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Summary and Conclusion

*H. pylori* is a commonly prevalent human specific pathogen and causative bacteria in chronic gastritis, gastric duodenal ulcer and gastric adrenocarcinoma in humans. The World Health Organization and International Agency for Research on Cancer have assigned that *H. pylori* is a class I carcinogenic because of its relation to gastric cancer. This finding aroused the well directed interest among gastroenterologists, clinicians, therapeutic research scientists and pharmacists to develop effective therapeutic strategies for its eradication. Survival of *H. pylori* in the hostile environment of the stomach and its ability to reside at the interface of mucous layer and epithelial cells of the antral region of the stomach make treatment of *H. pylori* a challenging position. Although *in vitro* it is highly sensitive to most antibiotics but their eradication from patients is difficult even with the current best therapies. The reason for incomplete eradication may be the degradation of antimicrobial agents by gastric acid and their poor ability to penetrate the mucosa layer and short residence time of drug in the stomach. Therefore it is difficult to deliver minimum inhibitory concentration of drug from conventional drug delivery system in gastric mucous where *H. pylori* colonize.

The aim of the treatment of *H. pylori* infection in any clinical situation is complete reepithelization of gastric mucosa and eradication of *H. pylori* from the foregut. Clinical trials have shown incomplete eradication of *H. pylori* with single antimicrobial agent therapy. In 1944 National Conference on Health Consensus Development has recommended combination of antibiotic and antisecreatory drugs for the treatment of peptic ulcer diseases associated with *H pylori*. Combination therapy leads to increased eradication rate of *H pylori* and increased patient compliance.

To alleviate the problems associated with incomplete eradication of *H. pylori* and increased patient compliance, a new concept of drug delivery system which is able to localize the drug in the vicinity of bacteria is desirable. It has been proposed that as other human pathogens *H. pylori* species express multiple adhesions that would allow bacterial interaction with various target cell epitopes on mucin components including phosphatidylethanolamine and fucosylated glyco-conjugates. The drug delivery system which could plug and seal the phosphatidylethanolamine
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(PE) surface receptor as well as continuously release the antibiotic at the infection site, in effective concentration and fully active form may lead to better eradication of *H. pylori*.

To overcome these shortcomings it was thought worthwhile to develop double liposomes and hydrogel anchored lipid vesicles system *i.e.* nanolipobeads formulation. Monotherapy is not recommended for the treatment of *H. pylori* because of its unacceptably and low eradication rate therefore treatment usually involve the combination of antibiotic with gastro cytoprotective agents. The purpose of the work was to develop dual drug delivery system which could target the PE surface receptor on *H. pylori* and release the combination of antibacterial and antisecretory drug in optimum concentration in the vicinity of organism. For the present work amoxicillin trihydrate (AMOX) was selected as antibacterial drug and ranitidine bismuth citrate was chosen for its gastric mucosal protective action.

The gift sample of ranitidine bismuth citrate provided by Glaxo Smith Kline, Bombay (India) was identified by the official method (I.P., 1996). The solubility of the drug was determined in various solvents at room temperature. Among the solvents used, highest solubility of drug was found in water, methanol and ethanol (95%). It was found to be slightly soluble in ethyl acetate and isopropanol and practically insoluble in chloroform and dichloromethane. The partition coefficient of ranitidine bismuth citrate was found to be 0.0213, 0.0269 and 0.0201 in n-octanol / water, n-octanol/ PBS (pH 7.4) and n-octanol /SGF (pH 1.2) respectively. The partition coefficient value of drug also suggested its polar and hydrophilic nature. The UV spectrophotometric for the estimation of ranitidine bismuth citrate by measuring absorbance at $\lambda_{\text{max}}$ 228 nm in SGF (pH 1.2), 313 nm in SIF (pH 6.8) and 313 nm in PBS (pH 7.4) was found to be reproducible and highly sensitive. The calibration curves were prepared within the concentration range of 2-20 $\mu$g / ml for quantitative analysis of ranitidine bismuth citrate which are recorded in Table 4.1.4, 4.1.5, and 4.1.6 graphically shown in Fig. 4.1.2(b), 4.1.3 (b) and 4.1.4 (b). They were found to obey Beer’s law within the specified range as indicated by the statistical analysis. The data was found to have nearly perfect correlation (correlation coefficient greater than 0.999 was observed in all.
the cases). Hence it could be concluded that the estimation procedure was convenient, fairly reliable, sensitive, less expensive and reproducible.

