Chapter 12
Summary and Conclusions

12.1 SUMMARY

12.1.1 INTRODUCTION

Cancer is the second leading cause of death behind heart disease for the past few decades. According to World Health Organization (WHO), every year more than 10 million people are diagnosed with cancer, and more than 6 million die from cancer worldwide. In the next 20 years these numbers will increase by 50%. Due to the high mortality rate, chemotherapy of cancer has been under vigorous investigation. The rapid metastasis to other organs makes cancer more dreadful disease and extends a lot of challenges in providing cure. Currently, the development of techniques which could selectively deliver drug molecules to diseased vasculature, without concurrent increase in its levels in the healthy tissues of the body, is one of the most enthusiastically and exhaustively pursued areas of research in experimental pharmacology and therapeutics.

There are many potential barriers to the effective delivery of a drug in its active form to cancer site. Most small molecule chemotherapeutic agents have a large volume of distribution on i.v. administration. The result of this is often a narrow therapeutic index due to a high level of toxicity in healthy tissues. Through encapsulation of drugs in a macromolecular carrier, such as liposomes, the volume of distribution is significantly reduced and the concentration of drug in the tumour is increased. This results in a decrease in the amount and types of non-specific toxicities and an increase in the amount of drug that can be effectively delivered to the tumour.

Current research is aimed at the development of drug delivery system with maximum therapeutic benefit for safe and effective management of diseases. The encapsulation of pharmacologically active compound in micro vesicles offers attractive possibilities in future therapy. These include the use of such vesicles *in vivo* where an encapsulated drug may be protected from premature degradation, inactivation and excretion. Liposomes are one of the preferred drug delivery systems due to its composition from the natural biological lipids and structural resemblance to cell membranes. The major advantages of liposomes are biodegradability, low toxicity, low immunogenicity and ability to trap both hydrophilic and hydrophobic drug

molecules. The utility of liposomes for delivering therapeutic agents has been severely limited due to a rapid removal by the phagocytic cells of mononuclear phagocyte system (MPS) particularly in liver and spleen, thereby provides low circulation half-life in the bloodstream.

Development of pegylated or sterically stabilized liposomes has been proved to overcome the above disadvantage. The coating of liposomes with polymers like polyethylene glycol (PEG) renders steric stability to the liposomes by providing a hydrophilic surface, which is thought to limit binding of serum opsonins as well as direct interactions with cells, most importantly, of the reticuloendothelial system. This results in enhanced circulation times and increased localization in the tumour. It has been suggested that sterically stabilized liposome formulations, which have the ability to bind to target ligands in situ, have potential applications for drug targeting to tumour tissues.

The aim of the present study was to develop targeted delivery systems for cancer therapy by exploring the potential of sterically stabilized liposomes for the delivery of anticancer drugs. The increase in blood circulation time of the sterically stabilized liposomes due to the avoidance of MPS uptake provides enhanced accumulation of the liposomes at the target (tumour) site due to leaky vasculature of the tumour (enhanced permeation and retention (EPR) effect). Thus anticancer drugs entrapped in sterically stabilized liposomes would, when injected into the body, be released from the liposomes in a sustained manner along with increasing the drug accumulation in tumour. This combination of sustained and targeted delivery not only would minimize interactions of these drugs with other tissues of the body, thereby reducing side effects commonly associated with this type of therapy, but also would lead to significant dose reduction. Thus, overall increase in therapeutic efficacy would be achieved.

The present investigation involves the preparation, characterization and *in vivo* evaluation of conventional and sterically stabilized liposomes containing three anticancer drugs namely Methotrexate (MTX), Tamoxifen citrate (TMX) and Cytarabine (CYT).

