over the pH range from 5.5 to 7.0, hydroxyzine was present in mainly the non-ionic form, which had a higher affinity for liposomes than its cationic form, resulting in the drug retention within the liposomes after entrapment and stable long-term PETH.

The long-term stability data of SUV and MLV of cetirizine is shown in Fig. 5. Following storage of liposomes containing cetirizine at 10±2°C for up to 24 months, PEC of SUV prepared using buffers of pH values ranging from 5.0 to 6.0 decreased minimally and there was no subjective visual change in the physical appearance of the SUV liposomes. The 10% to 20% loss of PEC in MLV was accompanied by slight discoloration, suggesting minor hydrolysis and/or oxidation of the PC.

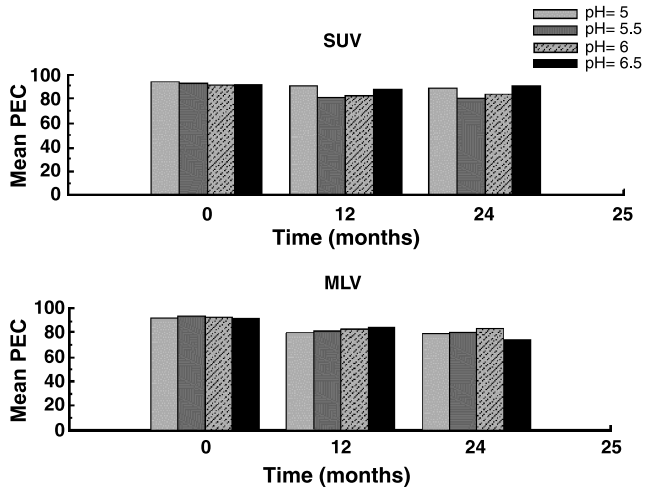

FIGURE 5 **Effect of Changing pH on the Stability of the Percent Entrapment of Cetirizine (PEC) in SUV and MLV Stored at 10±2°C. SUV = Small Unilamellar Vesicles, MLV = Multilamellar Vesicles.**

The use of buffers at different pH values did not affect the initial PEC of SUV and MLV or the stability of SUV, while all MLV formulations lost about 10% to 20% PEC after 24 months at 10±2°C. This could be explained by the fact that MLV are multilamellar and the loss of some outer lamella of the MLV liposomes might result in a reduction of PEC. Also, the decrease in PEC of MLV by storage may be due to the formation and leakage of the zwitterionic form of cetirizine, which is reported (Balen et al., 2001) to have a minimal binding affinity to the liposomes. Cetirizine-containing SUV had long-term stability at pH 5.0 to 6.5, as evidenced by no change in PEC, compared to the lower stability in cetirizine-containing MLV. After 24 months storage at 10±2°C, the maximum PEC retained in SUV and MLV was 90% and 84% at pH 6.5 and 6.0, respectively.

The freshly prepared hydroxyzine and cetirizine liposomes at pH 6.5 were further evaluated after initial formation by determining particle size from TEM (not shown). The freshly prepared SUV and MLV containing hydroxyzine had mean±SEM particle size diameters of 51.7±7.9 nm and 264.0±22.3 nm, respectively Elzainy et al., 2003. The freshly prepared SUV and MLV containing cetirizine had mean±SEM of particle size diameters of 153.5±17.8 nm and 358.3±22.0 nm, respectively (Elzainy et al., 2004). It was therefore concluded that the ethanol injection method resulted in liposomes of the size range of SUV (<100 nm) and the lipid film hydration resulted in liposomes of the size range of MLV (100 nm to 20 μm).

In summary, the use of hydrating buffers at a pH range from 5.0 to 7.0, and from 5.5 to 7.0 had a minimal effect on the initial entrapment of hydroxyzine in MLV and SUV, respectively. Below pH 5.5, the initial entrapment of hydroxyzine in SUV was reduced. Over the pH range of 5 to 6.0, cetirizine initial entrapment was slightly higher than hydroxyzine initial entrapment in both SUV and MLV. The change in the pH of buffer from 5.0 to 6.5 had a minimal effect on the extent of entrapment of cetirizine in both SUV and MLV. In all formulations, independent of pH, PETH was stable at 10±2°C for up to 24 months while PEC of MLV decreased.

From these results, it is proposed that the buffer of pH 6.5 and 7.0 and hydration time for 1 h without freeze-thawing treatment are optimal for initial entrapment of hydroxyzine in SUV and MLV prepared using PC. These conditions of preparation are optimal for long-term stability of SUV and MLV containing hydroxyzine stored at 10±2°C for 24 months. The buffers of pH range from 5.0 to 6.5 and hydration time for 1 h without freeze-thawing processes are optimal for initial entrapment of cetirizine in SUV and MLV prepared using PC. These conditions of preparation are also optimal for long-term stability of SUV containing cetirizine stored at 10±2°C for 24 months.

The liposome formulations containing hydroxyzine or cetirizine may be useful for the topical treatment of the symptoms of allergic skin disorders, while reducing the systemic absorption of these H_1-antihistamines. Using freshly prepared liposome formulations containing hydroxyzine or cetirizine applied to the skin in a rabbit model, the suppression of histamine-induced wheals was superior to that achieved when these two H_1-antihistamines were applied in a conventional o/w cream base, while systemic absorption of the medications was reduced (Elzainy et al., 2003, 2004).

GLOSSARY

CH	Cholesterol
HPLC	High-performance liquid chromatography
MLV	Multilamellar vesicles
O/W	Oil in water
PC	Egg L-α-phosphatidylcholine
PC-MLV	Multilamellar vesicles prepared using egg L-α-phosphatidylcholine
PC-SUV	Small unilamellar vesicles prepared using egg L-α-phosphatidylcholine
PEC	Percent entrapment of cetirizine
PETH	Percent entrapment of hydroxyzine
PL	Phospholipids
SUV	Small unilamellar vesicles
TEM	Transmission electron micrograph

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