CHAPTER 7

Mucoadhesive Liposomal Periodontal Gels

7.1 INTRODUCTION

Till date numbers of methods of preparation of liposome are reported. Pharmaceutically the preformulation study of liposome mainly include drug: lipid ratio, entrapment efficiency and drug retention property (Betagiri et. al., 1993).

Entrapment efficiency is the most important criteria for selection of method of preparation of liposome. An optimum loading procedure would achieve 90% entrapment or more in case of water insoluble and about 40-50% entrapment in case of water soluble drugs, which obviates the need of removal of unentrapped free drug from the formulations as a maximum of 10% of free drug is tolerated. The removal of unentrapped drug by dialysis or by passage through exclusion columns is usually time consuming, tedious, expensive and difficult to recover.

Pharmaceutically many lipid compositions can be employed for liposomal delivery systems. However, the less cost and more stable nature of acidic (negatively charged) lipids such as phosphatidyl choline (PC), and hydrogenated soya phosphatidyl choline (HSPC) are may be employed for liposomal delivery systems.

Considering the drug retention, it is unlikely that most drug loaded liposome formulations can exhibit sufficiently low leakage rates to allow retention times of one year or more. However, sufficiently high (90% or more) entrapment efficiency favors the non-removal of unentrapped drug as no leakage of drug would then occur on extended storage, due to the absence of the transmembrane drug concentration gradients. From a pharmaceutical point of view, high drug: lipid ratios are more economical and the optimum drug: lipid ratio of a liposomal formulation may be dictated by the biological efficacy and toxicity of the preparation.

In summary, optimum liposome formulations would exhibit high drug entrapment efficiency, employed in expensive and relatively saturated lipids such as hydrogenated soya phosphatidyl choline and cholesterol and exhibit the highest possible drug: lipid ratio which is consistent with maintained efficacy of the preparation.

Apart from these factors, other factors which need to be considered in selection of the methods of preparation include selection of methods avoiding use of organic solvents and

detergents (which are difficult to remove), well defined and reproducible liposomal yield with rapid and amenable scale up procedures.

Any special application of the liposome to be prepared also may contribute in the selection of the appropriate method.

7.2 EXPERIMENTAL

7.2.1 Preparation of minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposome

Unilamellar vesicles (ULVs) of minocycline hydrochloride/ clindamycin phosphate were prepared by the thin lipid film hydration technique (New, 1990). Briefly, required amount of soya phosphatidyl choline (soya PC), suitable amount of cholesterol (varying molar ratio of 1:0.2, 1:0.4, 1:0.6) were dissolved in a mixture of chloroform and methanol (ratio 2:1 by volume) in a 250ml round bottom flask. The flask was rotated in the rotary flask evaporator at 120 ± 10 rpm for required time period in a thermostatically controlled water bath at 45^0C under vacuum (600mm of mercury). The thin dry lipid film formed was hydrated using suitable amount of distilled water in which the required quantity of the drug (100mg) was dissolved to meet the required strength and the flask was rotated once again for complete hydration of the prepared thin film liposomes. A flow chart depicting the above process is shown in figure no.7.01. The major process parameters were optimized using the percentage drug entrapment as the response parameter.

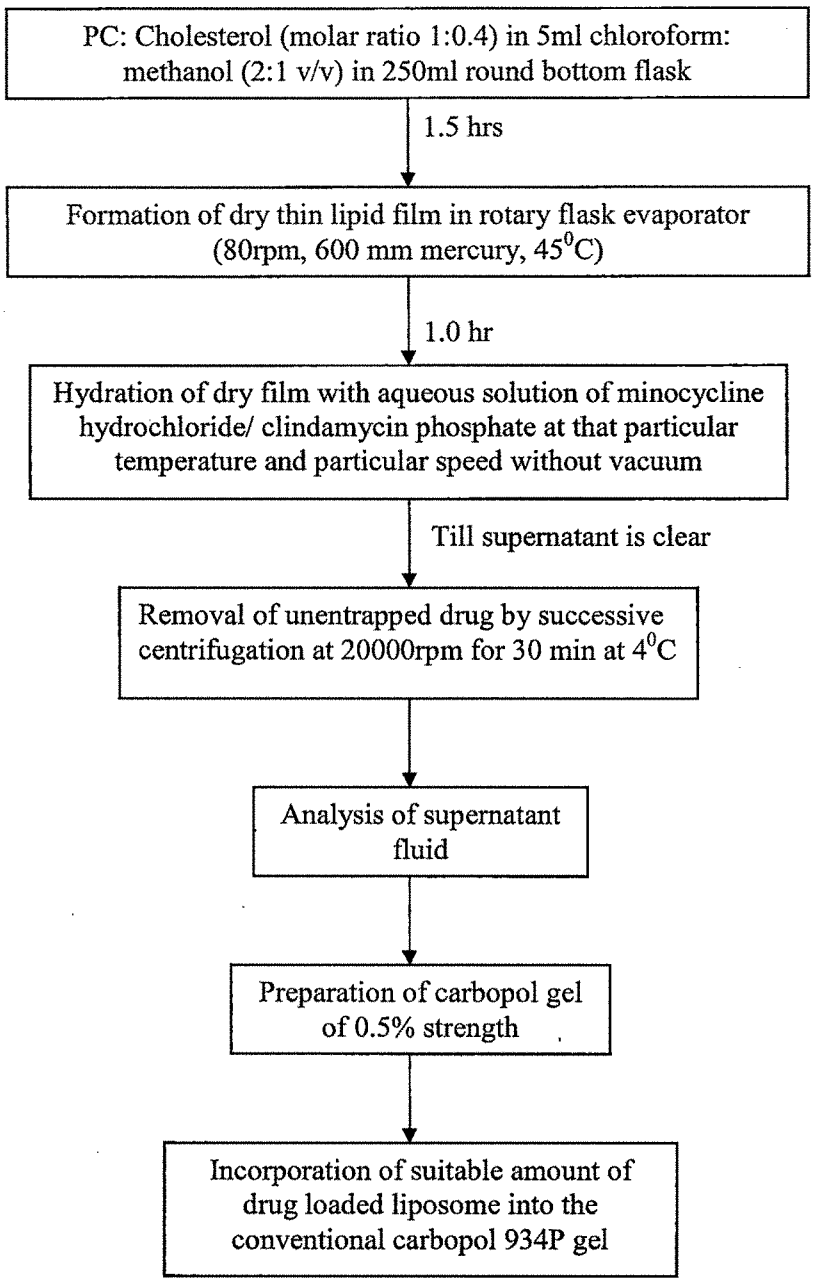
7.3 CHARACTERIZATION OF DRUG LOADED LIPOSOME

Both physical and chemical characteristics of liposomes influence their behavior in vivo and in vitro. There are several examples demonstrating the importance of proper selection of liposome structures to obtain optimum and reproducible therapeutic effects (Section 2.7.6, literature review). Physical and chemical characterizations are very important for a meaningful comparison of different liposome preparations or different batches prepared according to the same protocols. Biological considerations help to ensure safety of use in humans. As a rule, combinations of various characterization methods are used, as none of the existing techniques alone is able to describe liposomes adequately. The various techniques

used in characterization have been extensively discussed previously (Section 2.7.6, literature review). Liposome characterization should be performed immediately after preparation. One should also ensure that no major changes occur on storage so that a well characterized product is injected and the liposome dispersion warrants optimal reproducibility of clinical effects.