12.1.2 PREPARATION OF CONVENTIONAL AND STERICALLY STABILIZED LIPOSOMES CONTAINING METHOTREXATE, TAMOXIFEN CITRATE AND CYTARABINE

Conventional liposomes of MTX, TMX and CYT were prepared by the lipid film hydration method. Multilamellar vesicles (MLVs) of MTX were prepared using PC: Chol: MTX (molar ratio 1: 0.4: 0.1) and DL- αtocopherol (0.5 ml of 0.1 % w/v solution in chloroform). All the lipids and the drug, MTX were dissolved in a mixture of chloroform and methanol (ratio 2:1 by volume) in a round bottom flask. The flask was rotated in the rotary flash evaporator at 100 rpm for 20 min in a thermostatically controlled water bath at 37°C under vacuum (600mm of mercury) in order to remove the traces of organic solvents. The thin dry lipid film formed was hydrated using distilled water at room temperature for 30 min to produce liposomal suspension. The liposomal suspension so formed was then transferred to a suitable glass container and sonicated for 30 min using a probe sonicator in an ice bath for heat dissipation. The sonicated dispersion was then allowed to stand undisturbed for about 2 h at room temperature for annealing. The multi lamellar vesicles formed were sequentially extruded through 2 stacked Nuclepore polycarbonate track-etch membrane filters of 1 μm and 0.2 μm pore size for 5 times at 40°C. The unentrapped drug was removed by centrifugation and the pellet was resuspended in distilled water. The liposomal suspension was freeze dried along with sucrose which was used as cryoprotectant (3 times by weight of total lipids) overnight at -20°C, lyophilized for 24 h and stored in vials at 2 - 8°C. The lyophilized powder was rehydrated with distilled water before use.

Liposomes of TMX were prepared in the similar manner. The CYT liposomes were also prepared in the same manner with the difference that the drug was introduced into the system as a part of the hydration medium and not included initially in the solution of lipids used for drying down.

Phosphatidyl choline was estimated in the liposomes using Stewart assay in conjunction with a modified version of the Bligh-Dyer two phase extraction method. Cholesterol was estimated using the Zlatkis, Zak and Boyle method involving color development with ferric chloride and concentrated sulphuric acid. The modified Bligh-Dyer two-phase extraction method was used for

extracting cholesterol from the liposomes. MTX and TMX were determined using reported ultraviolet spectrophotometric methods. CYT was estimated using a developed, ultraviolet spectrophotometric method. All the methods were subjected to statistical analysis such as regression analysis, correlation coefficient etc. Important parameters for method validation such as accuracy, precision, selectivity and stability were also investigated and found satisfactory.

The proportion of the system components i.e. PC, Chol and the drug as well as the process parameters such as the volume of the solvent system, volume of hydration medium, hydration time, vacuum applied, speed of the rotary flash evaporator, sonication time were optimized based on the percent drug entrapment. It was observed that a molar ratio of PC: Chol: MTX: 1: 0.4: 0.1 and PC: Chol: TMX: 1: 0.67: 0.067 gave excellent liposomal dispersions.

In case of CYT containing liposomes, the process parameters like volume for solvent system, hydration time, rotation speed, temperature, sonication time and vacuum applied did not play significant role in influencing drug entrapment. But the other factors like Drug / Lipid [PC and Chol] ratio, PC / Chol ratio and the volume of hydration medium were found to affect the drug entrapment. So, a 3^3 factorial design was used to investigate the combined influence of three variables in the preparation of Cytarabine liposomes by thin film hydration method. The process variables like vacuum applied and hydration time were kept constant through out the study. Based on the factorial design, twenty seven batches of cytarabine liposomes were prepared. The liposome batches were evaluated for its percent drug entrapment (PDE) within the liposomal vesicles. By using the factorial design we could achieve a maximum drug entrapment of 83.5 % with the molar ratio of PC: Chol: CYT:: 1: 0.625: 0.125 and could predict the PDE for various combinations of the formulation variables using the contour plots and response surface plots.

