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DESIGN AND EVALUATION OF SUSTAINED RELEASED LIPOSOMAL PREPARATIONS CONTAINING ADENOSINE MONOPHOSPHATE

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Adenosine mono-phosphate (AMP) is an effective drug for the treatment of pain, which is scientifically known as post-herpetic neuralgia (PHN). The objective of this study is to achieve desired entrapment of AMP in liposome and sustain the release of AMP. Multi-lamellar vesicles (MLV) were prepared from different phospholipids using freeze-thawing method. The encapsulation efficiency percentage, particle size, size distribution, and in vitro release rate of AMP were investigated. The average particle size was 5.081± 1.72, 4.266± 1.06 and 2.791± 1.03μm for liposomes composed of Ph 90H, DSPC and DMPC respectively. AMP liposomes showed encapsulation efficiency in Ph 90h, DSPC and DMPC MLV of 88.6±2.71, 84.7± 2.37 and 57.4± 0.98 respectively. The % of AMP entrapped in liposomes showed a sustained release pattern over a period of 21 days. This indicates the usefulness of the liposomal delivery system for sustaining the in vitro release of AMP. Amongst the different storage conditions, liposomes stored at 4°C were found to be the most stable and only 1% of the drug was lost over the storage period of 30 days. Formulation of AMP in MLV is useful for sustained delivery of AMP.

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Liposomes are structures consisting of one or more concentric spheres of lipid bilayers separated by aqueous buffer compartments. These spherical structures can be prepared in diameters ranging from 80nm to 100 nm. The lipid vesicles are formed spontaneously when phospholipids are hydrated in aqueous medium [1]. Liposomes are useful in terms of biocompatibility, biodegradability, and low toxicity, and can control biodistribution by changing the size, lipid composition, and physical characteristics [2-4]. Because of their entrapping ability and high degree of biocompatibility, liposomes are suitable for many routes of administration [5-6].

Liposomes are classified as important potential drug delivery vehicles for all types of drugs, such as large and small drug molecules. The therapeutic activity of drugs can be dramatically improved when circulation longevity and disease site accumulation properties of liposomes are increased through liposome encapsulation [7].

AMP is a nucleoside that is found in RNA. It is an ester of phosphoric acid with the nucleoside adenosine. AMP consists of the phosphate group, the pentose sugar ribose, and the nucleobase adenine. AMP may play a role in limiting post-herpetic neuralgia, which is the pain that sometimes lingers after a bout of shingles (herpes zoster). One double-blind study involving 32 adults with shingles found that injections of AMP given three times per week for a month following a flare-up of shingles relieved the pain more quickly than placebo. AMP also helps healing the lesions and prevents recurrence of pain or lesions [8].

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The majority of liposome preparation techniques involve the application of volatile organic solvents (mainly chloroform, ether, or methanol), as a first step, to dissolve or solubilize the lipids. These solvents not only affect the chemical structure of the entrapped substance but will also remain in the final liposome formulation and contribute to toxicity and influence the stability of the vesicles [9].

In the current work, the freezing-thawing method was chosen for preparation of the liposomes because of its simplicity and absence of a need to use organic solvents [10].

The aim of the present study is to design a depot delivery system of MP using multilamelar vesicles (MLVs) of liposomes to overcome the limitations of conventional therapies (where intravenous administration could cause life-threatening arrhythmias of the heart [11]) and improve the patient compliance.

1. Materials and methods

1.1 Materials

AMP was purchased from Sigma-Aldrich GmbH, Germany. Phospholipon 90H® (hydrogenated soy-lecithin) (Ph 90H), Distearyl Phosphatidyl Choline (DSPC) and Dimyristyl Phosphatidyl Choline (DMPC) were purchased from Phospholipid GmbH, Germany. Sodium dihydrogen phosphate monohydrate and Triton® X-100 were obtained from Merck KGaA, Germany. Phosphate buffered saline was purchased from Biochrom AG, Germany. Acetonitrile HPLC grade was purchased from Fisher Scientific, Germany. All chemicals were of analytical grades.