The prepared conventional liposome containing minocycline hydrochloride/ clindamycin phosphate were characterized for the following attributes.

Figure 7.01: Flow chart for the preparation of conventional liposomes containing minocycline hydrochloride/ clindamycin phosphate



7.3.1 Morphology and lamellarity

Morphology and lamellarity of the conventional liposomes were ascertained from photomicrographs taken using an Olympus BX40 microscope at a magnification of 2500X. Figure 7.02 and 7.03 shows the photomicrographs of liposomal suspension formulations containing minocycline hydrochloride/ clindamycin phosphate respectively. The Transmission Electron Microscope study (figure no. 7.04 and 7.05) of the same formulations conformed to the unilamellarity of the liposome formulations.

Figure No. 7.02:
Photomicrograph of conventional liposome containing minocycline hydrochloride

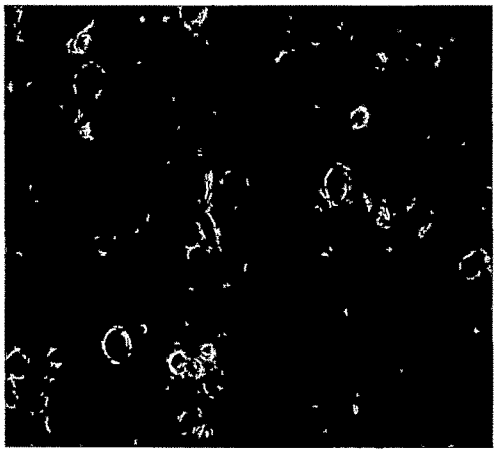


Figure No. 7.03:
Photomicrograph of conventional liposome containing clindamycin phosphate

7.3.2 Particle size

The mean particle size of the prepared liposomes was obtained by using Malvern Zetasizer nanoZS (Malvern Instruments, Zeta sizer, Nano Series, Nano ZS, Model No. ZEN 3600). Liposome suspension was filled in the specified cell and inserted with its integral gold electrodes close to the lid. Readings were recorded in triplicate.

Figure No. 7.04:
TEM of conventional liposome containing minocycline hydrochloride

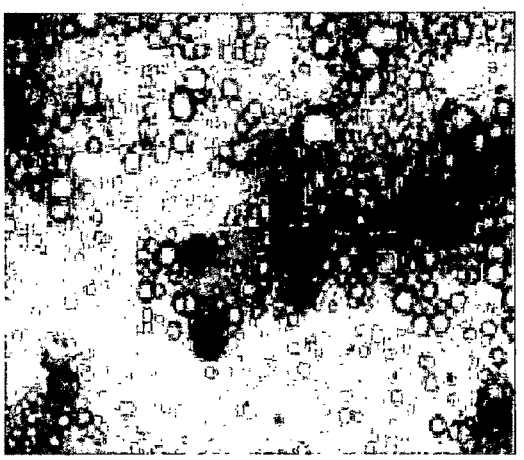
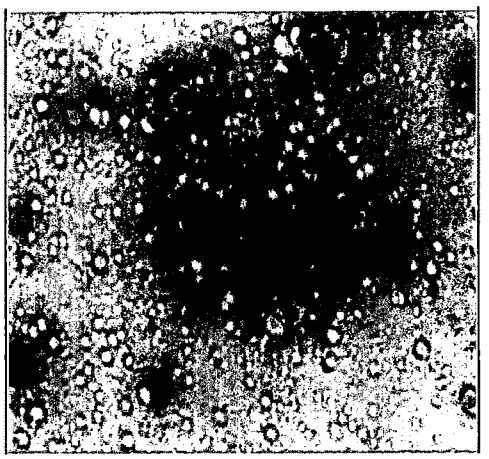


Figure No. 7.05:
TEM of conventional liposome containing clindamycin phosphate



7.3.3 Drug entrapment efficiency

The entrapment efficiency of minocycline hydrochloride/ clindamycin phosphate in the prepared liposomes was studied using the methods as described by New in 1990 for the estimation of the drugs in liposomes.

7.3.4 Preparation of the liposome loaded periodontal gel formulation

Minocycline hydrochloride/ clindamycin phosphate loaded liposome were loaded to the gel formulation. Carbopol 934P was used as the gel forming polymer. 0.5% Carbopol 934P was soaked in the distilled water for 1 hour. After hydration of the polymer, the dispersion was neutralized using 0.5 % w/v sodium hydroxide. To the gel formulation calculated amount of the drug loaded liposomal suspension was added to get 1% w/w liposomal periodontal gel formulation.

7.3.5 Drug content of the liposome loaded periodontal gel formulation

The weighed samples of minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposomes (30 mg) were dissolved in 100 ml phosphate buffer saline pH 6.75 and centrifuged at 20000 rpm at 4°C for 30 min. The supernatant fluid was collected and

analyzed for their quantification at 246nm and 210 nm respectively against blank using spectrophotometer. The noted drug content was the mean of three readings.

7.3.6 Syringeability of drug loaded periodontal liposomal gel formulation

Syringeability of the drug loaded periodontal liposome formulations was measured using the Universal Testing Machine (UTM) at room temperature with a 6mm diameter probe (Model, LF Plus, Lloyd Instruments, U.K) by filling the sample in a syringe. Before filling the syringe, the opening of the syringe was sealed. Samples were filled in a 3.00 ml glass syringe up to 2 ml mark from the back of the syringe and stoppered by the help of forceps. To the rubber stopper the plunger of the syringe was attached. The syringe containing the sample was placed in a holder for holding the syringe. The probe was attached to the load shell of the UTM. The compression test was done with a 150 N weight beam, utilized with a cross head and chart speed of 2.66 mm/min up to a maximum of 40 mm. Force recorded was the mean of three readings.

7.3.7 Evaluation of the mucoadhesive strength of drug loaded periodontal liposomal gel formulation

The mucoadhesive strength of the drug loaded periodontal liposomal gel formulations were determined by measuring the force required to detach the formulation from mucosal tissue using a modified method described by Jones et al (Jones et. al, 2000). Briefly, mucosal tissues were carefully removed from the cheek of the sheep obtained from the local slaughter house. Tissues were immediately used after separation. At the time of experiment, a section of cheek tissue was secured, keeping the mucosal side out, to upper probe using cyanoacrylate adhesive. Upper probe was attached to the precalibrated force transducer (UTM, Model, LF Plus, Lloyd Instruments, UK) connected to data acquisition system. The surface area of each exposed mucosal membrane was kept constant (0.80 cm^2). At room temperature, fixed amount of the sample was uniformly spread on lower probe using double sided adhesive tape, Upper probe was lowered until the tissue contacted the surface of the sample. Immediately, a force of 0.1 N was applied for 2min to ensure the intimate contact between the tissues and the sample. The probe was then moved upwards at a constant speed of 0.1 mm/s and the force in terms of detachment stress in dynes/cm^2, was determined from

the weight required to detach the tissues from the surface of each formulation, which was determined as the peak value in the resultant force versus time plot, using the following equation (Chang et. al., 1985);

Detachment stress (dyne/ cm^2) = m.g/ A

Where, m is the weight added in gram, g is acceleration due to gravity taken as 980cm/sec^2 and A is the area of tissue exposed.