Sterically stabilized liposomes of MTX, TMX and CYT were then prepared by suitably modifying the conventional liposomes prepared above. The modifications were done by introduction of appropriate sterically stabilizing agents into the liposomal bilayers. The agents investigated were

methoxypolyethylene glycol (mol wt. 5000) activated with cyanuric chloride coupled with phosphatidyl ethanolamine (mPEG5000-CC-PE), methoxypolyethylene glycol (mol wt.2000) activated with cyanuric chloride coupled with phosphatidyl ethanolamine (mPEG2000-CC-PE) and methoxypolyethylene glycol (mol wt.5000) activated with carbonyl diimidazole coupled with phosphatidyl ethanolamine (mPEG5000-IC-PE). In the synthesis of mPEG-CC-PE, first the methoxypolyethylene glycols (mPEG-5000 & mPEG-2000) were activated using cyanuric chloride (2,4,6-trichloro-s-triazine) in presence of potassium carbonate as per the previously reported method, which was modified suitably. The second step involved the conjugation of the activated mPEG with phosphotidyl ethanolamine in presence of triethylamine based on the earlier reported procedure. mPEG5000-IC-PE was synthesized by a previously reported method. The course of the reaction was monitored by thin layer chromatography. In both the steps, the products were characterized by infrared spectroscopy, nuclear magnetic resonance spectroscopy and by ultra violet spectroscopy.

The amount of the agents required for steric stabilization was optimized using the electrolyte induced flocculation test. Here, a fixed quantity of the liposomal suspension was diluted to a predetermined volume using different molar concentrations of an electrolyte, sodium sulphate. The absorbance of the resulting suspensions was measured spectrophotometrically. The concentration of the steric stabilizing agent, which did not allow any changes in the absorbance when different molar concentrations of the electrolyte were added to the liposomes was selected as the optimum concentration and used for further studies. 5mol% of the total lipids of both mPEG5000-CC-PE and mPEG5000-IC-PE and 7mol% of mPEG2000-CC-PE were found to be adequate for steric stabilization of the liposomes.

The presence of these agents led to significant differences in the entrapment of MTX and TMX as compared to the conventional liposomes. The entrapment of CYT was not affected much by the presence of these steric stabilizing agents. The significant reduction in entrapment of MTX and TMX was noticed which may be due to the presence of steric stabilizing agents which was incorporated in the bilayer where the drug was also

incorporated. There may be a competition between the drug molecule and the polymer molecule for occupying the lipid bilayer, which led to reduction in drug entrapment. Whereas in case of CYT, the drug was incorporated in the aqueous compartment of the liposomes, the inclusion of the steric stabilizing agents did not cause much reduction in the entrapment.

In all the above cases, the PC and Chol content was also analyzed and the ratio of PC: Chol in the liposomes was almost the same as taken initially for the drying down process.

12.1.3 CHARACTERIZATION OF THE LIPOSOMAL FORMULATIONS

The conventional and sterically stabilized liposomes of MTX, TMX and CYT were characterized for various aspects.

The particle size distribution of the conventional as well as sterically stabilized liposomes was estimated by the laser diffraction Malvern particle size analyzer that follows Mie's theory of light scattering. Morphology and lamellarity of the liposomes prior to extrusion through polycarbonate filters were ascertained through photomicrographs of the suspensions taken using an Olympus microscope. After lyophilization, the lyophilized liposome powder was coated with gold and then kept in the sampling unit as a thin film and then the photographs were taken at various magnifications using Jeol Scanning Electron Microscope.

DSC thermograms were obtained by differential scanning calorimetry of the pellets of liposomal formulations on a Mettler DSC 20 Differential Scanning Calorimeter.

Entrapped volume of the liposomes was determined by measuring the water content of a tight pellet of the prepared liposomes. Pelletization was achieved by centrifugation at an appropriate 'g' value.

Surface charge of the liposomes was ascertained by carrying out paper electrophoresis as per a modified version of a reported procedure.

Steric stabilization of the liposomes was studied by subjecting them to the electrolyte induced flocculation test. The electrolyte induced flocculation test was carried out by using different concentrations of sodium sulphate solutions. The liposomal formulations were mixed with the electrolyte

solutions and the resulting dispersions were measured at 400nm to check the extent of steric barrier present around the liposomes.

The amount of incorporated polyethylene glycol derivatized lipid present in the liposomes was determined by the earlier reported method using sodium nitrate picrate solution.