1.2 Preparation of MLV

MLV were prepared by freezing-thawing method [10]. 100 mg of the lipid (Ph 90H, DSPC and DMPC) was added to 2 ml of AMP solution (20 mg/ml) in phosphate buffered saline (PBS). The dispersion was rotated at 10 rpm (Neolab-Reagenzglas-Mischer, Neolab GmbH, Germany) for 30 min and then vortexed intermittently for 30 min (2 min vortex, 3 min interval in a water bath at 68 °C; the temperature above the phase transition temperature of Ph 90H, followed by six freeze-thaw cycles). The freeze-thaw cycles involve the immersion of the MLV in liquid nitrogen for 3 min followed by 6 min of thawing at 68 °C. The freezing process is used to aid the encapsulation of the drug inside the liposomal vesicular structure whereas the thawing process is used to break multilamellar vesicles and to promote the mixing of the enclosed contents with the release medium [12]. Thus the repeated freezing and thawing processes enhance the encapsulation efficiency of the drug. After the freeze-thaw treatment, the liposomal dispersion was left for an hour to allow for the liposomes to be formed and to further encapsulate the drug.

1.3 Separation of the free AMP

The free AMP could be separated from liposomes by centrifugation [13]. Briefly, the liposomes were diluted in 10 ml PBS and centrifuged at 10,000 g for 30 min. After the supernatant was removed, the residual liposomal pellet was resuspended in 10 ml of PBS and centrifuged again as described above. This cycle was repeated three times. The final liposomal pellet was resuspended with 4 ml of PBS for further investigations**.**

1.4. Determination of Encapsulation Efficiency (%)

A sample of 50 µl of the prepared MLV liposomes before removal of unencapsulated AMP was taken and lysed with 0.5% (v/v) Triton X-100 in distilled water (A). After removal of the unencapsulated AMP by washing and centrifugation, 50 µl were taken from the resuspended pellets and treated with triton x-100 as before (B). AMP content was determined in both A and B by HPLC. The encapsulation efficiency (%) was determined by the following equation:

$$\%\,EE = \frac{AD_b}{ADa}\,x100$$

Where, AD_a and AD_b are the amounts of AMP in liposome before and after the process of washing and centrifugation, respectively.

1.5. Characterization of particle shape by optical microscopy

The morphology of MLV liposomes was estimated by a Digital Biological Microscope, equipped with a computer controlled image analysis system (DMBA 450, Motic China Group Co. Ltd.).

1.6. Characterization of particle shape by Transmission Electron Microscope

The morphology of hydrated liposomes was determined using transmission electron microscopy (Jeol JemDos electron microscopy, Japan). The prepared sample was stained with 2% potassium phosphotungstate.

1.7. Particle size measurement by laser diffraction Analyzer

The vesicle size and size distribution of MLV liposomes were determined using laser diffraction analyzer (Beckman-Coulter LS 13 320, Inc.) configured with the universal liquid module. Run conditions were as follows: sample refractive index, 1.50; circulation speed, 50%; measurement time, 1 minute; and carrier fluid, water (refractive index, 1.33).

1.8. In-vitro release of AMP

AMP release was determined in vitro by resuspending the final liposome pellet in an equal volume of buffer (about 2 ml), and rotating 0.5 ml samples in 2.0 ml Eppendorf vials at 10 rpm in an incubator at 37 ºC. Vials were removed at time intervals and centrifugated at 10,000g for 15 min, and the amounts of AMP were determined in pellets. The pellet, about 0.25 ml, was dissolved in 5.0 ml Triton X-100 10% (w/v) and distilled water. The dissolved pellet was analyzed by HPLC.

1.9. Analysis of AMP using HPLC

The amount of AMP was analysed using HPLC. The HPLC system (Merck-Hitachi) was equipped with an HPLC System Manager Chromatography Data Station Software® Model D-7000, a D-7000 interface, a programmable L-7250 autosampler, a pump model L-7100 and a UV variable wavelength detector model L-7420 (MERCK HITACHI, Germany). The chromatographic separation was performed on a LiChrospher® 100 RP-18e column (250 × 4 mm, 5 μm). The mobile phase is composed of phosphate buffer (0.067 M $NaH_2PO_4.H_2O$ with 0.2 % triethylamine) and acetonitril (90:10) with flow rate of 1 ml per minute and measured using UV detector at 254 nm.