7.3.8 In vitro release of minocycline hydrochloride/ clindamycin phosphate from the conventional periodontal liposomal gel

The in vitro release study of liposomal gel formulations was performed by using sigma dialysis bag (MWCO 3500 and diameter 2.4 cm), which was filled with 500 mg of formulation. The bags were individually immersed in a beaker containing 25 ml of a receiver phosphate buffer solution pH 6.75.The temperature was maintained at 37 ± 1°C and the receptor medium was constantly stirred at 100 rpm to maintain the sink condition. At appropriate time intervals, samples were withdrawn from the receiver solution and an equal volume of pre-warmed buffer was replaced and the samples were assayed spectroscopically after appropriate dilution to quantitate the amount of minocycline hydrochloride/ clindamycin phosphate (246nm and 210nm respectively) release through the membrane. The results were plotted as cumulative amount released (Q) versus time (t).

7.3.9 In vitro permeation studies of minocycline hydrochloride/ clindamycin phosphate loaded conventional periodontal liposome gel

In vitro permeation studies were done as described by many research groups (Ceschel et.al, 2000; Pisal et.al, 2004). Cheek mucosal tissues were prepared from fresh sheep cheek mucosal membrane as described earlier and was fixed onto the Franz Diffusion cell. The 500 mg of gel was spread uniformly on to the mucosa previously fixed in between the donor and the receptor compartment of Franz Diffusion cell. The receptor compartment contained phosphate buffer, pH 6.75. The temperature of the elution medium was thermostatically controlled at 37±1°C by a surrounding water jacket and the medium was stirred with a bar magnet at 500 rpm, using a magnetic stirrer (Kakkar and Gupta, 1992). Aliquots withdrawn

at predetermined intervals over 8hr. were spectroscopically estimated to quantitate the amount of minocycline hydrochloride/ clindamycin phosphate permeated through the membrane. The results were plotted, as cumulative amount permeated (Q) versus time (t).

7.3.10 Data analysis of permeation studies of drug loaded periodontal liposomal gel

The steady state permeation flux was determined from the slope of the linear portion of the cumulative amount permeated (Q) versus time (t) plot. The lag time (t_L) was determined by extrapolating the linear portion of Q versus t curve to the abscissa. The partition coefficient of minocycline hydrochloride/ clindamycin phosphate was calculated as described by the equation (Saket et.al, 1984);

$$\text{Partition coefficient} = \frac{Cs - Ceg}{Ceg} \times \frac{1000}{We}$$

Where, Cs, Ceg and We are the initial concentration of minocycline hydrochloride/ clindamycin phosphate in phosphate buffer solution ($mg.ml^{-1}$), equilibrium concentration ($mg.ml^{-1}$) and weight (mg) of mucous membrane respectively. The dry weight of the mucous membrane was considered for calculating the partition coefficient.

The permeability coefficient (P) was calculated using the relation derived from fick's first law of diffusion (Aslani and Kennedy, 1996);

$$P = \frac{J.h}{C}$$

Where J is the steady state permeation flux, c is the initial concentration; h is the thickness of the mucous membrane.

Diffusion coefficient was calculated using the relation derived from fick's second law of diffusion (Pefile et.al,, 1998);

$$D = \frac{h^2}{6L}$$

Where h is the thickness of the mucous membrane and L is the lag time.

7.3.11 Stability study

The optimized batches were subjected to the stability studies. Formulation was stored in tightly closed vials at room temperature and at 4^0C for six months. The change in percentage entrapment efficiency and particle size was determined after six months. The data noted are the mean of three observations.

7.4 RESULTS AND DISCUSSION

7.4.1 Preparation of minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposome

Periodontal liposomes loaded with minocycline hydrochloride/ clindamycin phosphate were prepared by the thin lipid film hydration technique (New, 1990). Accurately weighed 83mg of soya PC, 17mg of cholesterol (molar ratio 1:0.4) were dissolved in a mixture of chloroform and methanol (ratio 2:1 by volume) in a 250ml round bottom flask. The flask was rotated in the rotary flask evaporator at 120 rpm for 1.0 hr in a thermostatically controlled water bath at 45^0C under vacuum (600mm of mercury). The thin dry lipid film formed was hydrated using 2ml of distilled water (in which the required quantity of the drug (100mg) was dissolved to meet the required strength) and the flask was rotated once again at the same speed as before at that particular temperature for 1hr for complete hydration. The major process parameters were optimized using the percentage drug entrapment as the response parameter. The compositions tried for the preparation of liposome formulations are tabulated in table no.7.01 and 7.02 respectively.

Table no. 7.01: Composition of various conventional liposomes containing minocycline hydrochloride

Batch No.	Molar ratio (PC: Chol: MnHCl)	Hydration volume (ml)	Hydration time (min)
ML1	1:0.2:0.1	1	30
ML2	1:0.2:0.1	1	60
ML3	1:0.2:0.1	1	90
ML4	1:0.2:0.1	2	30
ML5	1:0.2:0.1	2	60
ML6	1:0.2:0.1	2	90
ML7	1:0.2:0.1	3	30
ML8	1:0.2:0.1	3	60
ML9	1:0.2:0.1	3	90
ML10	1:0.4:0.1	1	30
ML11	1:0.4:0.1	1	60
ML12	1:0.4:0.1	1	90
ML13	1:0.4:0.1	2	30
ML14	1:0.4:0.1	2	60
ML15	1:0.4:0.1	2	90
ML16	1:0.4:0.1	3	30
ML17	1:0.4:0.1	3	60
ML18	1:0.4:0.1	3	90
ML19	1:0.6:0.1	1	30
ML20	1:0.6:0.1	1	60
ML21	1:0.6:0.1	1	90
ML22	1:0.6:0.1	2	30
ML23	1:0.6:0.1	2	60
ML24	1:0.6:0.1	2	90
ML25	1:0.6:0.1	3	30
ML26	1:0.6:0.1	3	60
ML27	1:0.6:0.1	3	90

Table no. 7.02: Composition of various conventional liposomes containing clindamycin phosphate

Batch No.	Molar ratio (PC: Chol:CP)	Hydration volume (ml)	Hydration time (min)
CL1	1:0.2:0.1	1	30
CL2	1:0.2:0.1	1	60
CL3	1:0.2:0.1	1	90
CL4	1:0.2:0.1	2	30
CL5	1:0.2:0.1	2	60
CL6	1:0.2:0.1	2	90
CL7	1:0.2:0.1	3	30
CL8	1:0.2:0.1	3	60
CL9	1:0.2:0.1	3	90
CL10	1:0.4:0.1	1	30
CL11	1:0.4:0.1	1	60
CL12	1:0.4:0.1	1	90
CL13	1:0.4:0.1	2	30
CL14	1:0.4:0.1	2	60
CL15	1:0.4:0.1	2	90
CL16	1:0.4:0.1	3	30
CL17	1:0.4:0.1	3	60
CL18	1:0.4:0.1	3	90
CL19	1:0.6:0.1	1	30
CL20	1:0.6:0.1	1	60
CL21	1:0.6:0.1	1	90
CL22	1:0.6:0.1	2	30
CL23	1:0.6:0.1	2	60
CL24	1:0.6:0.1	2	90
CL25	1:0.6:0.1	3	30
CL26	1:0.6:0.1	3	60
CL27	1:0.6:0.1	3	90