All the liposomal preparations, both conventional and sterically stabilized liposomes were found to have a mean particle size diameter between 150-250nm after repeated extrusion through 0.2μm Nuclepore polycarbonate track-etch filters. However, as compared to the conventional liposomes, sterically stabilized liposomes were found to exhibit a smaller mean particle size and a narrower distribution. These observations could be correlated with the surfactant properties associated with these steric stabilizing agents.

Photomicrographs of the formulated liposomal suspensions before extrusion indicated that the liposomes prepared were spherical in shape. It was observed that the majority of the liposomes formed in each case were multilamellar. However, small unilamellar vesicles (SUVs) were also seen and the presence of these vesicles is due to the sonication process used for size reduction during preparation. The scanning electron microscopic photographs of the lyophilized liposomal formulations indicated that the liposomes were spherical in nature and the particle size were in the range of 200nm after extrusion through 0.2μm Nuclepore polycarbonate track-etch filters.

The entrapped volumes of sterically stabilized liposomes containing methotrexate and tamoxifen citrate were found to be similar to that of the corresponding conventional liposomes. This proved that the incorporation of steric stabilizing agents does not have any significant effect on the entrapped volume. When poly ethylene glycol derivatives used as steric stabilizing agents into the liposomes containing cytarabine, a slight increase in the entrapment volume was found. The most probable reason for this increase seems to be the association of water molecules with the polyethylene glycol molecules. Since, this phenomenon is not observed with corresponding liposomes containing methotrexate and tamoxifen citrate (hydrophobic drugs), the presence of hydrophilic cytarabine seems to be a

major factor in this association. Cytarabine may be forming some kind of association with poly ethylene glycol molecules, which leads to a microenvironment of water around the system and a concomitant increase in entrapped volume.

Differential scanning calorimetry was used to ascertain the phase transition temperature of the prepared liposomes and to ensure that no phase separation of the various components took place. Results indicated that there was no major difference between the phase transition temperature of the conventional and sterically stabilized liposomes. The thermograms did not prove any evidence of phase separation of the membrane components indicating their complete miscibility.

Paper electrophoresis of the conventional and sterically stabilized liposomes showed no mobility confirming the absence of surface charge. Reports of earlier experiments indicate the requirement of neutral surface charge for sterically stabilized liposomes for better performance *in vivo.*

The electrolyte induced flocculation test was used for investigating the steric stabilization and to distinguish between conventional and sterically stabilized liposomes. The amount of polyethylene glycol required to produce steric stabilization to the liposomes was optimized using this test.

12.1.4 STUDIES OF RELEASE KINETICS OF DRUGS FROM LIPOSOMES

Drug release kinetic studies from the liposomes were performed by subjecting the liposomes to dialysis using Sigma dialysis membrane (which will retain materials with molecular weight greater than 12,000) under controlled conditions of temperature and agitation. The data obtained was analyzed using the equation of Eyring.

The raw data was plotted on semi log paper and the curve was subjected to curve stripping to get initial estimates of the unknown variables f_l, f_f, k_l, k_f and $t_{1/2}$ (time required for 50% of drug to be released from liposomes). These initial estimates were then taken for non-linear regression analysis performed using Quickcalc software (Plexus supporting systems, Ahmedabad) to obtain the final regressed values.

Both conventional and sterically stabilized liposomes of all the three drugs showed biphasic release pattern – a relatively more rapid initial phase

extended over a few hours, followed by a relatively slower phase. Due to its solubility in phosphate buffer (pH 7.4), a faster release profile was obtained for MTX and CYT containing liposomes. In case of TMX containing liposomes, the release was slower compared to other two drugs, MTX and CYT. The second phase of release extended over to a time even up to several days. Between the conventional and the sterically stabilized liposomes containing CYT, conventional liposomes exhibited a slower rate of release as against that of the sterically stabilized liposomes. In case of MTX and TMX, there was no significant difference in the release of drug from conventional and sterically stabilized liposomes. This observation proves the importance of location of entrapped drug within the liposomes and its relation with drug release. Being a water-soluble drug, CYT localizes in the aqueous compartments within the liposomes. Introduction of steric stabilizing agents tends to increase the bilayer hydrophilicity making it more permeable to CYT accounting for the faster release of the drug from these types of liposomes. MTX, which is entrapped in the bilayer being soluble in phosphate buffer accounted for faster release from the bilayer of the liposomes. The incorporation of steric stabilizing agents does not appear to alter the release pattern of the MTX from the liposomes. On the other hand, TMX, a water insoluble drug, localizes in the bilayers. Therefore, any increase in the hydrophilicity of the bilayers does not have any pronounced effect on release of TMX.

