SUMMARY
SUMMARY

Gastric cancer is the second most common cancer and cause of cancer related death world wide. Gastric cancer is the leading cause of cancer death in men and third leading cause of cancer of women. India is a developing country with one of the most diverse populations and diets in the world. Cancer rates in India are lower than those seen in the western countries, but are rising with increasing migration of rural population to the cities, increase in life expectancy and changes in the life style. In India there seems to be a difference in the distribution frequency of gastric cancer incidence. The southern and eastern parts of the India have higher frequency of gastric cancer than rest of the country. The large vegetarian population in northern India is at a lower risk of gastric cancer. But the times are changing; rapid flourish of post globalization, fast food brought by corporate culture, germ free bottled water, pasteurized milk and preserved meat items to the present day life in big Indian cities could contribute as risk factors for gastric cancer. However, it will be too early to link it with rising gastric cancer incidences in cities in India.

Stomach cancer treatment plans vary from patient to patient and depend on the stage and location of the cancer, the patient's age, and general health state. The three main treatment options for stomach cancer are surgery, chemotherapy and radiotherapy. Presently cancer patients are treated with a combination of chemotherapy, radiotherapy and immunotherapy.

Combination of active agents has been used since the late 1970's, aiming to improve the results of single agent chemotherapy. 5-FU has almost been universally used as the basis in the designing of combination treatment (combination chemotherapy). Advances in basic research resulted in better understanding of the mechanism of action of many chemotherapeutic agents, including 5-FU, the main drug used in advanced gastric cancer. Based on these data several second generation regimens were developed.
in the late 1980s. FAMTRX (5-FU, adriamycin, and high dose methotrexate) showed response rates ranging from 33-50% in phase II studies. The two most effective regimens FAMTX and EAP (cisplatin, etoposide and doxorubicin), were also directly compared in a prospective randomized study. FAMTX showed higher activity with significantly lower toxicity. So authors concluded that FAMTX should be the standard chemotherapy in advanced gastric cancer. Another combination ECF (epirubicin, cisplatin and 5-FU) was compared with FAMTX in a recent randomized trial which suggested that both regimens are highly cost effective. The uses of second generation regimens which combine 5-FU with other agents that modulate the activity of 5-FU have improved response rates in inoperable gastric cancer and in certain cases have resulted in a small increase in survival. FAMTX has shown considerable efficacy in advanced gastric cancer. In vitro studies have shown that methotrexate can enhance the activity of 5-FU by blocking the pyrimidine salvage pathway, thus leading the increased intracellular phosphoribosyl pyrophosphate. This shifts 5-FU into the RNA pathway, increasing destruction of cancer cells. On the other hand doxorubicin is responsible for inhibition of DNA and RNA synthesis by intercalation between DNA base pairs by inhibition of topoisomerase II and by steric obstruction. Doxorubicin intercalates at points of local uncoiling of the double helix. It appears that direct binding to DNA (intercalation) and inhibition of DNA repair (topoisomerase II inhibition) result in blockade of DNA and RNA synthesis and fragmentation of DNA. Doxorubicin is also a powerful iron chelator; the iron-doxorubicin complex can bind DNA and cell membranes and produce free radicals that immediately cleave the DNA and cell membranes. Use of combination of neoplastic agent is responsible for synergism and increase in cancerous cell destruction. It is designed to treat only the cancer cells and minimize damage to normal cells, healthy cells. Cancer treatments that deliver drug at tumor site may offer the advantage of reduced treatment related side effects and improved outcomes. The majority of drugs
used in the cancer treatments are administered systemically, orally, or locoregionally. Of these, only locoregional delivery presumes restriction of an administered drug to the site or location of the tumor. Thus because the concentration of antineoplastic agent at the tumor site is enhanced, systemic exposure is avoided or significantly minimized. Consequently it is assumed that the therapeutic benefits as well as therapeutic window of the drug are improved upon. The basic principle of regional administration of the antineoplastic agent is to deliver a higher concentration of the agent to the tumor present within a particular region of the body and to expose the tumor to the active drug for longer period of time that are safely possible with systemic administration.

The delivery of drugs currently used in cancer treatment is performed mostly through intravenous drug infusion. Due to unfavorable pharmacodynamics, the infusions need to be performed frequently, exacerbating the potential for side effects.

Oral delivery of drugs is by far the most preferable route of drug delivery due to ease of administration, patient compliance and flexibility in formulation etc. Oral sustained drug delivery formulations show some limitations connected with the gastric emptying time variables and too rapid gastrointestinal transit which could result in incomplete drug release from the device into the absorption window leading to diminished efficacy of the administered dose. To overcome this problem several attempts have been made to develop oral dosage forms capable of having prolonged retention time in the stomach to extend the duration of drug delivery. It is evident from the recent research and patent literature that interest in novel dosage forms is unexpectedly increasing. Example of such systems are gastro retentive dosage forms, these dosage forms are based on different mechanisms which include floatation, mucoadhesion, sedimentation, expansion, modified shape system or by simultaneous administration of pharmacologic agents that delay gastric emptying. Drug candidate suitable for this system are 1] which have site specific absorption in the stomach, or upper part of

