CHAPTER - 7

SUMMARY AND CONCLUSION
Liposomes have shown great potential in topical drug delivery. Topical route is often found to be inefficient and unreliable because of the inability of the drug to penetrate through the stratum corneum, and reach and maintain effective concentration in viable epidermis due to rapid penetration of drug reaching systemic circulation. It often results into systemic toxicity if effective concentrations in skin layers are to be achieved through oral route. These shortcomings limit the treatment of a number of dermatological diseases and disorders such as cutaneous tumors, eczema, infections, and psoriasis by oral or topical administration of therapeutic agents. In recent years, the use of liposomes as drug delivery vesicles in topical treatment is rapidly becoming established. Literature reveals that topically applied liposomal products, in comparison to conventional products, exhibit potential in enhancing local effect, eliminating local irritation, reducing systemic effect, optimizing dosage, providing prolonged release action and being cosmetically more acceptable.

Amongst various skin diseases, Herpes is of great concern today because of the advent of AIDS. It is a viral disease caused by *Herpes simplex* (type 1 & 2) and *Varicella Zoster* viruses. *Herpes simplex* type 1 commonly causes herpes labialis and keratitis and *Herpes simplex* type 2 usually causes herpes simplex genitalis. The infection is characterized by the appearance of single or multiple clusters of small vesicles, filled with clear fluid, on slightly raised inflammatory bases on the skin or mucous membranes. *Herpes zoster* caused by the *Varicella-zoster* virus, is an acute CNS infection involving primarily the dorsal root ganglia and characterized by vesicular eruption and neuralgic pain in the cutaneous areas supplied by peripheral sensory nerves arising in the affected root ganglia. Antiviral drugs that have been commonly used to treat these infections are idoxuridine and acyclovir. Acyclovir, a synthetic analogue of guanine, is a drug of choice for the treatment and prophylaxis of infections due to herpes simplex or varicella-zoster viruses. Generally, it is well tolerated but when administered intravenously as its sodium salt, it may cause local reactions at the injection site with inflammation and phlebitis. Other adverse effects following i.v. administration includes increased values of liver enzymes, hematological changes, skin rashes, nausea, vomiting, and headache. Encephalopathic changes including lethargy, confusion, tremors, and seizures have also been reported. Idoxuridine is a synthetic pyrimidine nucleoside used in the topical treatment of *Herpes simplex* keratitis and cutaneous forms of *Herpes simplex* and zoster. It may produce irritation and hypersensitivity reactions when applied to the skin.
administration of idoxuridine may cause bone marrow depression and liver damage. Its topical penetration through skin is reported to be poor. Oral administration of drug causes adverse effects such as nausea, vomiting, diarrhea, headache and skin rashes. It is only 15 to 30% absorbed from the GIT. It requires high frequency of doses that ultimately add to the GIT disturbances. Similarly, Topical absorption of acyclovir is poor and its application to intact skin may produce stinging and burning sensation or erythema.

Idoxuridine and acyclovir are quite effective to treat Herpes simplex but their various systemic and GIT untoward effects compel us to target them to the site of infection i.e. epidermis and ganglia, by improving their topical absorption without any stinging and burning effects and facilitating drug reservoir formation in the skin for prolonged supply of drugs.

Hence, this investigation was undertaken to develop topical liposomal products of ACY and IDU with the objective to overcome limitations of topical therapy using these drugs and provide at least supplement to the oral therapy for faster and complete cure and avoid recurrence of the disease. It was also hypothesized that liposomal encapsulation of these drugs will also eliminate/reduce local side effects of the drugs like irritation, burning, inflammation, in Herpes Simplex (HSV-1 & HSV-2) and micturation due the plain drug in HSV-2.

The drug content and the excipients of liposomes were analyzed by the reported analytical method with suitable modification whenever necessary to meet the requirement of this investigation. The method was standardized for estimation of drugs (ACY & IDU) under study, phosphatidylcholine (PC) and cholesterol (CHOL) contents. Calibration curves of ACY and IDU were prepared by UV-spectrophotometric method. The method was found to be sensitive between 5-35μg/ml and 5-25μg/ml and the λ max used were 250 nm and 287 nm respectively. The ability of phospholipids to form a red colored complex with ferrothiocyanate in organic solutions was used to estimate PC. The method was found to obey beer's law between 10-150μg/3ml concentrations of PC in chloroform. Complexation of CHOL with ferric chloride and sulphuric acid was the basis of the colorimetric method used for estimating CHOL. The method was found to obey beer's law between 5-100 μg/ml in glacial acetic acid. Absorbance of the standard solutions was
measured at the absorbance maxima and plotted graphically to get calibration curve. Regression analysis of the data proved the linearity of plots in the concentration range used. The interference of formulation components were checked and found non-interfering at absorbance maxima of the drugs.