12.1.5 STABILITY OF THE PREPARED LIPOSOMES

The stability of the conventional and sterically stabilized liposomes containing MTX, TMX and CYT was investigated. The prepared liposomal dispersions were subjected to stability studies, in triplicate, at conditions according to ICH guidelines i.e. 2-8°C with ambient humidity and 30 ± 2°C/ 60 ± 5% RH. The prepared dispersions were taken in 5ml amber colored glass vials (USP Type I glass) and the vials were purged with nitrogen. The vials were then sealed using 20-mm grey bromobutyl rubber stoppers and 20-mm aluminium seals.

At predetermined intervals of time, samples were removed and studied for parameters such as change in size and size distribution, steric stabilization and percent drug leaching. Changes in the size and size distribution of the

liposome formulations were determined by Malvern particle size analyzer. The changes in the steric stabilization were determined by the electrolyte induced flocculation technique while the percent drug leached from the liposomes was determined by centrifuging the liposomal suspension and analysing the supernatant for the drug content.

Results of the stability studies indicated that none of the batches of the liposomal suspensions were stable enough for commercial preparation in suspension form. The liposomal dispersions were relatively more stable at refrigeration conditions in comparison to liposomal dispersion kept at RT.

The above results indicated that freeze-drying was the only alternative to formulate stable liposomal products. Various cryoprotectants like sucrose and maltose were used for the purpose of lyophilisation. Lyophilization optimization of lyophilized liposomes was performed for maximum drug retention. It was found that sucrose proved to be better cryoprotectant compared to maltose. The liposomal suspensions were freeze dried along with the sucrose (3 times by weight of total lipids) overnight at -20°C, lyophilized for 24h. The lyophilized liposomal products were also tested for its stability at two different temperatures viz. refrigerator (2-8°C) and room temperature (30 ± 2°C). At predetermined intervals of time, samples were removed; rehydrated using distilled water and studied for parameters such as change in particle size distribution, steric stabilization and percent drug leaching. It was found that liposomes were found to be stable for 6 months at refrigerator and at ambient conditions.

12.1.6 *IN VITRO* CYTOTOXICITY STUDIES OF THE PREPARED LIPOSOMES

The *in vitro* cytotoxicity of the drugs (MTX, TMX and CYT) and their liposome formulations were evaluated by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. MTT cell proliferation assay offers a quantitative, convenient method for evaluating a cell population's response to external factors, whether it is an increase in cell growth, no effect, or a decrease in growth due to necrosis or apoptosis. The yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of

a detergent. The color can then be quantified by spectrophotometric means. For each cell type a linear relationship between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation.

BMG-1 human cerebral glioma cancer cell lines were used for the study. Various concentrations of free drugs and liposomes containing the drugs were incubated with a specific number of cells for various time periods at 37°C in a humidified incubator (5% CO_2 and 95% air). The result of the MTT assay showed that there was a concentration dependant cytotoxic effect with all the anticancer drugs (MTX, TMX and CYT) used. The liposomes containing anticancer drugs also showed similar cytotoxicity effects as that of the free drug after a period of 48h.