In the fight against cancer, new drug delivery systems are attractive to improve drug targeting of tumors (locoregional delivery), maximize drug potency, and minimize systemic toxicity. We have studied a new drug delivery comprising porous microspheres, i.e. Stomach Specific Floating Drug Delivery System that has bulk density less than gastric fluids and so remain buoyant in the stomach without affecting the gastric emptying rate for a prolonged period of time. While the system is floating on the gastric contents, the drug is released slowly at the desired rate from the system. After release of drug, the residual system is emptied from the stomach. These results in an increased gastric retention time (GRT) and a better control of fluctuations in plasma drug concentration. The floating sustained release dosage forms present most of the characteristics of hydrophilic matrices and are known as ‘hydrodynamically balanced systems’ (‘HBS’) since they are able to maintain their low apparent density, while the polymer hydrates and builds a gelled barrier at the outer surface. The drug is released progressively from the swollen matrix, as in the case of conventional hydrophilic matrices. These forms are expected to remain buoyant (6- 8 hours) on the gastric contents without affecting the intrinsic rate of emptying because their bulk density is lower than that of the gastric contents.

Pectin is a structural heteropolysaccharide contained in the primary cell walls of terrestrial plants. It was first isolated and described in 1825 by Henri Braconnor. It is produced commercially as a white to light brown powder, mainly extracted from citrus fruits. Pectin has wide applications in a variety of food formulations as jellying and thickening agent. Since it sets into Jelly in sugar-acid solution, it is regularly used in the preparation of jams, jellies and marmalades. In
addition to this, pectin has many other uses in food and pharmaceutical industries. Pectin is also being recommended for use as fat replacer. On account of its ever-increasing use and demand pectin has become an indispensable ingredient in food industry. Pectin is an inexpensive, nontoxic product extracted from citrus fruits and has been used as food additive, a thickening agent, and a gelling agent. In addition, pectin can reduce interfacial tension between an oil phase and water phase and is efficient for the preparation of emulsion. Pectin is stable at low pH and it passes through the small intestine more or less intact. In the large intestine and colon, microorganisms degrade pectin and liberate short chain fatty acids that have positive influence on health (prebiotic effect). Thus pectin is a soluble dietary fiber.

Casein is the name for a family of related phosphoproteins (αS1, αS2, β, κ). These proteins are commonly found in mammalian milk, making up 80% of the proteins in cow milk and between 20% and 45% of the proteins in human milk. Casein has a wide variety of uses, from being a major component of cheese, to use as a food additive, to a binder for safety matches. As a food source, casein supplies amino acids; carbohydrates; and two inorganic elements, calcium and phosphorus. Casein-derived compounds are used in tooth remineralization products to stabilize amorphous calcium phosphate (ACP) and release the ACP onto tooth surfaces, where it can facilitate remineralization. It is also an excellent emulsifier. Casein paint is a fast-drying, water-soluble medium used by artists. Casein-based glues were popular for woodworking, including for aircraft. Also used for Cheese making, Protein supplements and Plastics-fibermaking.

In the light of this, the topic of this research work “Formulation and Evaluation of Porous Microspheres of Methotrexate, 5-FU and Doxorubicin: A Combination Therapy to treat Gastric Adenocarcinoma”, is chosen. The object of this research work is to provide not only safe and efficacious formulation but also to improve the therapeutic
index of the drug, reduce side effects and also to have better formulation characteristics in comparison to conventional dosage forms.

This thesis contains ten chapters. Chapter One – Introduction: an overview on gastric adenocarcinoma, combination chemotherapy, porous microspheres, literature surveyed and plan of work, Chapter Two – Drug profile, Chapter Three – Analytical profile: Standard curves of drugs on different pH, Chapter Four - Preformulation Study: Physical and chemical properties of drugs, Chapter Five – Optimization, Formulation and Characterization: to prepare stable, efficacious and consistent quality of products, Chapter Six – In vitro drug release studies, Chapter Seven – Stability study of formulations: to provide consistent quality of product, Chapter Eight – In vivo study: to prove the safety and therapeutic effectiveness of the formulated products, Chapter Nine – Summary and conclusion: An overview of this research work and Chapter Ten – Bibliography.

**Analytical Profile**

For accurate analytical work it is important to determine the absorption maxima of the substance under study. Absorption maxima are the wavelength at which maximum absorption takes place. Solution of methotrexate was scanned in the UV range 200-400nm using UV visible spectrophotometer. The spectrophotometric method of analysis of methotrexate at $\lambda_{\text{max}}$ 255.5 and 256.4 nm for pH 2.0 and 4.0 respectively was found to be reproducible and sensitive.

Solution of Doxorubicin was scanned in the UV range 350-550nm using UV visible spectrophotometer. The spectrophotometric method of analysis of doxorubicin at $\lambda_{\text{max}}$ 496.6 and 495.8 nm for pH 2.0 and 4.0 was found to be reproducible and sensitive.

Solution of 5-FU was scanned in the UV range 200-400nm using UV visible spectrophotometer. The spectrophotometric method analysis of 5-FU at $\lambda_{\text{max}}$ 265.6 and 266.2 nm for pH 2.0 and 4.0 was found to be reproducible and sensitive.
spectrophotometric method was selected for in vitro analysis of drug in different simulated fluids. The data was regressed to obtain a straight line. The correlation coefficient was found to be greater than 0.98 in all the cases, indicating that Beer’s Law was followed in concentration range of 2-20µg/ml.