7.4.2 Effect of molar ratio of soya phosphatidyl choline: cholesterol: drug on periodontal liposome

Three different molar ratios of soya phosphatidyl choline: cholesterol: drug (1:0.2:0.1, 1:0.4:0.1 and 1:0.6:0.1) was considered. The liposomal suspension was found to be turbid in the case of 1:0.6:0.1 molar ratios and were found to be discrete in case of other two molar ratios. With increase in the concentration of the cholesterol, the reduction of the particle size was observed, which may be resulted due to the slow diffusion or hydration of the external

aqueous phase into the phospholipid membrane. The average diameter of the liposomal vesicles ranged from 126.2 to 539.3nm for minocycline hydrochloride and 119.3 to 501.1 for clindamycin phosphate. The polydispersity index (the measurement of homogeneity of dispersion, ranging from 0.0 (homogeneous) to 1.0 (heterogeneous)) for the size distribution were found to be in the range of 0.591 to 0.137. Zeta potential being the overall charge a particle acquires in a particular medium was measured using zetasizer and was found to be negatively charged ranging within 4.1 to 13.6. Observation under optical microscopy and TEM further conform the morphology and formation of unilamellar spherical flexible vesicles.

7.4.3 Effect of hydration volume on periodontal liposome

Three different hydration volumes were tried for the hydration of the phospholipid films (1.00, 2.00 and 3.00 ml). The liposomal suspension was found to be turbid in the case of 3 ml and was found to be discrete in case of other two hydration volumes. With increase in the hydration volume, the reduction of the particle size and uniformity in the size distribution was observed, which may be resulted due to the proper hydration of the external aqueous phase into the phospholipid membrane.

7.4.4 Effect of hydration time on the periodontal liposome

Three different hydration times were tried for the hydration of the phospholipid films (30, 60 and 90 min). The liposomal suspension showed no significant difference in particle size and entrapment efficiency in case of both 60 and 90 mins. However, the formulations with 90 mins hydration time were found to be turbid and discrete. The results of various liposome formulations with varying concentrations of the molar ratios of soya phosphatityl choline: cholesterol: drug, hydration time and hydration volume are shown in the table no. 7.01 and 7.02. Hence 1:0.4:0.1 molar ratio of soya phosphatityl choline: Cholesterol: drug was selected as the optimum molar ratio with 30 and 60 mins of hydration time for formulation of periodontal liposome formulation.

7.4.5 Entrapment efficiency of periodontal liposomes

For determination of the entrapment efficiency, it was necessary to break the vesicles of the

liposome so that the drug association with the liposome could be determined. For breaking, vesicles were centrifuged at 20,000 rpm and the supernatant was collected and were diluted appropriately in phosphate buffer pH 6.75. Aliquots were spectroscopically estimated to quantitate the amount of minocycline hydrochloride/ clindamycin phosphate at 246nm and 210 nm respectively. Results were tabulated in table no 7.03 and 7.04 respectively.

As it could be seen from the table no 7.03 and 7.04, the drug entrapment was found to be significantly affected by the molar ratio of the soya phosphatityl choline: cholesterol: drug. The increase in drug entrapment with an increase in cholesterol concentration may be attributed to the formation of the thin layer of phospholipid films. However, the further increase in cholesterol concentration resulted to a decrease in the drug entrapment efficiency. Hence the method adopted for the preparation of liposome could be a suitable method for the preparation of minocycline hydrochloride/ clindamycin phosphate with higher encapsulation efficiency.

7.4.6 Morphology and particle size studies of periodontal liposomes

The morphology of the drug loaded periodontal liposomes prepared by thin film hydration method was investigated by optical microscopy. The representative photo micrographs of the minocycline hydrochloride/ clindamycin phosphate loaded liposome are shown in Figure 7.02 and 7.03 respectively, which confirms that the prepared drug loaded liposome were finely spherical and uniform.

From the results of particle size shown in table no 7.03 and 7.04, it can be concluded that the effect of the molar ratio of soya phosphatityl choline: cholesterol: drug on the particle size were found statistically significant ($p< 0.05$). The results showed that the larger liposome was obtained at the lower level of soya phosphatityl choline: cholesterol: drug ratio. The decrease in the particle size observed with decrease in the soya phosphatityl choline: cholesterol: drug ratio could be attributed to the formation of thin film. Hydration of the films also affect on the particle size of the liposomes. Increase in the hydration volume decreases the particle size, which may be due to diffusion of the hydration volume to the thin layer of the phospholipid film, similarly increase in hydration time forms more uniform and smaller vesicles.

Table no. 7.03: Characterization of conventional liposome containing minocycline hydrochloride

Batch No.	Mean particle size ± S.E. (nm)	Drug entrapment (% ± S.E.)*	Zeta potential	Poly-dispersity index (PDI)
ML1	539.3 ± 19.6	17.4 ± 1.59	-13.6	0.591
ML2	487.8 ± 29.4	23.2 ± 1.42	-12.9	0.487
ML3	423.6 ± 13.1	19.6 ± 1.03	-12.4	0.367
ML4	383.2 ± 11.2	18.1 ± 1.93	-11.8	0.532
ML5	374.5 ± 29.3	32.3 ± 1.58	-11.3	0.463
ML6	342.8 ± 12.5	22.5 ± 1.23	-10.8	0.348
ML7	311.8 ± 19.8	18.5 ± 1.11	-10.2	0.323
ML8	289.9 ± 20.7	33.8 ± 0.93	-9.7	0.289
ML9	241.3 ± 11.8	21.6 ± 0.47	-9.3	0.249
ML10	248.6 ± 14.8	27.2 ± 1.29	-8.5	0.237
ML11	147.9 ± 15.1	43.6 ± 0.94	-8.1	0.231
ML12	198.2 ± 13.6	31.9 ± 1.01	-7.6	0.276
ML13	215.3 ± 12.3	28.4 ± 0.79	-7.2	0.189
ML14	147.6 ± 17.78	51.5 ± 1.49	-6.8	0.176
ML15	141.3 ± 19.9	32.4 ± 1.21	-6.4	0.137
ML16	139.4 ± 18.3	29.1 ± 1.03	-6.0	0.212
ML17	132.6 ± 12.6	53.2 ± 1.89	-5.7	0.276
ML18	126.2 ± 13.9	33.5 ± 1.17	-4.6	0.321
ML19	427.1 ± 11.4	20.1 ± 0.91	-5.2	0.329
ML20	394.6 ± 21.3	25.8 ± 0.76	-7.5	0.287
ML21	367.3 ± 18.9	22.3 ± 1.11	-6.8	0.367
ML22	321.8 ± 11.9	21.8 ± 1.37	-6.3	0.382
ML23	319.3 ± 17.2	28.9 ± 1.20	-6.9	0.427
ML24	307.2 ± 17.6	23.2 ± 0.91	-5.8	0.311
ML25	299.4 ± 19.3	23.7 ± 1.36	-5.4	0.356
ML26	295.7 ± 11.5	31.5 ± 1.92	-7.1	0.427
ML27	289.7 ± 19.3	27.7 ± 0.78	-5.2	0.493

Table no. 7.04: Characterization of conventional liposome containing clindamycin phosphate