12.1.7 *IN VIVO* BIODISTRIBUTION STUDIES BY RADIOLABELING TECHNIQUE

The in vivo behaviour of the conventional and sterically stabilized liposomes was studied by carrying out biodistribution studies in balb/c mice and blood kinetic studies in New Zealand rabbits by radiolabeling technique. The tumour targeting studies were also carried out in ehrlich ascites tumour (EAT) bearing mice. The biodistribution of liposomes administered in vascular or extra vascular spaces might be studied by the administration of radiolabeled liposomes and followed by scintigraphic imaging. Liposomes were labeled with high efficiency by the direct labeling technique using reduced ^{99m}Tc. The pertechnetate used for the study was first reduced to its lower valency state using stannous chloride dihydrate and then pH was adjusted to neutral before mixing with the drug / liposome suspension. The radiolabeling was optimized by taking three factors into account. i.e. pH of the complex, incubation time and stannous chloride dihydrate concentration. The optimum conditions required for maximum labeling efficiency were established. The radiolabeling of MTX, TMX, CYT and their liposomes was done after reducing the technetium with stannous chloride dihydrate at pH of 7, incubated for a specific period of time yielded maximum labeling efficiency of more than 98%.

Stability of the ^{99m}Tc - labeled complexes (MTX, TMX, CYT and their liposomes) with time was studied in saline and in serum (rabbit) at 37°C for various time intervals up to 24h. The experimental data revealed that there was hardly any detachment of the radioisotope from the complex. Even after a period of 24h incubation, less than 5% degradation of the labeled product was noticed which signifies the high stability of the radio labeled product and its suitability for *in vivo* use. DTPA challenging test demonstrated the high binding affinity of the ^{99m}Tc with MTX, TMX, CYT and their liposomes. The *in vitro* plasma protein binding studies of the labeled complexes were carried out and the results showed that the percent protein binding for the conventional liposomes varied significantly to that of the sterically stabilized liposomes of MTX, TMX and CYT. This also accounts to the stealth liposomes for the enhanced circulation time in blood as the polymer coating over the liposomes cause reduction in opsonization.

The blood kinetic study of MTX, TMX, CYT and their liposomes studied in healthy New Zealand rabbits at different time intervals after intravenous administration of the labeled complex through the ear vein. The free drug was eliminated from the blood very quickly whereas the conventional liposomes showed increased circulation time compared to free drug. The sterically stabilized liposomes showed enhanced circulation compared to conventional liposomes and even after 24h, sterically stabilized liposomes showed higher blood concentration compared to conventional liposomes. The sterically stabilized liposomes prepared using polyethylene glycol of mol wt.2000 showed enhanced circulation compared to the liposomes prepared using polyethylene glycol of mol wt 5000, which showed that the polyethylene glycol of low mol wt provides equally, or little higher resistance to opsonization and also provides prolonged blood circulation.

Biodistribution studies were carried out for the free drugs (MTX, TMX, CYT) and also their liposomes in 2-3 months old ehrlich ascites tumour bearing balb/c mice (weighing about 25-30gm). Fixed amount of ^{99m}Tc-drug / liposome complex was administered through the tail vein of each mouse. The mice were sacrificed at different time intervals and blood was obtained by cardiac puncture. The blood was weighed and the radioactivity present in the whole blood was calculated by keeping 7.3% of the body weight as

total blood weight. Subsequently, tissues (heart, lung, liver, spleen, kidney, stomach, intestine and tumour) were dissected, washed with normal saline, made free from adhering tissues, weighed and their radioactivity was measured in a shielded well gamma scintillation counter. To correct for physical decay and to calculate radiopharmaceutical uptake in each organ as a fraction of the injected dose, aliquots of the injectate, containing 2 % of the injected dose, were counted simultaneously at each time point.

Results of the *in vivo* biodistribution studies indicated that all the agents used produced steric stabilization of the liposomes. Free drugs were found to be cleared from blood quite rapidly. Conventional liposomes also cleared from the blood stream quickly when compared to the sterically stabilized liposomes. The sterically stabilized liposomes showed enhanced blood circulation and also increase in accumulation in tumour. There was 100 - 150 times increase in tumour accumulation of drug from conventional liposomes compared to free drug and around 350 –450 times increase in tumour drug concentration from sterically stabilized liposomes compared to free drug.