**Preformulation Study**

To optimize the performance of drug products, it is necessary to have a complete understanding of the physical, chemical and mechanical properties of drug substance prior to formulating them into drug products in order to develop stable, safe and effective dosage form. The preformulation study was performed in order to ensure the authenticity of the procured drug. The drug methotrexate was purchased from Macromax Exports Ltd, Mumbai, India. The color of the crystalline drug was found to be yellow to orange brown with no odor, nature was 8-10% hydrated, melting point was found between 180˚C to 189˚C. The drug was stable if solution is not strongly acidic or basic. The pH of the drug solution was 7.0-7.9 in sterile water for injection. Solubility of drug was determined in various solvent, which showed that drug is soluble in dilute solution of alkali hydroxide and carbonates and mineral acids, insoluble in water and ethanol, and practically insoluble in ether. The partition coefficient of methotrexate in n-octanol/water, n-octanol/HCl buffer, and n-octanol/acetate buffer was found to be 0.28, 0.30 and 0.33.

The drug doxorubicin HCl was purchased from, RPG Lifescience Ltd. New Mumbai, India. The drug was found to be odorless, free flowing crystalline powder having orange red color, nature was hygroscopic, and melting point was 204˚C to 205˚C. The pH of the drug solution (0.5%) was 4.0-5.5. The drug was stable in solution having pH range in between 3.0 to 6.5. Solubility of drug was determined in various solvents, which showed that drug is soluble in water, dilute solution of alkali hydroxide and carbonates and mineral acids, slightly soluble in ethanol, and practically insoluble in
ether. The partition coefficient of methotrexate in n-octanol/water, n-octanol/HCl buffer, and n-octanol/acetate buffer was found to be 0.30, 0.12 and 0.18 respectively.

The drug 5-FU was purchased from, RPG lifescience ltd, New Mumbai, India. The color of drug was found to be odorless, white or almost white crystalline hygroscopic powder, nature was hygroscopic, melting point was 280°C to 284°C. Drug is stable in solution which is not strongly basic (pH less than 9). The pH of the drug solution (1%) was 4.0-5.0. Solubility of drug was determined in various solvents. The drug was found to be soluble in dilute solution of alkali hydroxide and carbonates and mineral acids, sparingly soluble in water, slightly soluble in ethanol, and practically insoluble in ether. The partition coefficient of methotrexate in n-octanol/water, n-octanol/HCl buffer, and n-octanol/acetate buffer was found to be 0.16, 0.18 and 0.25. Partition coefficient value of drugs revealed that each drug possessed hydrophilic nature.

All the above mentioned drugs were identified by using UV Spectrophotometric method and Infrared Spectroscopic method. UV and infrared spectrum of drugs provided was found to be concordant with the reference spectrum of drugs. Assay of drug sample according to U.S.P. procedure, confirmed purity of drugs to be 99.48%, 100.3% and 98.88% respectively for methotrexate, doxorubicin and 5-FU, respectively.

The drugs were characterized for micromeritic properties. After micromeritic analysis the particle size of the methotrexate, doxorubicin and 5-FU were 7μm, 5μm and 98.4μm, bulk density was 0.621±0.0120g/cm^3, 0.70±0.0023 cm^3, and 2.01±0.052 cm^3, and angle of repose was 45.63±1.23°, 49.44±1.91° and 30.00±1.46°, respectively.

To determine interference of polymer and emulsifier (pectin and casein) in estimation of drugs, each drug was incubated separately with pectin and casein; the absorbance data of drug alone and in presence of different additives revealed no significant change in the absorbance of drug solution, indicating no interference of the same in the estimation of drug. Drug interaction was also determined by Fourier
Transform infrared spectroscopy. Drug with pectin and casein was scanned; data showed that there were no significant changes in FTIR spectra of drugs when compared with standard.

**Preparation of Microspheres: Preliminary Batch**

Microspheres were prepared by emulsification extraction technique. Pectin was used as a polymer and casein as emulsifier. The microspheres were prepared by modification of method described by Bulgareli et al. In preliminary batches 10 ml of 15% w/v (in different ratio) casein and pectin solution were added to 60ml Soya oil. Both oil and polymer solution was preheated separately up to 60°C. Each drug was added to the polymer emulsifier solution in two different quantities (50mg and 100mg). The mixture was mechanically stirred at 1000rpm to form o/w emulsion, after 5 min the solution was rapidly cooled to 15°C. One fifty milliliter of acetone was added to dehydrate & flocculate coacervate droplets. The microspheres were isolated by filtration through sintered glass filter. Residual oil over the microspheres was removed by washing with 250 ml of acetone. After preparation of microspheres they were stored at room temperature in a dessicator at 8% relative humidity, otherwise drying conditions can influence microspheres release profile. Fifty millimeter diameter vessel, a three blade turbine rotator of 35 mm in diameter with digital stirring speed counter was selected for preparation. The effect of formulation variables on characteristics of the microspheres viz. particle size, % buoyancy and % drug entrapment was observed.