The gift sample of amoxicillin trihydrate (AMOX) provided by Cadila Pharmaceutical, Ahmedabad (India) was identified by the official method (I.P., 1996). The solubility of the drug was determined in various solvents at room temperature. Among the solvents used, the highest solubility of drug was found in methanol. It was found to be freely soluble in water, sparingly soluble in ethanol and practically insoluble in hexane, acetonitrile and benzene. The partition coefficient of AMOX was found to be 0.109, 0.103 and 0.132 in n-octanol/water, n-octanol/SGF (pH 1.2) and n-octanol/PBS (pH 7.4) respectively. Partition coefficient value of drug also revealed its polar and hydrophilic nature. The UV spectrophotometric method selected for the analysis was found to be reproducible and highly sensitive. The $\lambda_{\text{max}}$ was obtained at 228 nm in SGF (pH 1.2), 272 nm in SIF (pH 6.8) and at 272 nm in PBS (pH 7.4). The calibration curves were prepared within the concentration range of 2-20$\mu$g/ml for quantitative analysis of AMOX and the data are given in Table 4.2.4, 4.2.5, 4.2.6 and graphically shown in Fig. 4.2.2 (b), 4.2.3 (b) and 4.2.4 (b), Beer’s law was obeyed within the specified range as indicated by the statistical analysis of data. The perfect correlation (correlation coefficient greater than 0.999) was observed in all the cases. The reproducibility of the method was tested by repeating the procedure, resulting in a standard error of 0.005925 in SGF (pH 1.2), 0.013481 in SIF (pH 6.8) and 0.016231 in PBS (pH 7.4). Hence it could be concluded that the estimation procedure selected for the study was quick, convenient, fairly reliable, sensitive, less expensive and reproducible.

The lipids and polymers were selected on the basis of their non interference in the estimation of drugs and suitability to the formulation. The absorbance data of ranitidine and AMOX increase of different additives in PBS (pH 7.4) are shown in Table 4.2.7 and 4.2.8. The absorbance data had shown no appreciable interference of additives in the estimation of ranitidine and AMOX.

A study of overlain spectra of AMOX and ranitidine in acetate buffer (pH 5) shows that AMOX has maximum absorbance at 272 nm whereas ranitidine absorbs
minimum at this wavelength shows. This indicates that there is considerable difference in absorbance peaks (λmax) of both the drugs and hence there was no interference of ranitidine at 272 nm. The shows the statistical analysis of the experimental data i.e. the regression equation, standard error of the slopes, intercepts and correlation coefficient are recorded in Table 4.3.4. The absorptivity was found approximately same for all the concentrations hence both drugs obeyed Beer’s law in concentration range of 2-20 μg/mL. The high value of correlation coefficient (r²) nearly unity also indicated good linearity of calibration curve of the both drugs. The Sandell’s sensitivity (SS) of AMOX and ranitidine was found to be sufficiently low indicating that the method is highly sensitive.

The recovery of AMOX and ranitidine from the standard mixture solution was found to be 99.83% and 100.44% respectively as shown in Table 4.3.5. The recovery results indicated that AMOX and ranitidine could be quantified by this method simultaneously with good accuracy. The proposed simultaneous equation method was found to be very simple and does not require any other costly instrument equipped with special package. The results showed that in the simultaneous equation method could be useful for simultaneous determination of AMOX and ranitidine in the same dosage form.

Double liposomes were prepared by glass beads method in two steps. In the first step preparation of ranitidine bearing inner liposomes were prepared by reverse phase evaporation method and the second step involved the hydration of the AMOX bearing PE lipid film above phase transition temperature of lipids on glass beads by suspension of inner liposomes. The inner liposomes were optimized for of molar composition of lipid on the basis of vesicles size, shape, polydispersity index, zeta potential, number of vesicles/mm³, entrapment efficiency and drug release. Formulation IL1, IL2, IL3, IL4 and IL5 were found to have vesicles size of 183.3±6.5 nm, 174.4±5.2 nm, 169.3±4.6 nm, 156.4±4.1 nm, and 191.3±7.1 nm respectively. The optimized molar ratio of PC: CH for preparation of inner liposomes was found to be 7:3 (Table 5.1.2). Cholesterol provides rigidity to the vesicles and act as a fluidity buffer. However at higher ratio of cholesterol as in case of formulation IL5, deformity in vesicles was observed.
The polydispersity index of inner liposomes IL1, IL2, IL3, IL4 and IL5 was found to be 0.337, 0.223, 0.128, 0.083 and 0.281 respectively. The PDI of the inner liposomes IL4 was found to be 0.083 indicating that the formulation IL4 was a monodisperesed colloidal suspension.