Batch No.	Mean particle size ± S.E. (nm)	Drug entrapment (% ± S.E.)*	Zeta potential	Poly-dispersity index (PDI)
CL1	501.1 ± 19.6	18.1 ± 1.05	-11.7	0.579
CL2	469.3 ± 29.4	23.9 ± 0.89	-10.8	0.543
CL3	411.4 ± 13.1	20.2 ± 1.81	-10.3	0.529
CL4	347.6 ± 11.2	19.2 ± 1.27	-9.8	0.492
CL5	312.7 ± 29.3	34.9 ± 0.76	-9.5	0.471
CL6	289.1 ± 12.5	24.9 ± 0.76	-9.1	0.447
CL7	261.9 ± 19.8	17.5 ± 1.04	-8.6	0.409
CL8	241.2 ± 20.7	33.1 ± 1.16	-8.4	0.376
CL9	201.5 ± 11.8	24.1 ± 0.93	-7.9	0.358
CL10	178.5 ± 13.6	21.9 ± 1.19	-7.3	0.319
CL11	152.3 ± 15.1	41.8 ± 1.68	-6.5	0.268
CL12	201.6 ± 12.3	27.4 ± 0.66	-6.1	0.248
CL13	267.3 ± 14.8	24.3 ± 1.67	-5.7	0.219
CL14	149.2 ± 11.9	55.7 ± 1.11	-5.3	0.197
CL15	138.2 ± 18.3	31.3 ± 1.17	-4.9	0.191
CL16	127.6 ± 13.3	18.3 ± 1.95	-4.7	0.186
CL17	125.1 ± 27.2	42.1 ± 1.35	-4.4	0.182
CL18	121.3 ± 10.9	26.9 ± 1.83	-4.1	0.179
CL19	309.3 ± 11.1	19.3 ± 0.93	-5.2	0.239
CL20	319.5 ± 17.2	37.2 ± 2.16	-5.8	0.259
CL21	367.5 ± 18.2	22.7 ± 1.78	-6.2	0.293
CL22	389.2 ± 21.2	21.1 ± 1.69	-6.8	0.318
CL23	412.6 ± 19.8	38.6 ± 1.09	-7.1	0.334
CL24	447.9 ± 12.7	27.6 ± 2.30	-7.5	0.347
CL25	473.8 ± 15.3	19.6 ± 0.82	-7.9	0.372
CL26	482.3 ± 22.2	40.8 ± 1.34	-8.1	0.398
CL27	489.1 ± 17.2	23.8 ± 1.67	-8.3	0.428

7.4.7 Determination of Zeta Potential and Poly dispersity Index

The zeta potential of various minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposomes was performed with a Malvern Zetasizer nanoZS apparatus. Phosphate buffer pH 6.75 was used as environment. The results of the investigation for minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposomes are given in table no. 7.03 and 7.04 respectively, which showed that the increase in soya phosphatidyl choline: cholesterol:drug ratio leads to increase in the zeta potential and decrease in the poly-

dispersity index. However, the further increase in hydration time increases the PDI.

7.4.8 Preparation of the liposome loaded periodontal gel formulation

Depending on the percentage drug entrapment efficiency the minocycline hydrochloride/ clindamycin phosphate loaded liposome were loaded to carbopol 934P gel formulation. 0.5% Carbopol 934P was soaked in the distilled water for 1 hour. After complete hydration of the polymer, the dispersion was neutralized using 0.5 % w/v sodium hydroxide. To the above gel formulation calculated amount of the drug loaded liposomal suspension was added to get 1% w/w liposomal periodontal gel formulation. The composition of the drug loaded liposome loaded carbopol 934p gel was given in table no. 7.05.

Table no. 7.05: Composition of various liposome loaded periodontal gel formulations

Composition in % w/w	Formulation Code			
	ML11	ML14	CL11	CL14
Carbopol 934P	0.5	0.5	0.5	0.5
0.5% NaoH	2 ml	2 ml	2 ml	2 ml
MnHCl loaded liposome	4.6mg	3.9mg	-	-
$ClPO_4$ loaded liposome	-	-	4.8mg	3.6mg
Purified water	qs	qs	qs	qs
Drug content	99.56 ± 0.75	97.56 ± 1.25	98.52 ± 0.98	97.85 ± 0.92
pH	6.54	6.61	6.55	6.62

7.4.9 Syringeability of the drug loaded periodontal liposomes

The assessment of the syringeability may be performed in terms of force required to syringe the formulation to the application site. Syringeability of the formulations mainly depends on the particle size of the liposomes. The prepared optimized liposome possess a particle size ranging from 126.2 to 539.3 nm and 121.3 to 501.1 nm respectively for minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposomes, which enabled their easy injection into periodontal pockets. It was found that the mean particle size increased with the increase in the cholesterol concentration. The results of the syringeability shown in table no 7.06 is the mean of three observations.

Table no. 7.06: Determination of syringeability of various minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposomal gel

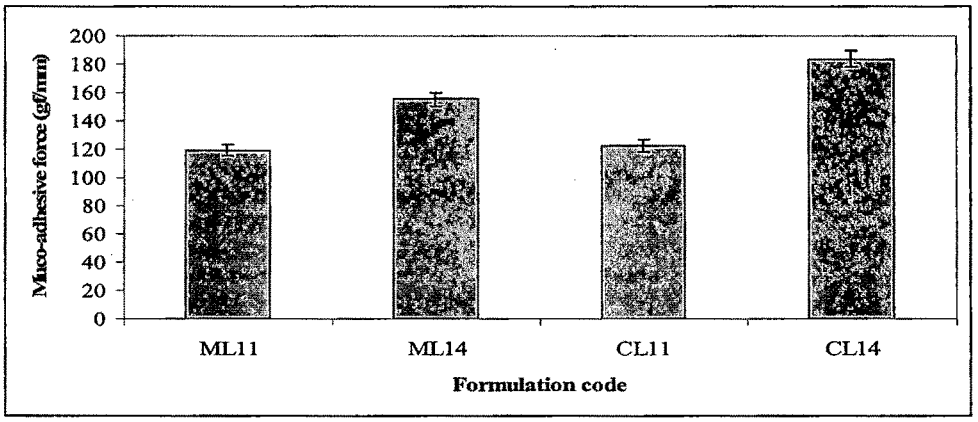
Formulation Code	Syringeability (gf)*
ML11	21.6 ± 0.32
ML14	24.1 ± 0.19
CL11	23.2 ± 0.47
CL14	26.8 ± 0.21

*** n=3**

7.4.10 Mucoadhesive strength of the drug loaded periodontal liposomes

Mucoadhesive studies were carried out to ensure the adhesion of the formulation onto the mucosa for a prolonged period of time at the site of absorption. The model used for mucoadhesive strength measurement was validated by studying effect of initial contact time of the tissues with the formulation. About 2 min was found to be the optimum time to achieve maximum detachment stress. At lower contact time, formulations did not have sufficient time to interact with mucosal membrane where as increase in contact time greater than 2min did not affect mucoadhesive strength significantly. Mucoadhesive studies indicated that (figure no. 7.06) the mucoadhesive strength was found to be significantly affected by the polymer concentration. Increase in the polymer concentration would result in increased migration of polymer to the surface. This would increase the probability of hydroxyl groups for binding with sialic acid residues at the mucosal membrane resulting in increased mucoadhesive strength.