In preliminary batches different ratio of polymer to emulsifier (1250:250, 1000:500, 750:750, 500:1000 and 250:1250) was used for preparing the polymer solution, the polymer solution was too viscous at ratio 1250:250 and 1000: 500 (pectin: casein) and difficult to pour in oil. As the quantity of polymer increased from 250-1250mg in polymer to emulsifier ratio, percentage entrapment efficiency of microspheres increased with low % buoyancy. On the other hand if quantity of emulsifier was raised
from 250 to 1250 in polymer to emulsifier ratio, microspheres showing irregular shape and large size with increased buoyancy and with less entrapment efficiency were produced. Therefore 750:750 (1:1) of pectin to casein was found to be optimum concentration of polymer and emulsifier which provided microspheres of small size with good % entrapment efficiency and increased % buoyancy.

In factorial design batches M1 to M9, D1 to D9 and F1 to F9, the polymer to emulsifier ratio (1:1) and quantity of drug (100mg) was kept constant which was selected from preliminary batches. The temperature and stirring speed were varied in batches. All other variables were used as mentioned in preliminary trial batches.

**Optimization of Process**

Traditional optimization methods generally study the effect of one variable at a time, because it is statistically easier to manipulate. However, in many cases, two factors may be interdependent, and it is impractical or false to attempt to analyze them in the traditional way. A $3^2$ randomized full factorial design was adopted to optimize the variables. In the present investigation two factors were evaluated, each at 3 levels (low, medium and high), and experimental trials were performed at all nine possible combinations. In the present investigation, temperature ($X_1$) and stirring speed ($X_2$) were selected as independent variables. The particle size, % drug entrapment, and % Buoyancy were selected as dependent variables.

The statistical analysis of the factorial design batches was performed by multiple polynomial regression analysis using Microsoft Excel. The data clearly depicts that the Particle size, % drug entrapment, and % Buoyancy values are strongly dependent on the selected independent variables. The polynomial equations can be used to draw conclusions after considering the magnitude of coefficient and the mathematical sign it carries (positive or negative). The value of the correlation coefficient indicates a good fit.
To demonstrate graphically the effect of the temperature and stirring speed, the response surface plots were generated for the dependent variables, particle size, %buoyancy and % drug entrapment using sigma plots software. Multiple polynomial regression analysis revealed that both factors had significant influence on particle size, %buoyancy and % drug entrapment. To evaluate the contribution of different levels of factor (X1) and factor (X2), 2-way ANOVA followed by Tukey test was performed using Sigma Stat Software. In case of methotrexate – for factor X1, it was found that statistically significant difference existed between levels -1.00 vs 1.00, -1.00 vs 0.00 and 1.00 and 0.00 levels (p<0.05).

But in case of factor X2 for entrapment efficiency, it was found that there is no significant difference between -1.00 vs 1.00, -1.00 vs 0.00 and 1.00 and 0.00 levels. From the results of tukey test, factor X1 and X2 had significant effect on all responses except X2 on % drug entrapment. This means % entrapment efficiency was not greatly affected by stirring speed.

In case of doxorubicin – for factor X1, it was found that there is statistically significant difference between levels -1.00 vs 1.00, -1.00 vs 0.00 and 1.00 and 0.00 levels (p<0.05). But in case of factor X1 for particle size, it was found that there is no significant difference between -1.00 vs 1.00, -1.00 vs 0.00 and 1.00 and 0.00 levels. From the results of tukey test, factor X1 and X2 had significant effect on responses except X1 on particle size which means particle size was not greatly affected by temperature.

In case of 5-FU – for factor X1, it was found that there is statistically significant difference between levels -1.00 vs 1.00, -1.00 vs 0.00 and 1.00 and 0.00 levels (p<0.05). From the results of tukey test, factor X1 and X2 had significant effect on all the responses.
Characterization of Microspheres

Microspheres were characterized for drug content. Drug content ranged from 95.40±0.45 to 97.54±0.48, 78.1±0.42 to 79.2±0.53 and 90.24±0.65 to 94.90±0.34%, for methotrexate microspheres, doxorubicin microspheres and 5-FU microspheres. The maximum % drug entrapment was found to be 95.40±0.89 to 97.54±0.53, 72.97±0.54 to 75.99±0.38 and 72.2±0.87 to 73.3±0.74 respectively for methotrexate microspheres, doxorubicin microspheres and fluorouracil microspheres, respectively.

Particle size and surface morphology were assessed by scanning electron photomicrographs which showed that microspheres are spherical with rough surface. Through optical microscopy it was observed that the particle size of methotrexate microspheres, doxorubicin microspheres and 5-fluorouracil microspheres were 107.00±0.23 to 59.60±0.95, 94.2±0.66 to 55.0±0.44 and 102.0±0.57 to 50.0±0.86, respectively.

The % Buoyancy was found to be 74.2±0.23 to 82.0±0.27, 73.2±0.47 to 82.0±0.29 and 38.0±0.63 to 76.0±0.36 % for methotrexate microspheres, doxorubicin microspheres and 5-fluorouracil microspheres, respectively.