Twenty-seven batches of liposomes of ACY and IDU were prepared by TLE and REV methods using $3^3$ (three variable three levels) factorial design. The three variables selected for TLE and REV methods were Drug/PC/CHOL (in molar ratio), hydration volume, hydration time and Drug/PC/CHOL (in molar ratio), volume of organic phase, and volume of aqueous phase respectively. Other processing variable like vacuum, rotation of flask, sonication time etc was kept same.

After dissolving the weighed quantities of PC, CHOL and α-tocopherol (antioxidant) in chloroform/methanol (2:1) solvent system and evaporating the solvent under vacuum using a rotary flask evaporator. The resulting lipid film was hydrated with 9μmol/ml solution of ACY and 5.4μmol/ml solution of IDU in both TLE and REV methods respectively. The liposomes were separated from free drug by dialysis method using dialysis sack (cut off mol. Wt. 12,000). All the variables were altered according to the experimental design and evaluated for the PDE, size, shape and lamellarity and PC and CHOL contents.

Liposomes of ACY prepared by TLE method gave maximum PDE of 79.5% (0.057) when Drug/PC/CHOL molar ratio, hydration volume, and hydration time was 1:20:10, 6.0 ml and 2hrs and respectively while in case of IDU, maximum PDE of 72% (1.021) was obtained with these were 1:20:10, 4.0 ml, and 2 hrs respectively. Liposomes of ACY and IDU were prepared by REV method gave maximum PDE of 77.2% (0.330) with drug/PC/CHOL (in molar ratio), volume of organic phase and volume of aqueous phase of 1:4:0.5, 6.0 ml and 1.0 ml respectively. Another batch of ACY liposomes prepared by REV method conceded for all studies gave PDE of 71.1% (0.188) when drug/PC/CHOL ratio, organic phase and aqueous phase volume of 1:2:0.5, 6.0 ml and 1.0 ml respectively. The reason of selecting this batch with PDE of 71.1% (0.188) was half amount of PC required as compared to the first batch for obtaining nearly same PDE 77.2% (0.330).
Liposomes of IDU prepared by REV method gave maximum PDE of 83.5%(0.694) when Drug/PC/CHOL molar ratio, organic phase volume and aqueous phase volume were 1:4:1, 6.0 ml and 1.0 ml. Another batch of IDU prepared by REV method gave nearly same PDE of 74.4%(0.347) when Drug/PC ratio, organic phase volume and aqueous phase volume of 1:2:1, 6.0 ml and 1.5 ml. The reason was the same as in the previous case i.e. PC quantity was reduced to half for nearly the same PDE. Thus, TLE method of preparation of ACY/IDU liposomes was rejected for the reason of use of high quantity of PC for preparing liposomes for almost same PDE. A 10-fold reduction in the PC requirement in REV method was the reason for selecting this method for preparing the liposomes for further studies. Hence, potential batches (Batch-2) and (Batch-14) with PDE 71.1%(0.88) and 74.4% (0.37) of ACY and IDU prepared by REV were selected for further studies.

Microscopic examination of the prepared liposomes revealed spherical and multilamellar nature of ACY/IDU liposomes when prepared by TLE and spherical and oligolamellar nature when prepared by REV method. Vesicle size during optimization of method of preparation of ACY/IDU liposomes was determined by observing liposomes of each batch under microscope at 1000X magnification, in terms of geometric mean diameter by plotting logarithm of size of liposomes versus cumulative percent frequency on log-probability graph. Finally, the volume mean diameter of optimized batches were determined Malvern Mastersizer. MLVs of ACY showed a range of dg between 3.2 to 5.4μm while that of MLVs of IDU showed a range of dg between 3.2 to 5.0μm. The volume mean diameters of optimized batches of ACY and IDU MLVs were found to be 4.23 μm and 5.29 μm respectively by Malvern Mastersizer. Similarly REVs of ACY showed a range of dg between 3.2 to 5.0 μm while that of REVs of IDU showed a range of dg between 2.7 to 4.9 μm. The volume mean diameter of optimized batches of ACY and IDU prepared by REV method were found to be between 3.27 μm and 3.47 μm respectively.