The zeta potential of IL1, IL2, IL3, IL4 and IL5 was found to be +10.6±0.3 mv, +9.4±0.4 mv, +8.2±0.5 mv, +6.1±0.2 mv and +5.2±0.2 mv respectively. The incorporation of higher molar ratio of cholesterol significantly affected the zeta potential at 25°C. The increase in molar concentration of cholesterol decreased the zeta potential of inner liposomes prepared using soya PC and stearylamine (SA). This may be attributed to the negative charge present on cholesterol which is due to the polar heads of cholesterol containing hydroxyl groups which can easily combine with the polar region of the PC to produce a kind of dipole tropism that decreased the liposomal surface charges (Table 5.1.2).

The vesicles count of inner liposomes IL1, IL2, IL3, IL4 and IL5 was found to have 25, 28, 29, 36 and 16 respectively. Maximum number of vesicles/mm³ X 100 i.e. 36 was obtained in case of formulation IL4. The entrapment efficiency of liposomes IL1, IL2, IL3, IL4 & IL5 was found to be 61.1±1.9 %, 64.7±1.7%, 68.3±1.6%, 72.5±2.1% and 57.6±1.8% respectively. The maximum entrapment efficiency (72.5±2.1%) was found to be in case of formulation IL4.

The surface morphology of inner liposomes formulation IL4 was observed by transimission electron microscopy. The TEM photomicrograph (Fig 5.1.1,b) shows multilamellar (multilayered) vesicles with the lamellae of vesicles evenly spaced to the core.

The percent cumulative ranitidine release from optimized inner liposomes formulation IL4 in SGF (pH 1.2), SIF (pH 6.8) and PBS (pH 7.4) was found to be 83.2±0.92%, 89.3±1.9%, and 91.3±2.7 % respectively in 72 hrs.

The inner liposomal formulation IL4 showing spherical shape, low PDI, higher number of vesicles / mm³ and high entrapment efficiency was selected for the preparation of double liposomes formulations. Furthermore double liposomes formulations were also optimized by keeping PC: CH ratio (7:3) constant and varying
the molar concentration of phosphatidylethanolamine (PE). The optimization was done on the basis of vesicles size, shape, PDI, zeta potential, number of vesicles/mm$^3$ and entrapment efficiency etc. The optimum PC: CH: PE ratio was found to be 7:3:0.2 (Table 5.1.4). The increase in the concentration of PE in outer liposomes preparation displayed deformed vesicles resulting into unspecified structures and aggregated liposomes which were seen on microscopic observation. The double liposomes formulations OL1IL4, OL2IL4, OL3IL4 and OL4IL4 were found to have vesicle size of 703.8 ±6.8 nm, 791.6±5.4 nm, 832.3±5.8 nm and 902.5± 6.5 nm respectively.

Polydispersity index of formulations OL1IL4, OL2IL4, OL3IL4 and OL4IL4 was found to be 0.172, 0.097, 0.218 and 0.291 respectively. The results indicated that formulation OL2IL4 was found to be monodisperesed colloidal suspension.

The zeta potential of formulations OL1IL4, OL2IL4, OL3IL4 and OL4IL4 was found to be +5±0.3 mv, +11±0.8 mv, +14±0.9 mv, and +18±0.7 mv respectively. The increase in molar concentration of PE in the preparation of liposomes resulted in outer bilayer of liposomes with significant increase in zeta potential which may be attributed to the cationic charge present on PE (Table 5.1.4).