The GI stability of the particles was investigated by suspending the particles in simulated gastric fluid for 8hrs and found to be quite stable under the study conditions and duration. This formed an important exercise, as stable particles would remain floated and results in increase in subsequent bioavailability of drug.

Drug polymer interaction was studied by FT-IR spectroscopy. The spectrum was recorded for pure drugs, loaded drug microspheres and unloaded microsphere (placebo). Samples were prepared by mixing 5% of drug or microspheres with 95% of KBr in glass pestle mortar. The scanning range was 4000 cm\(^{-1}\) to 400 cm\(^{-1}\) and resolution was 2 cm\(^{-1}\). The peaks which are present in spectra of placebo microspheres are similar to that of
drug loaded microspheres. FTIR analysis reveals that complete encapsulation of drug occurs in microparticles.

Flow properties of the formulations were determined and it is found that angle of Repose, Housner’s Ratio and Carr’s Index, for methotrexate microspheres was 20.32±0.432° to 27.36±0.690°, 11.01±0.342 to 17.17±0.241, and 1.135±0.035 to 1.164±0.015, respectively. For doxorubicin microspheres it was 22.67±0.325° to 29.43±0.432°, 11.63±0.362 to 15.13±0.343, and 1.139±0.054 to 1.166±0.042 respectively. For 5-fluorouracil microspheres it was 20.23±0.867° to 27.03±0.543°, 11.23±0.543 to 16.42±0.562 and 1.132±0.045 to 1.173±0.025, respectively. Angle of repose is defined as the maximum angle possible between the surface of a pile of the powder and the horizontal plane. The lower the angle of repose, better the flow property. The rough and irregular surface of particles gives higher angle of repose. The Hausner ratio and Carr’s index are both measures of the flow properties of powders. A Hausner ratio of <1.25 indicates a powder that is free flowing whereas >1.25 indicates poor flow ability. The smaller the Carr’s Index the better the flow properties. For example 5-15 indicates excellent, 12-16 good, 18-21 fair and > 23 poor flow. So for optimize formulation angle of repose is low, Hausner ratio was <1.25 and smaller Carr’s Index which means the formulations are free flowing.

**In Vitro Drug Release Study**

In Vitro dissolution studies were performed using US Pharmacopoeia XXIII Dissolution apparatus II (paddle type). For pharmacokinetic determination PCP-disso-V3 I software was used. It is a programme developed in Microsoft excel-97 for assessment of drug release kinetics and model fitting. This software is applicable

- For dissolution study,
- Transdermal diffusion study
- Prodrug hydrolysis study and
• All other studies in which drug release of a drug is to be studied.

In vitro drug release study of all the formulations was performed in pH 2.0 and pH 4.0 at 37±0.5°C. pH 4.0 was selected for study because the doxorubicin shows maximum stability at this pH. Pectin has a very complex structure; it is completely degraded by colonic bacteria but is not digested in the upper GI tract. Numerous studies contributed to elucidate the structure of pectin. Basically, it is polymer of \( \alpha \)-D-galacturonic acid with 1-4 linkages. This chain is regularly interrupted by some rhamnogalacturonan segments that combine galacturonic acid residues and \( \alpha \)-Lrhamnopyranose by a 1-2 linkage. The galacturonic acid of the backbone is partially methyl esterified. Low methoxy pectin with degree of esterification less than 50% can form rigid gels. This gel is stable in low pH solution and is being investigated as a carrier material for different controlled release systems. Casein by virtue of its emulsifying properties causes incorporation of air bubbles and formation of large holes in the microspheres that act as air reservoir in floating system and serve as a simple and non expensive material used in controlled oral drug delivery systems. Results indicate that all the formulations releases drug for 8hrs.

The optimized microspheres formulations were subjected to in vitro drug release studies. In case of methotrexate microspheres and 5-FU microspheres it was found that the formulation (M9 and F9) prepared at maximum temperature and stirring speed had highest release within 8hrs. This may be due to the fact that increase in temperature may cause increase in solubility of drug and polymer both and higher stirring speed produces particles of small and uniform size but in case of doxorubicin microspheres (D6) maximum release was obtained at medium temperature and maximum stirring speed. Because at higher temperature doxorubicin forms clot like structures which will not provide uniform size and shape of microspheres. So drug release was also affected.

To ascertain the drug release mechanism and release rate, data of all the formulations were model fitted by using PCP Dissolution software. The model selected
were Zero order, First order, Higuchi Matrix, Korsmeyer Peppas and Hixon Crowell. The
drug release data were fitted into various release equations to explain the kinetics of the
drug release microspheres. Methotrexate microspheres and 5-Fluorouracil microspheres
followed Korsmeyer Peppas model while model followed by doxorubicin microspheres
was matrix. The value of release exponent ‘n’ calculated for all the formulations
indicates that the methotrexate formulations and some of the doxorubicin formulations
released drug in Fickian manner (n<0.5) and most of the Doxorubicin and 5-FU
formulations followed non Fickian (anomalous) release mechanism (n>0.5) i.e. erosion
followed by diffusion. Another important parameter is the pH of drug release. The drug
release was better at pH 4.0 as compared to pH 2.0 in all formulations.