Figure 7.06: Mucoadhesive strength of minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposomes



7.4.11 In vitro release of minocycline hydrochloride/ clindamycin phosphate from the drug loaded periodontal liposome gel

The in vitro release studies indicated that the main factors affecting release of minocycline hydrochloride/ clindamycin phosphate from the liposomal gel formulations are shown in table no 7.07 and 7.08 respectively. There was a decrease in the drug release with an increase in the concentration of the cholesterol was observed. The liposome periodontal gel prepared using 1:0.4:0.1 molar ratio of soya phosphatidyl choline: cholesterol: drug ratio was taken for release study. It was observed that the initial release was resulted to be 0.8 % in case of minocycline hydrochloride and 0.9 % in case of clindamycin phosphate.

The release of minocycline hydrochloride/ clindamycin phosphate has been examined keeping the soya phosphatidyl choline: cholesterol: drug molar ratio constant with varying hydration volume. The release rate increases with increase in the hydration volume which may be due to the proper wetting of the drug within the liposomal formulation. However a burst effect was observed in all the formulations, which also increases with increase in hydration volume and the effect may be attributed to the free drug present on the surface of the liposomes. There is a significant difference ($p < 0.05$) in the drug release rates of the liposomal gel formulations with improved hydration volume. However, change in hydration time showed no such significant change. The reason behind this finding may be the formation of smaller unilamellar vesicles. Figure 7.05 depicts Q versus $t^{1/2}$ plot of liposomal gel formulations with different hydration volume.

As shown in table no. 7.03 and 7.04 the formulations ML11 and CL11 showed very less amount of drug entrapment with a larger particle size. Data from table no. 7.07 and 7.08 also evidenced that the formulations ML11 and CL11 showed very less amount of drug release after 24hrs compared to that of ML14 and CL14. As evidenced from figure no. 7.06 and 7.07 all the four formulations showed linear increase in release of the drug contained with respect to hydration time. However, depending on the release characteristic of the periodontal formulations, due to the nearly similar amount of drug release, the formulations ML11, ML14, CL11 and CL14 are taken for the permeation study.

TABLE NO: 7.07: In Vitro Release Profile of minocycline hydrochloride from conventional periodontal liposome gel

Time in Hour	% Minocycline hydrochloride released ± SD	
	ML11	ML14
0.00	0.00 ± 0.00	0.00 ± 0.00
1.00	4.27 ± 0.021	5.07 ± 0.035
2.00	9.11 ± 0.011	11.71 ± 0.016
4.00	16.07 ± 0.026	18.62 ± 0.021
6.00	21.38 ± 0.029	24.20 ± 0.011
8.00	25.61 ± 0.019	27.63 ± 0.034
12.00	31.43 ± 0.013	33.46 ± 0.021
24.00	43.07 ± 0.024	49.98 ± 0.025

n=3

Figure 7.07: In Vitro Release Profile of minocycline hydrochloride from conventional periodontal liposome gel

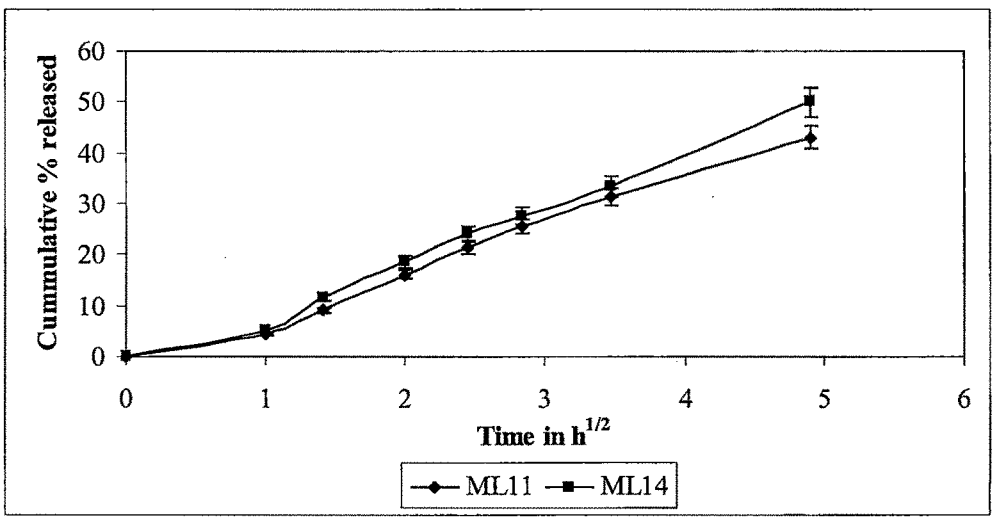
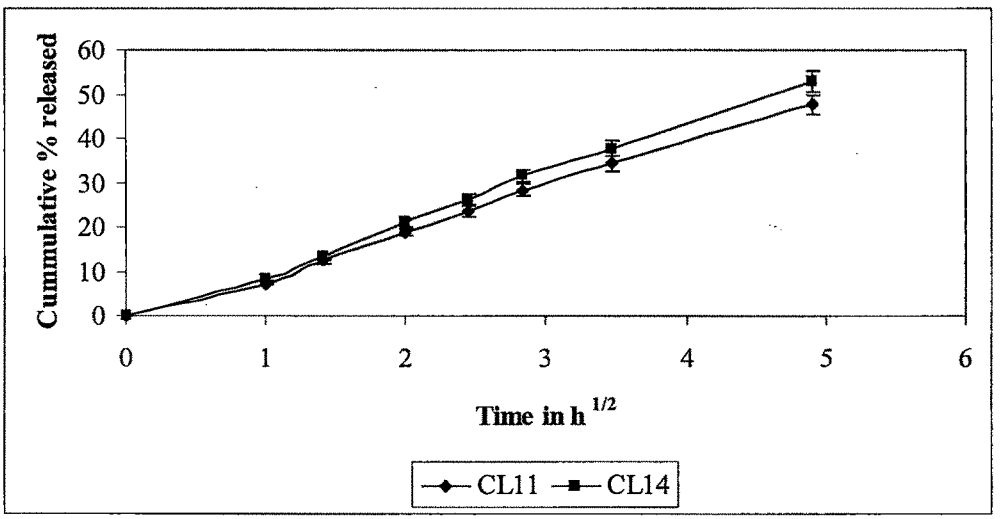


Table No: 7.08: In Vitro Release Profile of clindamycin phosphate from conventional periodontal liposome gel

Time in Hour	% Clindamycin phosphate released ± SD	
	CL11	CL14
0.00	0.00 ± 0.00	0.00 ± 0.00
1.00	7.23 ± 0.031	8.13 ± 0.063
2.00	12.36 ± 0.018	13.30 ± 0.052
4.00	18.75 ± 0.011	21.08 ± 0.027
6.00	23.43 ± 0.021	26.31 ± 0.019
8.00	28.42 ± 0.042	31.72 ± 0.028
12.00	34.35 ± 0.039	37.78 ± 0.017
24.00	47.71 ± 0.027	52.77 ± 0.026

n=3

Figure 7.08: In Vitro Release Profile of clindamycin phosphate from conventional periodontal liposome gel