The entrapment efficiency of double liposomes formulations OL1IL4, OL2IL4, OL3IL4 and OL4IL4 was found to be 59.3±.1.8, 67.9±1.3, 54.6±21 and 41.5±2.7 of amoxicillin trihydrate respectively and 68.4±3.3%, 72.3±2.6%, 69.8±1.9% and 59.3±2.8 % of ranitidine respectively. The results showed that a higher concentration of PE and cholesterol in outer liposomes after a certain extent destabilized the formulation with might have promoted the leaching of drug. The optimum ratio of PC: CH: PE for the preparation of double liposomes was found to be 7:3:0.2. Increasing concentration of PE in the preparation of double liposomes performed deformed vesicles with higher PDI and low entrapment efficiency resulting into unspecified structures and aggregated liposomes as seen by microscopic.

TEM photomicrograph of double liposomal formulation OL2IL4 showed thick dark outer layer depicting a coating of lipidic layer surrounding the inner liposomes as shown in Fig. 5.1.5(b).
The percent cumulative drug release from double liposomal formulation OL2IL4 was determined by dialysis tube method and found to be 77.2±2.1% of ranitidine and 91.4±1.8% of AMOX in 72 hrs. The linear regression coefficient value of % cumulative drug release was found very close to one indicating almost the zero order drug release. This may be attributed to the presence of additional barrier in the form of outer liposomes for the passage of ranitidine to external medium. The results clearly suggested that double liposomes could also be used for sustained release as well as dual drug delivery purpose.

The development of dual drug delivery system that combines complimentary properties of liposomes and polymeric nanoparticles i.e. nanolipobeads represent a new trend in the application of site specific and targeted drug delivery. Preparation of nanolipobeads involved three steps (a) preparation of PVA hydrogel nanoparticles by emulsification followed by freeze thaw cycling method (b) surface acylation of PVA hydrogel nanoparticles by treating with palmitoyl chloride and (c) preparation of nanolipobeads by formation of PE liposomes on acylated PVA hydrogel nanoparticles by vesicular fusion method.

The preparation of PVA hydrogel nanoparticles was optimized by various formulation and process variables e.g. PVA concentration, drug concentration, surfactant concentration, number of freeze thaw cycles, stirring speed and stirring time etc, in order to obtain nanosized particles with maximum drug entrapment efficiency.

The concentration of PVA employed in the preparation of PVA hydrogel nanoparticles was optimized by varying the concentration of PVA viz 2.5%, 5%, 10% and 15% w/v. The optimization was done on the basis of average particle size and PDI. Increasing the concentration of PVA, particle size increased and polydispersity index was slightly decreased. But above 10% w/v polymer concentration, the PDI was drastically increased probably due to swelling behavior of PVA. Formulation (NP-C3) showed an optimum size of 288.7±4.4 nm with low PDI of 0.096 imparting monodispersity, therefore selected for further optimization process (Table 5.2.1 and Fig 5.2.2).
The concentration of drug was optimized on the basis of average particle size and maximum entrapment efficiency. For this PVA hydrogel nanoparticles were prepared with varying concentration of ranitidine viz. 25, 50, 75 and 100 mg/100mg of PVA. It was observed that on increasing the concentration of drug, the entrapment efficiency increased upto 50/100 mg of PVA while on further increasing drug concentration, the entrapment efficiency gradually decreased. This could be due to the saturation of PVA with the drug and also the particle size does not significantly changed. Therefore formulation (NP-C\textsubscript{3}D\textsubscript{2}) was selected for further optimization process (Table 5.2.1 and Fig. 5.2.3).

The particle size was decreased upon increasing the concentration of span 80 in oil phase. This might be due to the increase in surface tension of aqueous phase which ultimately seem to allow the formation of nanosized particles. Optimum size of particles i.e. 284.7±4.3 nm with 89.6±1.8%, drug entrapment was obtained at surfactant concentration of 1.5% v/v. However on further increasing the surfactant concentration the particle size was decreased due to formation of micelles and entrapment efficiency was also decreased which may be due to the leaching out of the drug. Therefore formulation NP-C\textsubscript{3}D\textsubscript{2}E\textsubscript{3} was selected for further optimization process. (Table 5.2.1 and Fig. 5.2.4.)

The PVA: oil phase ratio was varied from 1:1 to 1:10 and optimized on the basis of particle size and % drug entrapment efficiency. The results showed that PVA: Oil phase ratio 1:10 yielded the smallest and more evenly distributed nanoparticles of 289.6±4.1 nm size with 90.7±1.5% entrapment efficiency. Therefore formulation NP-C\textsubscript{3}D\textsubscript{2}E\textsubscript{3}O\textsubscript{4} was selected for further optimization process (Table 5.2.1 and Fig. 5.2.5).