1.10. Storage stability studies

The drug retentive capacity of the vesicles was studied by keeping the liposomal dispersion at four different temperatures i.e., 4°±2°C, 25 ± 2°C, 37 ± 2°C/75% ± 5% RH and 45 ± 2°C/75% ± 5% for a period of 30 days in sealed ampoules after flushing with nitrogen. The drug content of vesicles was determined periodically.

2. Results and discussion

2.1. Characterization of particle shape by optical microscopy

The morphology of MVLs at 400× magnification with an optical microscope is shown in Fig. 1. The MLVs were spherical appeared with more than one lamellae.

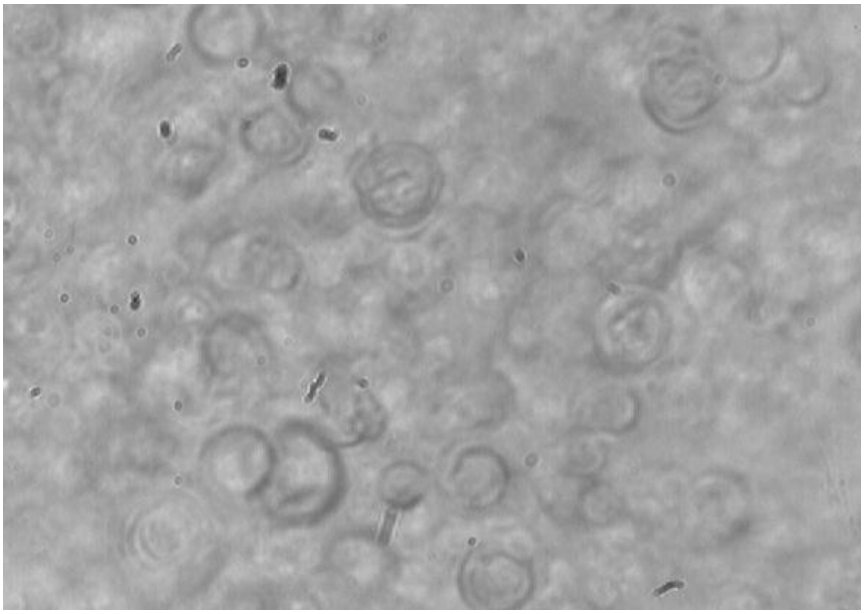

Fig. 1: A morphological picture of AMP MLV at 400×magnification taken by a light microscope, which is equipped with a computer controlled image analysis system.

2.2. Transmission Electron Microscopy

Fig. 2 shows the produced liposomes. As observed, they are well-identified, perfect spheres.

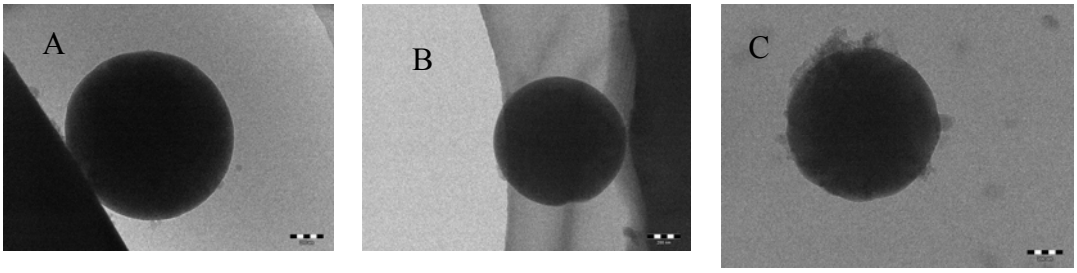

Fig. 2: Transmission electron microphotograph of AMP liposomes stained with 2% potassium phosphortungstate, DSPC (A), Ph 90 (B) and DMPC (C).

2.3. Particle size measurement by laser diffraction Analyzer

Fig. 3 shows the volume weighted size distribution profile of the MLV liposomes prepared using different lipids. All profiles showed a single narrow peak. The mean diameter measured was 5.081 ± 1.72, 4.266 ± 1.06 and 2.791 ± 1.03μm for liposomes composed of Ph 90H, DSPC and DMPC respectively. This could be due to the difference in acyl chain length.