It can be concluded from these studies that polysaccharide pectin releases drug in
stomach for prolong period due to floating and may be successfully used to achieve
stomach targeted delivery of drugs. The release of higher amount of drug at pH 4.0 in
comparison to pH 2.0 in dissolution medium clearly revealed that if the pH of stomach
can be increased slightly it will provide better release of drug from porous microspheres.

Formulation Study

The optimized formulations of microspheres were selected for further study. The
batches were selected on the basis of good drug content, finer particle size, better
entrainment efficiency, excellent buoyancy, reproducibility, and better in vitro drug
release. The microspheres M9 (methotrexate microspheres), D6 (doxorubicin
microspheres), and F9 (5-Flourouracil Microspheres) were selected for further study.
The drug content of M9, D6 and F9 was 97.54±0.48, 79.3±0.25, and 94.90±0.34,
respectively. The % entrapment efficiency of selected formulation was 97.54±0.48,
75.99±0.38, 73.3±0.34 respectively. The percentage buoyancy of optimized formulations
was 82.0±0.98, 82.0±0.29, 76.0±0.18, respectively. Particle size of M9, D6, and F9 was
59.60±0.95, 55.0±0.44, 50.0±0.28 micrometers respectively. The selected formulation M9, D6 and F9 released drug up to end of 8 hrs.

The n values of optimized formulation were M9 - 0.4522 and 0.4889, for D6 - 0.5168 and 0.5160 and for formulation F9 - 0.6119 and 0.5315, at pH 2.0 and 4.0 respectively.

The optimized microspheres were found to be stable and having good entrapment efficiency. The in vitro data suggest that release mechanism of M9 was Fickian and for F9 it was non Fickian and for both best fit model was found to be Korsmeyer Peppas. In case of D6 release mechanism was non Fickian and best fit model was found to be matrix.

**In Vitro Cytotoxic Activity**

The inhibitory potency of the three drugs on the Kato III cell line was compared by using the IC\(_{50}\) value. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. MTT assay was used to calculate the IC\(_{50}\), the principle of assay which is based on a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product.

Cell line was purchased and procured from National Centre for Cell Science, Pune, INDIA. Culture medium was removed with floating cells to a centrifuge tube, the cell layer was rinsed and suspended into six to eight ml of complete growth medium and cells were aspirated by gently pipetting. Supernatant was discarded and cells were resuspended in fresh growth medium. Aliquots of appropriate cell suspension were added to new culture vessels. Culture vessels were placed in incubator at 37°C. Briefly
the compounds were dissolved in DMSO and serially diluted with complete medium to get the concentrations in range of 0.001, 0.01, 0.1, 1.0 and 10.0µg/ml test concentration. DMSO concentration was kept < 0.1% in all the samples. Pure drug solution, porous microspheres of individual drug (optimized formulation) and different combinations of porous microspheres were selected for study. It were found that pure drug solution of methotrexate, doxorubicin and 5-fluorouracil showed less effect than microspheres of similar potency and combination of microspheres proved to be the most effective, especially of all three drugs. A comparison of the relative inhibitory potency of these combinations was made. Results indicate that combination of 5-flourouracil microspheres plus methotrexate microspheres and doxorubicin microspheres plus 5-flourouracil microspheres were approximately equally potent and combination of doxorubicin microspheres plus methotrexate microspheres approximately twice potent. Combination of three drugs i.e. doxorubicin microspheres plus methotrexate microspheres plus 5-flourouracil Microspheres were six and four times potent than the above combinations respectively. Therefore it may be concluded that combination of microspheres all three drugs are very effective in killing Kato III cells in very less quantity in comparison to combination of microspheres of two drugs or one drug alone.

**Stability Studies**

Stability is aimed at assuring that the drug/drug product remains within specifications established to ensure its identity, strength, quality and purity. It can be interpreted as length of time under specific conditions and storage that product will remain within the predefined limits for all its important characteristics. The main purpose of conducting stability testing of pharmaceutical products is

1. To ensure the efficacy, safety and quality of active drug substance and dosage forms
2. To establish shelf life or expiration period
3. To support label claim

The stability data on any dosage form includes selected parameters that together form the stability profile. This stability profile is the basis for assigning the storage conditions and shelf life to pharmaceutical products. The design of the stability program for the finished product should be based on the knowledge of the behavior and properties of the drug substance and the dosage form. Therefore medicines do not stay indefinitely. Some can be kept for only a short time. There are many reasons why this is the case? In 1984, Rhodes listed four general causes for the limited time for which medicines can be kept and these are -

1. Loss of vehicle (such as evaporation of water or volatile ingredients).
2. Loss of uniformity
3. Change in bioavailability
4. Change in appearance

It is important to recognize and be aware of the potential for instability in both manufactured and extemporaneous products. There is need to specify storage control. In the past it was the practice in many pharmaceutical manufacturing companies to evaluate the stability of pharmaceutical preparations by observing for a year or more, corresponding to the normal time that they would remain in stock and in use. Such a method was time consuming and uneconomical. Accelerated studies at higher temperatures were also used by most companies.