The stirring speed was also optimized in terms of average particle size and maximum drug entrapment efficiency. On increasing the stirring speed, the particle size of PVA hydrogel nanoparticles was decreased. At 4000 rpm, formulation NP-C\textsubscript{3}D\textsubscript{3}E\textsubscript{3}O\textsubscript{4}R\textsubscript{4} produced particles of i.e. 279.8±3.9 nm size with spherical shape and 90.4±2.3% drug entrapment efficiency (Table 5.2.1 and Fig. 5.2.6).
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The average particle size of PVA hydrogel nanoparticles was reduced with increase in stirring time. The particle size of 287.3±5.1 nm with 89.3±2.6% entrapment efficiency was found in case of formulation NP-C₃D₃E₃O₄R₄T₃ after stirring for 45 min. On stirring for more than 45 min caused the oil: PVA phase to separate due to agglomeration of the polymer PVA. Therefore this formulation was selected for further optimization (Table 5.2.1 and Fig. 5.2.7).

The physical method of cross linking *i.e.* number of the freez thaw cycles (FT cycles) for the preparation of PVA hydrogel nanoparticles was optimized on the basis terms of particle size and % drug entrapment efficiency. The study results showed that average particle size was increased with increasing FT cycles due to increased cross linking density. Formulation NP-C₃D₃E₃O₄R₄T₂FT₃ showed 274.6 ± 4.3 nm particle size with 91.6±1.5% entrapment efficiency after three FT cycles. More than three freez thaw cycles decreased the drug entrapment efficiency due to formation of rubbery gel. Therefore formulation NP-C₃D₃E₃O₄R₄T₂FT₃ was selected for the preparation of optimized PVA hydrogel nanoparticles (Table 5.2.1 and Fig. 5.2.8).

The scanning electron (SEM) photomicrograph of optimized PVA hydrogel nanoparticles shows the spherical shape of nanoparticles with slight roughness on the surface. This roughness may be due to the shrinkage of the PVA hydrogel nanoparticles during the drying process when water is evaporated. The photomicrograph is shown in Fig. 5.2.9 (a).

*In vitro* ranitidine release study from optimized PVA hydrogel nanoparticles (NP-C₃D₃E₃O₄R₄T₃FT₃) was carried out in SGF (pH 1.2), SIF (pH 6.8) and PBS (pH 7.4) by dialysis method. No initial burst release was observed in any medium which suggested that the ranitidine molecules were entrapped inside the PVA hydrogel nanoparticles. The nearly linear relationship between % cumulative ranitidine release and the square root of time was obtained for the first 10 hr which suggested that drug release from PVA hydrogel nanoparticles followed a diffusion controlled release mechanism. The % cumulative drug release from optimized nanoparticles was found to be 87.1±2.7% in SGF (pH 1.2), 92.8±2.9% in SIF (pH 1.2) and 95.2±3.1% in PBS (pH 7.4) upto 72 hrs. A slight increase in drug release was observed at higher pH,
this could be due to the increase in the degree of hydrolysis of PVA at higher pH (Table 5.3.4 and Fig. 5.2.10)

Surface acylation of PVA hydrogel nanoparticles was done by treating with palmitoyl chloride (1M) in hexane and addition of 2 ml (5 N) NaOH to induce the acylation at room temperature by keeping for 2-3 days and evaporating the solvent. The palmitic species were anchored on the surface of nanoparticles via esterification with surface hydroxyl groups. The completion of reaction was characterized by two methods (Jin et al., 1996). First method is based on the measurement of contact angle of acylated PVA hydrogel nanoparticles which aggregated and floated on water surface due to their hydrophobicity while PVA hydrogel nanoparticles sank to the bottom of the vessel and these observations were consistent with contact angle measurement. The contact angle of acylated PVA hydrogel nanoparticles was found to be 103° as compared to PVA hydrogel nanoparticles which is 90° indicating that the surface of hydrogel nanoparticles is hydrophobic. Second more promising method for characterization of acylation is the fourier transform infra red spectroscopy of nanoparticles. FTIR spectra of PVA hydrogel nanoparticles is shown in Fig. 5.2.11 (a) and that of acylated hydrogel nanoparticles is shown in Fig. 5.2.11 (b). In the FTIR spectrum of PVA hydrogel nanoparticles, a peak due to O-H streching appears at 3429.8 cm\(^{-1}\) which has been significantly suppressed in the spectrum of acylated PVA hydrogel nanoparticles. A new peak due to ester bond formation at 1710.5 cm\(^{-1}\) is observed in the spectrum of acylated PVA hydrogel nanoparticles. This peak confirmed the estrification of hydroxyl groups of PVA hydrogel nanoparticles with palmitic species. The peak due to ester C=O strech (1215 cm\(^{-1}\)) is prominent in the spectrum of acylated PVA hydrogel nanoparticles further confirmed the esterification of PVA hydroxyl groups. Peaks at 2831.1 cm\(^{-1}\) and 2925.2 cm\(^{-1}\) due to C-H strech in PVA hydrogel nanoparticles spectrum became sharp in the spectrum of acylated PVA hydrogel nanoparticles at 2848.9 cm\(^{-1}\) and 2922.3 cm\(^{-1}\). No noticeable change is observed in other peaks of both acylated PVA hydrogel nanoparticles spectra and non acylated PVA hydrogel nanoparticles spectra.