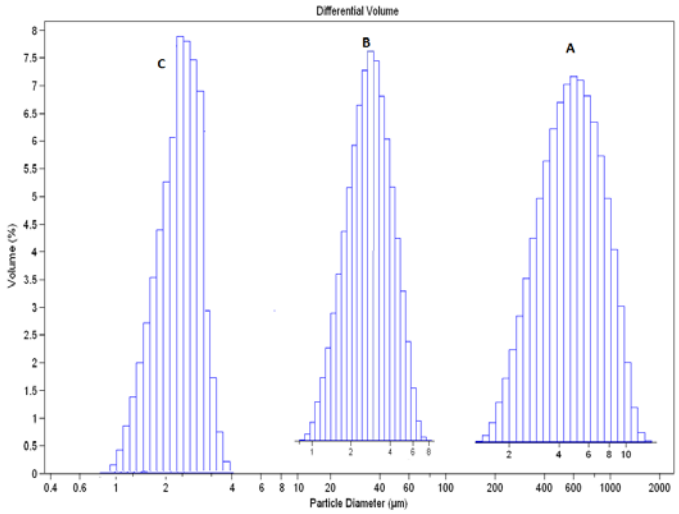

Fig. 3: Particle size distribution of AMP MLV: A for PH 90 H MLV; B for DSPC MLV; C for DMPC MLV.

2.4. Encapsulation efficiency (%)

2.4.1. Effect of lipid type on the % EE of AMP in liposomes

The concentration of both the total amount of AMP before washing and centrifugation and that of the non-encapsulated AMP in the pellet after washing and centrifugation was determined using the standard calibration curve. Consequently, the % of encapsulation efficiency of AMP in all liposomes formulations was easily calculated. The % of encapsulation efficacy (%EE) was obtained according to the above equation and plotted in Fig. 4. It is obvious that % EE of AMP had higher values in cases of liposomes prepared using Ph 90H (88.6±2.71) than DSPC (84.7±2.01) and DMPC (57.4±2.98). The lowest value of % EE was obtained with liposomes prepared from DMPC lipids. This may be due to the difference in acyl chain length and in the phase transition temperature (Tc) where Ph has longer acyl chain and higher Tc (54 °C) than DMPC which has a short acyl chain and a low Tc (23 °C). These findings are in agreement with Michael Anderson and Abdelwahab Omriere, who reported that the greatest encapsulation efficiency was found in DSPC liposomes compared to DPPC and DMPC liposomes [14]. This might also be due to that the longer acyl chain provides more space in the bilayer for partition of AMP molecules [15].

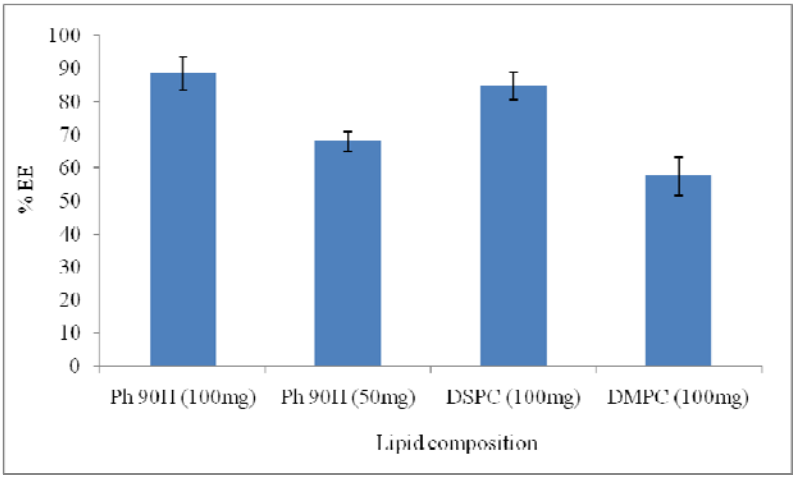

Fig. 4: % EE of Amp in MLV liposomes of different lipid compositions.

2.4.2. Effect of lipid concentration on the % EE of AMP in liposomes

Two concentrations of Ph 90H, 50 and 100 mg /ml were taken and the % EE of AMP in the prepared MLV was determined. It was found that % EE of AMP was increased with increasing Ph 90H content of the liposome (Fig. 4). This might be due to an increase in Ph 90H bilayer thickness as reported by Al-Angry, et al. [16].