When compared to sterically stabilized liposomes, conventional liposomes showed extremely significant increase in accumulation in liver and spleen. It could be accounted for as the combined activity of the circulating blood passing through the organs as well as that due to particle uptake by cells of reticuloendothelial system (RES). The presence of very low amount of radioactivity in stomach after 24 h proved the *in vivo* stability of the radiolabeled complexes.

Scintigraphic studies were carried out in EAT bearing mice after administration of fixed amount of ^{99m}Tc labeled complexes through the tail vein. The mice were fixed on a board and imaging was performed using a Single Photon Emission Computerized Tomography (SPECT) gamma camera. The gamma scintigraphic study of conventional and sterically stabilized liposomes showed very clearly the accumulation of labeled complexes in various organs including liver, spleen and tumour present in the mouse. The conventional liposomes showed increased liver and spleen accumulation with less tumour accumulation in comparison with the sterically stabilized liposomes because of increase in opsonization.

12.1.8 MYELOSUPPRESIVE STUDIES OF PREPARED LIPOSOME FORMULATIONS

The anticancer agents are generally potent myelosuppresants. They are known to cause haematological changes when administered even at therapeutic doses. So a comparison was made to find out the presence of any change in toxicity profile between the free drug and the liposomal formulations containing the drugs. Balb/c mice were used to analyse the changes in haematological parameters following administration of free and liposomal MTX, TMX and CYT. The mice were injected either with drug / liposomal formulations containing drug (MTX, TMX and CYT) with the dose equivalent to that of the human therapeutic dose for a period of 10 days and then the changes in haematological parameters like RBC count, total WBC and Hemoglobin were analysed. It was found that the free drugs (MTX and CYT) when injected for a period of 10 days caused severe reduction in total RBC and WBC count but produced no considerable change in Hemoglobin content. In case of free TMX, a slight reduction of RBC and WBC count was found. When the drugs were encapsulated in liposomes, the toxicity was reduced to a greater extent and even after injecting the same dose of drug containing liposomes; no significant change in the total RBC and WBC count was noticed. This proves the liposomes, an effective carrier of anticancer drugs for targeting the tumour site without causing systemic toxicity.

12.2 CONCLUSIONS

Liposomal drugs have been suggested to be the long awaited "magic bullet" cancer therapy due to their ability to accumulate selectively in the tumour. However, the problem remains that not all the cancers and not all patients respond to the "bullet" equivalently. The drug being delivered by liposomes plays an important role in the response achieved. Multi drug resistance has led to significant obstacles in the ability of standard chemotherapy regimens to cure cancer. Liposomal drugs have the advantage of continually bombarding the cancer cells with low doses of standard chemotherapy and possible overwhelming drug transporters responsible for pumping drugs out of the cells.

A systematic attempt was made to make the anticancer therapy more effective and safe by incorporating the anticancer drugs in sterically stabilized liposomes. It was hypothesized that sterically stabilized liposomes will provide a prolonged systemic circulation and enhanced tumour accumulation of the drug. This combination of sustained and targeted delivery would help to reduce side effects of these drugs and would also lead to a significantly lower dose being required for achieving therapeutic efficacy. The anticancer agents, methotrexate (MTX), tamoxifen citrate (TMX) and cytarabine (CYT) were chosen as the drugs for investigation. Multilamellar vesicles of the three drugs were prepared using the lipid film hydration technique. The important process parameters for the preparation of liposomes were optimized. It was found that the molar ratio of the components of the liposomal system played an important role in determining the percentage drug entrapped. The parameters such as speed of rotary flash evaporator, and vacuum applied for drying was important in the formation of thin dry film during the preparation of liposomes. The selection of hydration medium was critical in the preparation of MTX liposomes. The use of phosphate buffer as the hydration medium caused solubilization of MTX from the thin dry film and resulted in less percentage drug entrapped. In case of TMX liposomes, no other process parameters significantly affected the drug entrapment but in case of CYT, volume of hydration medium was of great importance next to the drug lipid ratio and ratio of the various lipids used in determining the amount of drug encapsulated within the liposomes.