Once the formulation is developed, the practical utility of the formulation depends on the maintenance of the therapeutic efficacy throughout the shelf life under different storage conditions. Various in vitro characterization parameters of microspheres were assessed after storage of the formulations for 3 to 6 months at 40°C ± 2°C, 75% RH ± 5% according to ICH guidelines and results were compared with those obtained before storage.
Minor change was recorded in particle size of all optimized formulation after storage for 3 and 6 months. Any change in surface morphology characteristics could have a remarkable influence on the performance of the formulation. The shape of microspheres was examined by SEM which was initially spherical with rough surface and there was no change in surface morphology after 6 months storage. In the analysis of drug content of microspheres statistically insignificant difference (p<0.05) was recorded in the drug content of the formulation after storage for 3 to 6 months in comparison to formulation before storage for stability. The % residual drug content was evaluated as a part of storage stability studies considering initial drug content as 100%. The degradation of microspheres was relatively very lower at room temperature. As temperature increases the degradation also increased. The shelf life (t_{10%}) was found to be 2 years 90 days, 2 years 63 days and 2 years 75 days, for M9, D6 and F6 formulations, respectively. The formulation adjuvants have negligible effect on drug stability during the storage period of more than 2 years.

Optimized formulations M9, D6 and F9 after 3 and 6 months storage were subjected to in vitro drug release study in order to check the efficacy of the formulation in maintaining its integrity in simulated gastrointestinal fluids. Results revealed that microspheres exhibited drug release in stomach for 8 hrs after 3 and 6 months storage, which was slightly decreased in comparison to release obtained from freshly prepared formulation. In vitro release study at pH 2.0 releases 72.80%, 76.42% and 69.94% respectively at 8hrs after 3 months and 73.11%, 76.76% and 70.27% respectively at 8 hrs after 6 months storage. The same formulations when freshly prepared releases 72.24%, 75.95% and 68.31% drugs at 8 hrs.

At pH 4.0 optimized formulations releases 85.31%, 89.09%, and 84.69% respectively at 8hrs after 3 months and 85.82%, 89.95%, and 85.11% respectively at 8 hrs after 6 months storage. The same formulations before storage for stability study
released 84.52%, 88.42%, and 84.03% drugs at 8 hrs. Thus the difference in drug release after storage for 3 and 6 months was insignificant (p>0.05). Best model followed by M9, D6 and F9 was same as before stability study. The similar percent of drug release before and after stability study confirmed the stability of porous microspheres after storage for 6 months and proves the efficacy of microspheres in site specific delivery of drugs in gastric cancer.

Therefore it may be concluded that all the formulations M9, D6, and F9 remained stable during 6 months stability period at 40°C ± 2°C/75% RH ± 5% RH. Shape and surface morphology of formulations did not alter after 6 months of storage in comparison to formulation before stability study. In all the formulations, there was minor reduction in drug content after storage, however that was insignificant. All the formulations maintained their integrity during stability study. After release study they showed release up to 8 hrs and best fit model was also the same before and after study.

In Vivo Studies

ZOral administration of dosage forms leads to dissolution, absorption and distribution of entrapped therapeutic moiety in various segments of GI tract depending on the characteristics of dosage forms and drug. Conventional dosage forms are orally administered with the aim of achieving systemic absorption for therapeutic action. However, some pathophysiologic conditions require stagnation of released drug in gastrointestinal environment rather than systemic absorption. The attainment of such a goal requires release of drug in that part of GI tract in a controlled manner where action of drug is required. Stomach specific drug delivery need to remain in stomach and at the same time exhibit its therapeutic action in stomach. With the advent of imaging techniques, it is now feasible to determine the release behavior of the formulation. On the basis of images having release behavior of formulation it is possible to alter the composition or characteristics of the formulation to achieve complete site specificity. In
vivo transit behavior of the porous microspheres was determined using gamma scintigraphic method. The microspheres were loaded with drug and stannous chloride then labeled with $^{99m}$Tc. In the present study, radiolabelled microspheres were orally administered to mice with the help of feeding tube with 0.5ml of water. The feeding tube was used for administration of formulation to avoid contamination and interference of radioactivity in oral cavity or throat. The gi transit path of microspheres through GI tract was monitored in mice using gamma camera. The oral administration of microspheres leads to retention of microspheres in stomach for more than 8 hrs. The scintigram was captured at different time intervals and retention time in stomach and gastric transit was recorded. The mean gastric retention time was found to be $472\pm6.2$min.

The integrity and retention of porous microspheres in the stomach was confirmed by scintigram just after administration of dosage form. After 1 hr small amount of drug was released in the stomach. The release of small proportion of drug may be due to the presence of drug at outer surface and when the formulation comes in contact with gastric fluid, it is easily dissolved and diffused out in fluid. The microspheres remained in the stomach for 2, 3, 4, 6, and up to 8 hrs. Drug release at 4 hrs to 6 hrs was highest. After 6 hrs small amount of microspheres enter to small intestine, large intestine, and to colon but maximum drug release occurs in stomach. Complete degradation of porous microspheres occurred after 12-14 hrs with consequent release of drug which was left in very minute amount. This sustained release pattern of microspheres would be highly beneficial for treatment of disease associated with the stomach. Release of drug and presence of microspheres could also be detected in stomach after 8 hrs. The $\gamma$ scintigraphic study of nonporous microspheres was also performed. The nonporous microspheres have shown gastric transit time of $105\pm4.6$ min. After 2 hrs, the formulation entered into small intestine and after 4 hrs it entered into large intestine where complete degradation of microspheres occurs. The nonporous microspheres did
not release complete drug in stomach, in all the animals indicating that porous microspheres successfully releases maximum drug in stomach.