2.5. Effect of lipid type on the % AMP retained in liposomes

Fig. 5 illustrates the % AMP retained in liposomes of different lipids in phosphate buffer (PBS, pH 7.4) at 37ºC. This figure shows that there is an initial burst drug release at zero time, then a sustained release pattern over 21days period was exhibited for all MLV formulations. The % of AMP retained in the prepared liposomes follows the order PH 90H > DSPC > DMPC. It may be noted that DMPC liposomes are capable of delivering higher amount of AMP with respect to both PH 90H and DSPC liposomes. This result could be due to a higher bilayer permeability of DMPC liposomes compared with that of both PH 90H and DSPC lipids. The ability of liposomes to retain AMP is correlated with the alkyl chain length of the lipid component. Drug release from liposomes is known to be influenced by the phase transition temperature of the lipid excipients within the bilayer. There are several factors which directly affect the phase transition temperature including alkyl hydrocarbon chain length: as the hydrocarbon chain length is increased, van der Waals interactions between the lipid chains become stronger requiring more energy to disrupt the ordered packing, thus the phase transition temperature increases. Therefore at 37 ◦C DMPC (*T*c = 23◦C) liposomes will be in the fluid state resulting in increased drug loss compared with the higher transition temperature lipids DSPC (*T*c = 55◦C) and PH 90H (*T*c = 58◦C) [17]. The release of AMP might be sustained due to the higher aqueous volume-to-lipid ratio of MLV [18]. Therefore, AMP is mainly encapsulated in the internal aqueous chambers. So the release of drug from MLV becomes sustained. Moreover, the sustained release of encapsulated AMP from MLV may also be attributed to the unique structure of MVL particles. The MVL particles are composed of numerous lamellae containing encapsulated drug. Thus, these several lamellae of lipid membranes will not lead to a total drug release from the internal aqueous content. It also includes surface erosion of exterior vesicles causing release of encapsulated drug to the external medium [19].

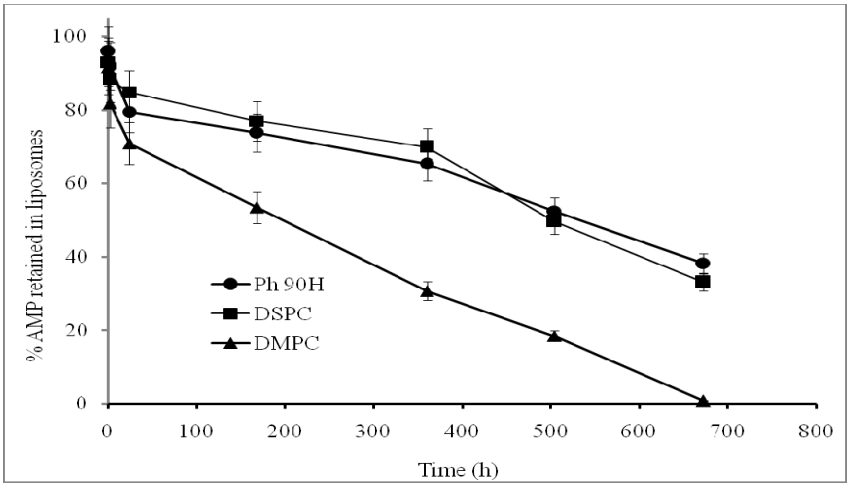

Fig.5: Percentage retained of vesicle-encapsulated AMP in MLV after incubation in PBS at 37 ºC. Each point represents mean ± standard deviation of four independent experiments (bars represent standard error). ■ DSP; ●, Ph 90H; and ▲, DMPC.

2.6. Effect of Ph 90H concentration on AMP release

The in-vitro release of AMP from two liposomal vesicles with different Ph 90H concentrations was exhibited in Fig. 6. From the result, it was concluded that the release of AMP can be influenced by the concentration of Ph 90H in the matrix. The overall tendency observed was a decrease of release rate with increasing lipid concentrations between 50 and100 mg/ml of

PBS. The release rate of AMP from 50 mg/ml Ph 90H is higher than that from 100 mg/ml Ph 90H. This might be due to the increased viscosity and the large vesicle size of the liposomes, which occurred with high concentration of the lipid. These results are in accordance with those obtained by E. Esposito, et al. [20].