The sterically stabilized liposomes were prepared in the similar method using agents like methoxypolyethylene glycol (mol wt.5000) activated with cyanuric chloride coupled with phosphatidyl ethanolamine (mPEG5000-CC-PE), methoxypolyethylene glycol (mol wt.2000) activated with cyanuric chloride coupled with phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE) and methoxypolyethylene glycol (mol wt.5000) activated with carbonyl diimidazole coupled with phosphatidyl ethanolamine conjugate (mPEG5000-IC-PE). The concentration of these agents, which provide steric stabilization, was optimized using the electrolyte induced flocculation test. 5mol % of the total lipids of both mPEG5000-CC-PE and mPEG5000-IC-PE

and 7mol% of mPEG2000-CC-PE were found to be adequate for steric stabilization of the liposomes. The presence of these agents led to significant difference in the entrapment of the drugs as compared to that in the conventional liposomes in case of MTX and TMX.

The conventional and the sterically stabilized liposomes were characterized for size and size distribution, morphology, lamellarity, surface charge, polyethylene glycol content incorporated in bilayers, and steric stabilization. Results of the study indicated no major changes in the above properties between the liposomes except for steric stabilization.

Drug release kinetics from the liposomes was studied by subjecting the liposomes to dialysis under controlled conditions of temperature and stirring, and the data analysis was done using the equation of Eyring. It was observed that drug release, from both the types of liposomes for all the three drugs was of biphasic nature with an initial, rapid phase followed by a subsequent slow release phase. Location of the drug within the liposomes and the solubility characteristics of the drugs were shown to play important role in the rate of drug release.

Stability studies of the formulated liposomes indicated that none of the prepared liposomal suspensions were stable enough for commercial preparation on suspension form. Freeze drying was selected as an alternative to obtain the required stability. The liposomal suspensions were freeze-dried and lyophilized using sucrose as cryoprotectant. The lyophilized liposome product was found to be quite stable for more than 6 months at both refrigeration and room temperatures.

The MTT assay corroborates that the drug encapsulated in liposomes showed similar cytotoxic effects on the *in vitro* BMG-1 cell lines when compared to that of the free drug.

Radiolabeling of the three drugs and their liposomes were carried out successfully with high labeling efficiency. The radiolabeled complexes were found to be highly stable in both *in vitro* and *in vivo* conditions. The biodistribution studies of both the types of liposomes revealed that sterically stabilized liposomes led to increased residence times in blood with significant reduction in the distribution of the drugs to the organs of the

mononuclear phagocyte system such as the liver and the spleen. The sterically stabilized liposomes also offered increased drug accumulation in tumour compared to conventional liposomes containing drug and the free drug. The tumour accumulation of the sterically stabilized liposomes was also proved by γ imaging studies. The toxicity study regarding the haematological changes due to the anticancer drugs was performed and the results suggested that there was a significant reduction in the toxicity caused by the drugs when entrapped in liposomes as compared to the free drug.

Thus the present study demonstrates some new findings, which may be exploited in improving the therapeutic efficacy of anticancer agents using liposome systems. However, other methods of preparation for conventional and sterically stabilized liposomes need to be investigated further so that the drug lipid ratio can be increased to reduce the cost of the formulation. Other methods of characterization of the sterically stabilized liposomes need to be developed so that they can be included in the regular quality control tests used for ensuring batch-to-batch reproducibility in the production of this novel drug delivery system. Various other cryoprotectants can be tried to formulate more stable liposome formulation that retains its properties upon reconstitution and ensures maximum drug retaining capability. Extensive clinical trials need to be performed to establish the efficacy and safety of these liposomal delivery systems in clinical practice.

Based on the above study, we believe that these systems have the potential to improve the chemotherapy of certain neoplasms. Their effective use will not only reduce the dose of the chemotherapeutic agents for a given degree of therapeutic response, but due to their inherent tendency to undergo cellular uptake, they may also provide opportunities for drugs which are normally resistant to some cancer cells. It is appreciated that targeted drug delivery systems may not be useful in the management of all type of clinical neoplasms. However, the application of these systems for the selective in situ destruction of tumour tissue may obviate the need for major surgical intervention or at least reduce its urgency.