Optimized batch of ACY liposomes (Batch 2) was subjected to stability studies for 3 months period and PDR was determined in liposomes at 3 different storage conditions (2-8°C, 25±2°C and 37°C). Vesicle size of samples withdrawn at definite time intervals was also determined. Maximum stability of liposomes in liposomal suspension was observed.
at 2-8°C. An attempt was made to enhance the PDR by increasing CHOL molar ratio keeping the total lipid same. Liposomal surfaces of these modified batches were adsorbed with cross-linked BSA to impart rigidity to liposomal membrane and thereby reducing the permeability of lipid bilayers to improve the PDR at different storage conditions.

In case of Batch-2 (ACY), when composition of Drug/PC/CHOL was changed from 1:2:0.5 to 1:1.2:1.3, PDE reduced from 71.1% (0.163) to 20.6% (0.816). However, stability at 2-8°C in terms of PDR increased from 91.5% (0.617) to 99.0% (0.206), at 25±°C, PDR increased from 49.1% (0.555) to 70.4% (1.630) and at 37°C, PDR increased from 19% (0.879) to 35% (0.897).

In case of Batch-14 (IDU), when composition of Drug/PC/CHOL is changed from 1:2:1 to 1:1.5:1.5, PDE reduced from 74.4% (0.735) to 37.4% (0.735). However, stability at 2-8°C, in terms of PDR increased from 97.5% (0.298) to 98.7% (0.206), at 25±°C, PDR increased from 38.4% (0.973) to 58.5% (1.007) and at 37°C, PDR increased from 25.8 (0.837) to 42.4% (0.761).

Increasing the molar concentration of CHOL decreased PDE reduced from 71.1% (0.163) to 20.6% (0.816) in case of ACY liposomes; while from 74.4% (0.735) to 37.4% (0.735) in case of IDU liposomes. Therefore, adsorption of BSA on liposomal surface followed by cross-linking it with glutaraldehyde technique was used for rigidization of liposomal surface for preventing drug leakage on storage. It was observed that modified batches of ACY (Batch-2) i.e. A, B, C, and D and modified batches of IDU (Batch-14) i.e. E, F, and G; adsorbed with cross-linked BSA, showed a significant improvement (p<0.05) in PDR at 25±2°C and 37°C temperatures; while improvement in PDR was found to be insignificant (p>0.05) at 2-8°C.

Therefore, cross-linked BSA adsorption technique was found to improve PDR maximum upto 1.3 to 2.6 fold. Thus, to avoid decrease in initial drug entrapment due to increase in CHOL molar concentration in order to improve drug retention, cross-linked BSA adsorption technique was found to be better for the selected drugs.

Since, the aim of this investigation was to develop ACY and IDU liposomal gels and their clinical evaluation on human patients; therefore surface adsorbed stable liposomes of ACY and IDU could not be taken further studies because these liposomes need testing on
animal for the toxic effects of glutaraldehyde cross-linked BSA. Thus, the optimized ACY liposomal batch (2) and IDU liposomal batch (14) were taken for further studies.

1%w/w plain and 1%w/w liposomal gels of ACY and IDU were prepared using 2%w/w and 5%w/w HPMC K4M gel bases were prepared by the method of levigation. Plain gels of ACY with 2% and 5%w/w HPMC K4M bases were coded as PAG-1 and PAG-2; while in case of IDU as PIG-1 and PIG-2 respectively. Similarly, liposomal gels were coded as LAG-1 and LAG-2 in case of ACY and LIG-1 and LIG-2 in case of IDU gels. Plain gels of either of drugs contain required amount of plain drug along with the required amount of components (PC, CHOL and α-tocopherol) required to prepare 1% w/w liposomal gels of either drug.

The stability of liposomal gels (LAG-1, LAG-2, LIG-1 and LIG-2) was conducted at three storage conditions (2-8°C, 25±2°C and 37°C). The results were obtained in terms of PDR in liposomes over three months period at different storage conditions. Maximum PDR (>96%) was found in LAG-2 and LIG-2 formulations after the storage for three months at 2-8°C. At higher temperatures (25±2°C and 37°C), a significant decrease (p<0.05) in PDR was observed. However, higher reduction in PDR was observed in LAG-2 and LIG-2 compared to LAG-1 and LIG-1. Thus, LAG-2 and LIG-2 formulations were taken for diffusion studies across HCS.