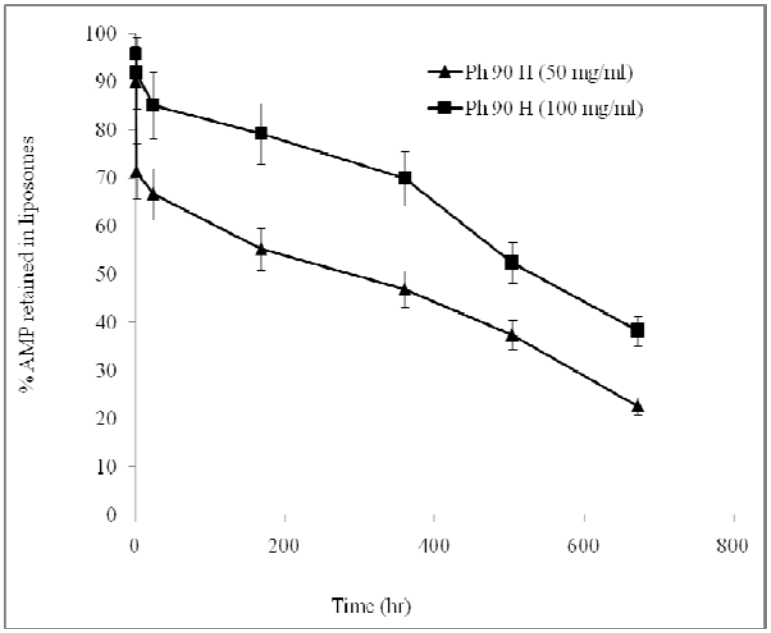

Fig.6: Effect of lipid concentration on the percentage retained of AMP in MLV after incubation in PBS at 37 ºC. Each point represents mean ± standard deviation of four independent experiments (bars represent standard error). ■ *Ph 90H (50 mg/ml) and* ▲*, Ph 90H (100 mg/ml).*

2.7. Stability of AMP in liposomes

Ph 90H MLV was selected as the formulation for stability study, which exhibited higher concentrations of AMP that retained at 3 different temperatures 4°±2°C, 25 ± 2°C/75% ± 5% RH and 40 ± 2°C/75% ± 5% RH for a period of 30 days. Fig. 7 showed the % retained of AMP in Ph 90 H MLV at 3 different temperatures. It was found that more than 99% of the drug was retained after 4 weeks during storage at 4°±2°C. While 82% of the drug was found to be entrapped in the liposomal formulations after two weeks, 58% of the initial drug was present after 30 days of storage at 25 ± 2°C75% ± 5% RH. However, at 40 ± 2°C/75% ± 5% RH, MLV could retain 10% of their initial drug after 30 days. This could be attributed to the fact that higher temperatures increase the propensity of lipids to undergo a transition to a nonbilayer phase. It is known that the oxidative deterioration of the liposome's phospholipid constituents plays a fundamental role in its stability. The main factors that are deleterious to liposome integrity are the presence of aldehydes produced in the per-oxidation process and the splitting of the phosphor-lipid into an acyl chain and the respective lysophospholipid. These processes occur simultaneously during liposome storage [21].

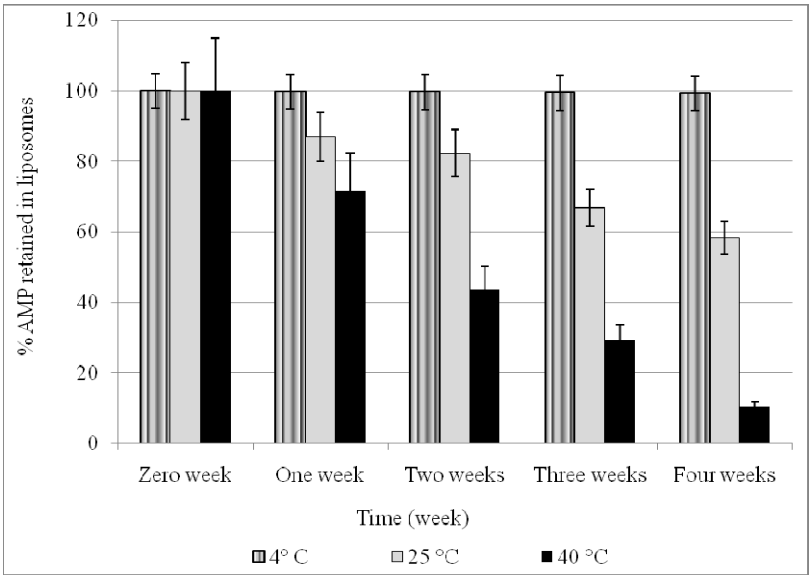

Fig. 7: % AMP retained in MLV liposomes (Ph 90H) at different storage conditions (4, 25 and 40 °C). Data shows mean ± S.D. (n=3).