The porous nature of microspheres might be responsible for gastroretention of dosage form in stomach for more than 8hrs. Porous microspheres are responsible for floatation for a prolonged period of time on gastric content. After 8 hrs, the formulation entered into the small intestine. This sustained release pattern of porous microspheres would be highly beneficial for treatment of disease associated with stomach. Release of the drug from microspheres could also be detected in colon after 14-16hrs.

Gamma scintigraphy confirmed the site of drug release in stomach from porous microspheres. In vivo performance of developed stomach specific delivery system was further endorsed by conducting antitumor activity on mice.

Swiss albino mice male/female aged 8 to 9 weeks were used. The animals were kept under a standard 12/12 light/dark cycle and were given food and water ad libitum. The animals were administered 2 doses of 3 mg of benzo(a)pyrene in 0.25ml of corn oil orally with 2 weeks between the doses i.e. on 1st and 15th day. The animals were sacrificed after 10 weeks using lethal chloroform anesthesia. Abdominal cavity was opened and the stomach and duodenum were isolated. Organs were flushed with PBS to remove the gastric contents. Organs were cut longitudinally to expose the lumen and were observed under stereo zoom microscope for the presence of tumors. Tumors were observed in the stomach and duodenal lumen. The B(a)P-treated mice were divided into 6 groups (n = 10). The treatment groups were administered 1000 mg/m² of methotrexate, 2000 mg/m² of 5-fluorouracil and 30 mg/m² of doxorubicin drug in a corn oil suspension or equivalent (in the case of microspheres) orally. The dosage regimen was repeated till the end of the experiment. All the data were statistically analyzed by one-way analysis of variance
These studies indicated that treatment of mice with benzo(a)pyrene resulted in 100% forestomach tumors after 10 weeks with an average of 2.47 with 6 mm size tumors per mouse compared with corn oil treated control animals. The mice were divided in twelve groups (n=6).

*Normal Dosage Regimen - Administration of 5-FU after 1hrs of methotraxate, administration of doxorubicin 15 days after administration of methotraxate and 5-FU.

The animal of first to six groups were kept empty stomach before administering drug – first group was control group and other five groups were treated with different dosage regimens i.e pure drug treatment (normal dosage regimen), Floating microspheres treatment (normal dosage regimen), floating microspheres treatment (combination of three microspheres administered at a time), floating microspheres treatment (combination of three microspheres administered at a time with half dose), floating microspheres treatment (three microspheres administered at a time with three forth dose), seven days after the last dose of B(a)P. During the initiation period (first dose) results indicated that 40.0, 53.0, 62.0, 68.0 and 46.31 percentage inhibition of tumors size in group 2, group 3, group 4, group 5, and group 6, respectively. The percentage inhibition of tumors size after second dose was 56, 68.20, 83.83, 81.14, and 54.97 for same groups, respectively.

The animal of seven to twelfth groups feed with standard diet before administering drug – seventh group was control group and rest of the groups were treated with same dosage regimens as above that is pure drug treatment (normal dosage regimen), Floating microspheres treatment (normal dosage regimen), floating microspheres treatment (combination of three microspheres administered at a time), floating microspheres treatment (combination of three microspheres administered at a time with half dose), floating microspheres treatment (combination of three microspheres administered at a time with three forth dose), seven days after the last dose of B(a)P. During the initiation period (first dose) results indicated that 45.0, 53.2, 67.0, 64.0 and
45.01 percentage inhibition of tumors size in group 8, group 9, group 10, group 11 and group 12, respectively. The percentage inhibition of tumors size after second dose was 60.14, 72.10, 89.65, 85.44 and 54.86 respectively for same groups.

The statistically significant (p<0.05) percentage inhibition in average size of tumors were obtained with each treatment group as compared with control group and pure drug solution. Results indicated that the percentage inhibition in tumor size is more in group seven to twelfth as compared to first to sixth group and concluded that if this combination therapy administered after meal will provide more inhibition of tumor.

Reduction in body weight as a result of chemotherapeutic drug induced toxicity is the basis selected for this study. The ability of porous microspheres in lowering drug induced toxicity was assessed. Healthy albino female rats of uniform body weight (150-170g) with no prior drug treatment were used. Body weights were significantly lowers in all treatment groups compared to that of control, whereas in groups administered blank microspheres body weights were comparable to control suggesting that the effect was due to drug treatment. Moreover, the body weights displayed statistically significant differences among different treatment groups, which were conclusive in showing efficacy of microspheres formulation in reducing toxicity associated with antineoplastic agent especially with doxorubicin. The microspheres evidence a statistically different protective of drug toxicity, when compared to pure drug solution which was quite evident from changes in the body weights of animals. However, the microsphere formulation could not completely eradicate this problem.