The in vitro diffusion studies of SSA, PAG-1, PAG-2, LAG-1, and LAG-2 of ACY and SSI, PIG-1, PIG-2, LIG-1 and LIG-2 of IDU were performed using validated self-designed diffusion cell and the results were compared. The diffusion medium was analyzed at specific time intervals for 72 hours for its drug content. The mean flux values of PAG-1 and PAG-2 were compared statistically with mean flux values of LAG-1 and LAG-2 respectively by student ‘t’ test, a significant reduction (p<0.05) was observed in the mean flux values of LAG-2 and LAG-2 gels. A 3.5 fold reduction in mean flux value of LAG-1 compared to PAG-1 and a 2.9 fold reduction in mean flux value of LAG-2 compared to PAG-2 was observed. Liposomal entrapment of the drug may be the reason for this steep reduction in flux values.
Similarly, a 3.5 fold reduction in mean flux value of LIG-1 compared to PIG-1 and a 2.2 fold reduction in mean flux value of LIG-2 compared to PIG-2 was observed. Liposomal entrapment of the drug may be the reason for this steep reduction in flux values. After 72 hours the drug retained on the skin was found to be approximately two fold in case of liposomal gels compared to plain gels. This confirms the role of liposomes in the enhancement of retention of drug in skin layers. On the basis of the diffusion studies LAG-2 and LIG-2 were selected for the clinical evaluation on human patients for the treatment of HSV-1 and HSV-2 infections. For clinical study, products were coded as PAG, PIG for plain ACY and IDU gels and LAG and LIG for liposomal gels respectively.

A comparative double blind clinical evaluation of plain PAG with LAG and PIG with LIG was conducted over a period of eight weeks in New civil Hospital, Govt. Medical College, Surat.

For clinical evaluation of PAG and LAG, 26 patients of HSV-1 and HSV-2 were selected. Out of twenty-six patients selected for clinical evaluation of PAG and LAG, ten patients (six males, four females aging between 21-34 years) were suffering from HSV-1 infection and sixteen patients (all males, aging between 24-40 years) were suffering from HSV-2 infection. Patients suffering from both the diseases were equally divided in two groups (i.e. group of five patients each in case of HSV-1 and group of eight patients in case of HSV-2). However, numbers of female patients were homogeneously distributed in case of HSV-1. One group in both the diseases received topical treatment with PAG and other with LAG. About 0.5 g of gel was applied for five times a day on four or five selected lesions in affected areas after washing with lukewarm water. Measurements of the lesions were taken in millimeters by the resident doctors using metric scale in the New civil Hospital, Department of Dermatology, Surat, (Gujarat) India. Since, all the patients were out-patients, they were explained the benefit of these studies and their written consent was obtained to participate in this investigation. They were directed to apply the test samples as explained. All were advised to visit every week for observations during the treatment. Patients with widespread lesions and serious infections were not considered for this study. All other therapy was stopped one week before starting the clinical studies. But anti-inflammatory tablets (Ibuprofen- 400 mg) three times a day was orally administered during the course of clinical studies.
Similarly, for clinical evaluation of PIG and LIG, 20 patients of HSV-1 and HSV-2 were selected. Out of twenty patients selected for clinical studies of PIG and LIG, ten patients (five males, five females aging between 30-50 years) were suffering from HSV-1 infection and ten patients (all males, aging between 25-36 years) were suffering from HSV-2 infection. Patients suffering from both the diseases were equally divided in two groups (i.e. group of five patients each in case of HSV-1 and HSV-2). However, numbers of female patients were homogeneously distributed in case of HSV-1 infection. One group in both the diseases received topical treatment with PIG and other with LIG. About 0.5 g of gel was applied five times a day on four or five selected lesions in affected areas after washing with lukewarm water. Measurements of the lesions were taken in millimeters by the resident doctors using metric scale in the New civil Hospital, Department of Dermatology, Surat, (Gujarat) India as mentioned in case clinical evaluation of PAG and LAG.

Average of the three of four cross-sectional lengths of all the lesions are summed up and divided by the number of lesions yielded the average reduction in the infected length of the lesions. Effectiveness of the test samples of gels (PAG, LAG, PIG and LIG) was calculated in terms of average percent improvement in the healing of the lesions (APIHL). APIHL was the decrease in the summed up average length of lesions per week. The therapy was continued for eight weeks.