Also the high stability of liposomes during storage at 4° C might be due to the presence of more than one lamella which prevents the leakage of drug from vesicles. The leakage of drug from the vesicles during storage at elevated temperature might be due to the effect of high temperature on the gel to liquid transition of lipid bilayers together with possible chemical degradation of the phospholipid component [12]. Therefore this study suggests the storage of liposomal formulation in refrigerator in order to prevent loss of drug from the liposomes [22].

3. Conclusions

Incorporation of AMP into MLV liposomes was shown to be influenced by lipid alkyl chain length, and the concentration of the lipid. Retention of AMP was enhanced by employing long alkyl chain length PC (Ph 90H and DSPC) but reduced by incorporation of AMP in short alkyl chain length PC (DMPC). Therefore the current study suggests that using Ph 90H and DSPC lipids would be beneficial for sustained release pattern of AMP.

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References

[1] S. S. Bhalerao, A. Raje Harshal. Drug Dev. Ind. Pharm. **29**(1), 451–467(2003).
[2] S. Anabousi, M. Laue, CM. Lehr, U. Bakowsky, C. Ehrhardt. Eur. J. Pharm. Biopharm. **60,** 295–303(2005).
[3] S. Abraham, D. Waterhouse, L. Mayer, P. Cullis, T. Madden, M. Bally. Methods Enzymol. **391,** 71–97(2005).
[4] S. Vyas, S. Quraishi, S. Gupta, K. Jaganathan. Int. J. Pharm. **29**, 62–25(2005).
[5] R. Fielding. Clin. Pharmacokinet. **21,** 155–164(1991).
[6] S. Siler-Marinkovic, L. Mojovic, V. Davinic, B. Bugarski. Drug Dev. Ind. Pharm. **23**, 483–488(1997).

[7] A. Jennifer, Shabbits, N. Gigi. Chiu, D., Lawrence Mayer. Journal of Controlled Release. **84**, 161–170(2002).
[8] S. Sklar, W. Blue, E. Alexander, et al. JAMA. **253**, 1427-30(1985).
[9] M. Mozafari Reza.Cellular and Molecular Biology Letters, **10**, 711-719(2005).
[10] G. Shazly, T. Nawroth, P. Langguth. Dissolution Technol. **15**, 7-10(2008).
[11] A.Gaby, J. Wright, Nutritional Therapy in Medical Practice. Proceedings from Nutritional Therapy in Medical Practice Conference, Seattle, WA, Oct 25–8(1996).
[12] J. P. Philipus, B. J. Frisken. Biophys. J. **85**, 996–1004(2003).
[13] W. Liang, T. Levchenko and Torchilin. J. Microencap. **21(3)**, 251-261(2004).
[14] M. Anderson and A. Omri. Drug Delivery. **11**, 33-39(2004).
[15] T. Vassilios, M. Spyridon, and G. Sophia. Drug Delivery **14(7)**, 441-445(2007).
[16] A. Al-Angary, M. Bayomi, S. Khidr, M. Al-Meshal, M. Int. J. Pharm **114**, 221–225(1995).
[17] A. Mohammed, N. Weston, A. Coombes, M. Fitzgerald, Y. Perriea. Int. J. Pharm. **285**, 23–34(2004).
[18] M. Langston, M. Ramprasad, T. Kararli, G. Galluppi, N. Katre. J. Contr. Release **89**, 87–99(2003).
[19] M. Ramprasad, A. Amini, T. Kararli, N. Katre. Int. J. Pharm. **26**, 193–103(2003).
[20] E. Esposito, C. Zanella, R. Cortesi, E. Menegatti, C. Int. J. Pharm.**172**, 255–260(1998).
[21] G. Minakshi, D. Tathagataa, K. Narendra. AAPS PharmSciTech. **8(2)**, E1-E8 (2007).
[22] A. Bhatia, R. Kumar, O. Katare. J. Pharm. Pharm. Sci. **7**, 252–259(2004).