To examine the kinetics of the drug release and mechanism, the release data were fitted to models representing zero order, first order, Higuchi's square root of time (Sankar and Mishra, 2003) and korsemeyer and peppas model. The coefficient of correlation values (Calculated from the plot of Q vs t for zero order, Log (Qo-Q) vs t for first order and Q vs $t^{1/2}$ for Higuchi model, Log (Q/Qα) vs log t for peppas model where Q is the amount of drug

release at time t, Qα is the amount of drug release at time α and Qo-Q is the amount of drug remaining after time t) as shown in table no 7.09. Thus it can be concluded that the minocycline hydrochloride/ clindamycin phosphate release from the liposomes is best explained by Higuchi model. The mechanism of the drug release is further investigated by the well-known exponential equation, which is often used to describe the drug release behavior from polymeric systems;

$$Mt / M\alpha = kt^n$$

Where Mt/Mα is the fractional drug release at time t; k is a constant incorporating the properties of the macromolecular polymeric systems and the drug and n is a kinetic constant which depends on and is used to characterize the transport mechanism. When n 0.5, this indicates a quasi diffusion mechanism, when n > 0.5, an anomalous non-fickian diffusion is observed, when n = 1 indicates a zero order release (Sankar and Mishra, 2003). This values of n and K was obtained from the plot of Q vs $t^{1/2}$. The values of n obtained for all the batches are less than 0.5, which indicates that the drug release followed quasi fickian diffusion.

From the in vitro release data of minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposome gel it may be concluded that the main factor affecting the drug release from the liposome gel are may be the hydration time and concentration of cholesterol in the liposome formulation.

Table no. 7.09: Release kinetics parameters of minocycline hydrochloride/ clindamycin phosphate loaded conventional periodontal liposome gel

Batch Code	Correlation coefficient				N (Release exponent)	K (Release rate constant)
	Zero order	First order	Higuchi	Peppas		
ML11	0.894	0.0243	0.9857	0.3275	0.9434	0.356
ML14	0.9144	0.0115	0.9905	0.3065	1.0576	0.395
CL11	0.897	0.0167	0.9961	0.3000	1.0088	0.456
CL14	0.8938	0.0074	0.9957	0.3011	1.1161	0.478

7.4.12 Ex vivo permeation study of minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposome gel

7.4.12.1 Determination of saturated drug concentration

A saturated minocycline hydrochloride/ clindamycin phosphate solution in phosphate buffer pH 6.75 was prepared separately by equilibrating the excess minocycline hydrochloride/ clindamycin phosphate with the vehicle for 2 hours. The temperature of the solution was maintained at 25^0C using a circulating water bath. The sample was filtered and appropriately diluted for estimation of saturation solubility of minocycline hydrochloride/ clindamycin phosphate. The saturated concentration of minocycline hydrochloride/ clindamycin phosphate in phosphate buffer pH 6.75 was found to 106.994 mg ml^{-1} and 103.900mg.ml^{-1} respectively.

7.4.12.2 Preparation of mucosal tissue

The animal was sacrificed in the slaughter house and the sheep cheek pouch was excised. It was washed thoroughly with distilled water. The mucosal membrane so separated was cut into pieces of 3×3 cm. A piece of the mucosal membrane was washed with isotonic phosphate buffer pH 6.75 and kept in the phosphate buffer pH 6.75 in order to remove any soluble components. The integrity of the mucosal surface was tested microscopically (Raykar et.al, 1998) before to confirm the absence of any significant change.

7.4.12.3 Measurement of thickness of sheep cheek mucosal membrane

The mucosal thickness of cheek mucous membrane was measured microscopically in the similar manner as given earlier. The average thickness was found to be $1.52 \pm 0.325 \times 10^{-2}$ μm, which is the mean of three measurements.

In vitro permeation studies of various minocycline hydrochloride/ clindamycin phosphate loaded mucoadhesive periodontal liposomes were done as described by Caschel et.al, 2000 and Pisal et. al, 2004. From the results of permeation it is evidenced that all the periodontal liposome gel formulations posses sustain release of drug, which may be due to low lipid permeability of drug.

Cumulative amount of drug permeated as function of inverse of square root of time is given in figure 7.08 and 7.09. Different drug permeation kinetics is presented in table no 7.11. It was evidenced that cumulative amount of drug permeated with time was reduced for all the formulations compared to the pure drug solution. Effective permeability (Permeability coefficient) of minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposomes was found to be within 3.349 to 4.945 which is much more less than that of pure drug.

Table No: 7.10: In Vitro Permeation Profile of minocycline hydrochloride/ clindamycin phosphate from conventional periodontal liposome gel

Time in Hour	% minocycline hydrochloride/ clindamycin phosphate permeated ± SD			
	ML11	ML14	CL11	CL14
0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1.00	1.70 ± 0.015	4.59 ± 0.011	6.63 ± 0.018	7.38 ± 0.021
2.00	6.91 ± 0.011	10.08 ± 0.015	11.43 ± 0.021	12.21 ± 0.009
4.00	12.66 ± 0.026	16.92 ± 0.025	16.42 ± 0.034	19.50 ± 0.011
6.00	16.23 ± 0.031	22.27 ± 0.031	21.61 ± 0.041	24.81 ± 0.028
8.00	20.42 ± 0.017	26.54 ± 0.019	25.48 ± 0.028	28.81 ± 0.021
12.00	26.20 ± 0.039	32.23 ± 0.037	29.48 ± 0.016	34.45 ± 0.018
24.00	33.63 ± 0.023	48.07 ± 0.027	44.15 ± 0.019	50.82 ± 0.023

Figure 7.09: In Vitro Permeation Profile of minocycline hydrochloride from conventional periodontal liposome gel

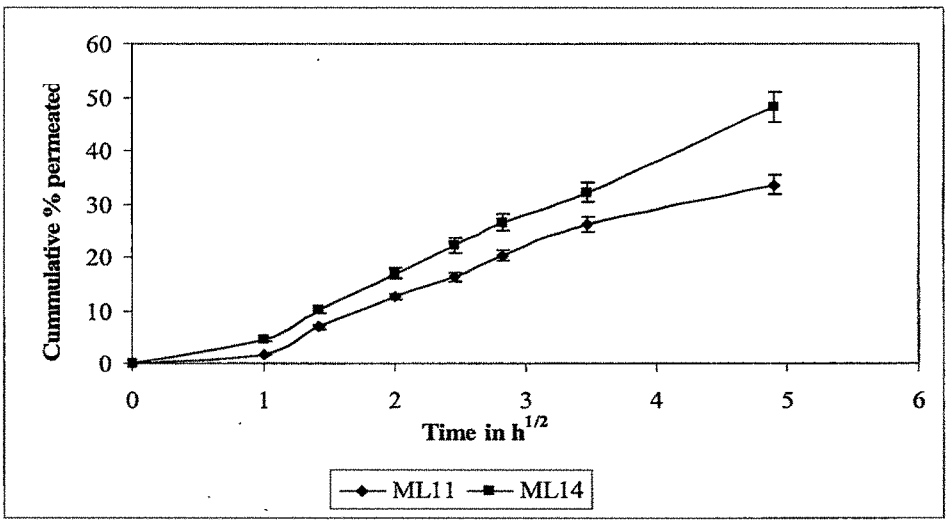


Figure 7.10: In Vitro Permeation Profile of clindamycin phosphate from conventional periodontal liposome gel