The findings of the clinical studies were significant increase in APIHL of HSV-1 and HSV-2 lesions and significant reduction in the local adverse effects following topical treatment with LAG compared to PAG. PAG showed 80 – 95% improvement in healing of the lesion in patients of HSV-1 & HSV-2 even after the eight weeks treatment; while LAG showed complete healing of lesion after five weeks treatment. This significant improvement in treating the disease with LAG may be due to higher ACY retention and reservoir effect after liposomal encapsulation of ACY. Higher drug retention and reservoir effect of ACY liposome may be due to the components of liposome (phospholipids) which provides increased retention of the ACY in skin layers and thereby higher drug deposition and retention were achieved. This may be the possible reason for better therapeutic efficacy of LAG.
The findings of the clinical studies are significant increase in APIHL of HSV-1 and HSV-2 lesions and significant reduction in the local adverse effects following topical treatment with LIG compared to PIG. PIG showed 25-40% improvement in healing of the lesion in patients of HSV-1 & HSV-2 even after the eight weeks treatment; while LIG showed 45-68% healing of lesion after eight weeks treatment. Significant difference in APIHL was found when results of PIG compared with the LIG, but complete healing of the lesions was not observed in either of the diseases. This difference in the APIHL may be due to the reservoir effect of IDU liposomes in the skin layers and high drug deposition and retention on skin.

The local side effects observed with PAG gel i.e. itching, inflammation, pain and burning for HSV-1 and itching, inflammation, micturation and burning for HSV-2 were significantly decreased with the use of LAG. All the symptoms were completely abolished after three weeks treatment with LAG.

Topical side effects observed with PIG gel i.e. itching, inflammation and burning for HSV-1 and itching, inflammation, micturation and burning for HSV-2 were significantly decreased with the LIG but could not be completely eliminated even after liposomal encapsulation.

CONCLUSIONS

The liposomal gels of antiviral drugs ACY and IDU were developed, characterized and evaluated with the objective to overcome the limitations the topical therapy of herpes simplex using these drugs. It was hypothesized that liposomal encapsulation of drugs will enhance the drug deposition and retention onto skin and hence will improve therapeutic index of the drugs in treatment of herpes simplex. It was also assumed that liposomal encapsulation will either reduce or eliminate reported side effects and will increase patient compliance. Usually the worsening of lesions in initial period of topical therapy is most common reason for noncompliance.

The results of the investigation reveal following significant conclusions:

- Liposomes of the both the drugs under study were prepared using TLE and REV methods. In TLE method, liposomes obtained were spherical and multilamellar. While in REV method liposome obtained were spherical and oligolamellar.
REV method was found to be better of the two methods for preparing liposomes of ACY and IDU for the reason of having nearly same PDE with one tenth of PC in liposomal membrane compared to liposomes prepared by TLE method.

Stability of liposomal suspension of these drugs showed that increase in CHOL molar concentration can improve the PDR but, showed decrease in PDE. Liposome adsorbed with cross-linked BSA can improve the PDR without increasing the CHOL molar concentration in lipid bilayers. Thus avoiding decrease in PDE, PDR can be improved by regidization of liposomal membrane by adsorption of cross-linked BSA.

A comparative *in vitro* diffusion study of plain drug gels and liposomal drug gels showed enhanced drug retention in skin and decrease in mean flux values for liposomal drug gels as compared to the plain drug gels.

A comparative double blind clinical evaluation of plain and liposomal gel of ACY for HSV-1 and HSV-2 patients showed significant improvement in therapeutic response and remarkable decrease in the local side effects of the disease with liposomal drug gels as compared to plain drug gels. Complete remission of lesion was observed in five weeks after topical application of liposomal ACY gel.

However, liposomal gel of IDU could not treat HSV-1 and HSV-2 patients completely even in eight weeks in spite of two fold improvement in AIPHL compare to plain IDU gel. The side effects of IDU also could not be fully eliminated after liposomal encapsulation.

The studies conclusively demonstrated the use of ACY liposomal gel developed in this investigation in treatment of both the types of Herpes Simplex with reduced or no side effects. However, similar extent of improvement in treatment of same disease could not be obtained by liposomal encapsulation of IDU. Concentrated efforts with changed liposomal composition may result into a formulation having enhanced therapeutic index with limited or no side effects. Clinical use of developed liposomal gel formulations of ACY and IDU, although has been demonstrated in this study, but must be confirmed by conducting clinical trials on larger number of subjects.