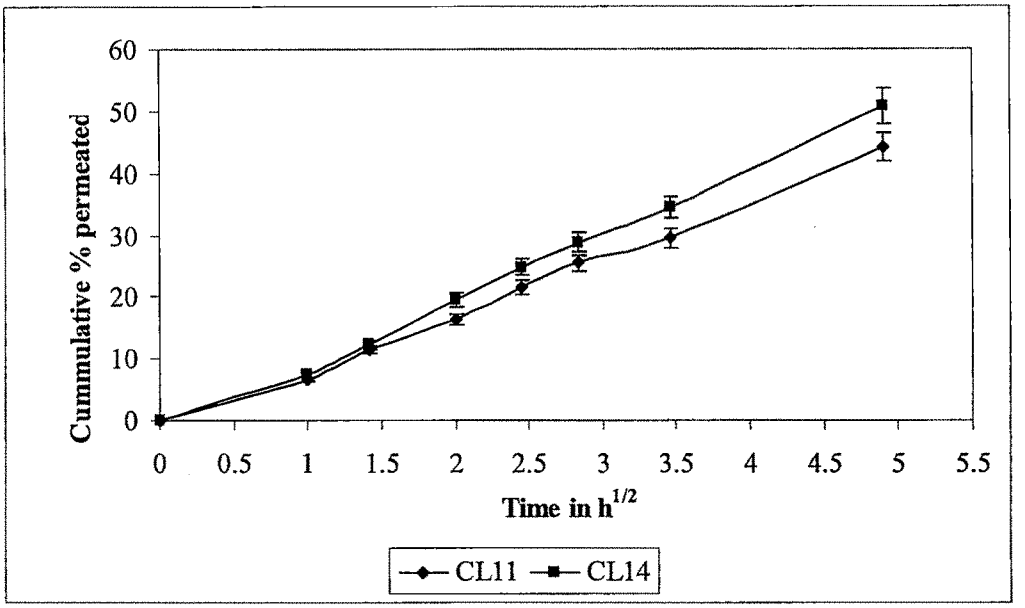


Table no. 7.11: Permeation kinetics parameters of minocycline hydrochloride/ clindamycin phosphate loaded conventional periodontal liposome gel

Formulations	Permeation flux $J(mcg.cm^{-2}.hr^{-1})$	Lag time ($t_L$hr)	Diffusion coefficient ($D\times10^{-8}cm^2.sec^{-1}$)	Permeability coefficient ($P\times10^{-7}cm.sec^{-1}$)
ML11	1.29	0.50	2.13	3.349
ML14	1.75	0.25	4.28	4.543
CL11	1.53	1.00	1.06	4.090
CL14	1.85	0.75	1.43	4.945

7.5 STABILITY STUDY

The results of the short term stability at room temperatures for 3 months were done for the optimized formulations, which indicated that the prepared formulations are highly stable. The changes in the drug content of minocycline hydrochloride/ clindamycin phosphate loaded liposome gels are given in table no.7.12. From the results it is evident that there was no significant change observed in percentage of drug content of the liposome gel formulation on storage at 4°C and at room temperature for 3 months.

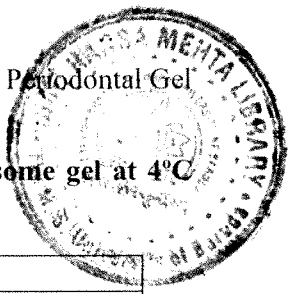


Table no. 7.12: Stability study of various drug loaded periodontal liposome gel at 4°C and at room temperature

Formulation Code	Particle Size Distribution	Drug Content	
		At 4°C	At room temperature
ML11	152.3 ± 13.1	99.56 ± 1.23	98.21 ± 1.36
ML14	150.6 ± 11.3	98.52 ± 1.11	99.17 ± 1.17
CL11	151.6 ± 12.7	97.52 ± 0.98	98.5 ± 1.31
CL14	150.9 ± 14.3	98.67 ± 1.01	99.23 ± 0.97

7.6 CONCLUSION

All the proportions of soya phosphatidyl choline: cholesterol: drug tried showed very less amount of drug entrapped which may be due to the high solubility of minocycline hydrochloride and clindamycin phosphate in distilled water. As evidenced from table no. 7.03 and 7.04, increase in the percentage of drug entrapment was observed with increase in cholesterol concentration. However, a sudden decrease in percentage of drug entrapment was observed with further increase in cholesterol concentration along with increase in hydration time. Hence, the molar ratio of soya phosphatidyl choline: cholesterol: drug was optimized at 1:0.4:0.1 with 1hr of hydration time and 2 ml of hydration volume.

The drug loaded liposomal gel formulations were prepared depending on the amount of drug entrapped (43.6% and 51.5% for minocycline hydrochloride and 41.8% and 55.7% for clindamycin phosphate). The amount of drug loaded liposome formulation to be incorporated into the conventional carbopol 934P (0.5%) gel was calculated to get 1% w/w of the liposome periodontal gel strength and is shown in table no. 7.05.

The close examination of the photomicrographs of the prepared liposomes (Figure 7.02 and 7.03) indicates that the majority of the prepared liposomes were spherical and unilamellar in nature. The TEM photographs also conformed to the unilamellarity of the liposomes.

Table 7.03 and 7.04 shows the mean particle size of the prepared liposome formulations. The entrapped efficiency of all the periodontal liposome formulations was obtained by centrifugation of the systems. The less amount of drug entrapment of the liposome formulations obtained may be due to the purely water soluble nature of both the drugs.

The in vitro release data of minocycline hydrochloride/ clindamycin phosphate loaded periodontal liposomal gel indicated that the increase in the concentration of cholesterol along with increase in hydration volume increases the drug release significantly. However, the decrease in particle size with increase in cholesterol concentration was observed.

An initial burst of release was caused by the minocycline hydrochloride/ clindamycin phosphate at the surface of the periodontal liposomal gel which was followed by a release rate approaching higuchi order. The release rates of minocycline hydrochloride/ clindamycin phosphate after the initial burst occurs within 4.27- 49.98μg $ml^{-1}h^{-1}$ and 7.23-52.77μg $ml^{-1}h^{-1}$ respectively. The permeation kinetics parameters conforms the release of drug following higuchi order of release indicating the similar pattern as that of the release of the formulation.

In vitro permeation data of minocycline hydrochloride and clindamycin phosphate loaded periodontal liposomal gel formulations showed that as the polymer concentration increases, lag time, permeability coefficient and diffusion coefficient decreases, which may be because of the increase of the path of the drug molecule.

Results of the stability study indicated that the periodontal liposomal gel formulations containing the minocycline hydrochloride/ clindamycin phosphate showed stability for three months in room temperature and at 4^0C. Morphology of the liposomal gel formulations did not change in the accelerated storage conditions.

From the above study this can be concluded that the controlled and sustained release of minocycline hydrochloride/ clindamycin phosphate over a period of 24hrs may be achieved by using soya phosphatidyl choline: cholesterol: drug with biocompatibility. Therefore, the minocycline hydrochloride/ clindamycin phosphate loaded mucoadhesive periodontal liposomal gel formulations may be suggested as an effective therapeutic modality for the treatment of periodontitis.

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