Final formation of nanolipobeads was accomplished by treating acylated PVA hydrogel nanoparticles with equal part of suspension of phosphatidylethanolamine
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(PE) liposomes bearing AMOX by vesicle fusion method. The effectiveness of method used for nanolipobead preparation was evaluated by fluorescence microscopy. The nanolipobeads were prepared by treating acylated PVA hydrogel nanoparticles with rhodamines 123 labelled PE liposomes using vesicle fusion method. The photomicrograph was taken using fluorescent microscope. The photomicrograph (Fig. 5.2.12) shows that PE liposomes associated with hydrophobic fatty acid anchored on the surface of the polymer and self organised into a membrane distributed over the surface through hydrophobic interactions.

The fusion of PE liposomes on the acylated PVA hydrogel nanoparticles was also observed by confocal laser scanning microscopy (CLSM) when viewed as a 1mm slice at the focal depth at the equator of a nanolipobeads. The photomicrograph not only shows that the homogenous phospholipid membrane surrounded on the surface of acylated PVA hydrogel nanoparticles but biocompatible lipid bilayer also provided protection for the sensing fluorophore. The CLSM photomicrograph is shown in Fig. 5.2.12 (c).

Nanolipobeads formulations were prepared by fusion of varying molar concentration of phosphatidylethanolamine (PE) liposomes on acylated PVA hydrogel nanoparticles and characterized for particle size, PDI, zetapotential and drug entrapment efficiency. The nanolipobeads formulations LB1 LB2 and LB3 were found to have the vesicle size of 832.7±6.1 nm, 779.2±5.5 nm and 773.3±4.3 nm respectively.

Polydispersity index of formulations LB1 LB2 and LB3 was found to be 0.257, 0.213 and 0.153 respectively. The result shows that the formulation LB3 having minimum value of PDI is monodisperesed in nature.

The zeta potential of formulations LB1 LB2 and LB3 was found to be +11.6±0.7, +8.1±0.9 and +5.6±0.3 mV respectively. The increase in molar concentration of cholesterol in the preparation of PE liposomes decreased the zeta potential which may be attributed to the negative charge present on the cholesterol. Further it may be due to the polar heads of cholesterol containing hydroxyl groups, which can easily combine with the polar region of the PE to produce a kind of dipole tropism that decrease the charge of nanolipobeads (Table 5.2.7).
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The maximum entrapment efficiency 65.3±1.2% of amoxicillin trihydrate and 86.1±3.2% of ranitidine was found in case of formulation LB3 which might be due to formation of stable multilayer lipid vesicles on the polymer support (Table 5.2.8 and Fig. 5.2.13).

The degree of swelling of optimized PVA hydrogel nanoparticles and nanolipobeads formulation was found to be 3.36 and 1.42 respectively. Reduction in degree of swelling in nanolipobeads formulation indicated that lipid layer fusion on the hydrogel nanoparticles acts as a barrier for water permeation could affect the release rate (Table 5.2.9 and Fig. 5.2.14).

In vitro drug release study of amoxicillin trihydrate and ranitidine from optimized nanolipobeads formulation (LB3) in acetate buffer (pH 5) was carried out by dialysis method. The amount of drug released was calculated by using simultaneous equation. The % cumulative drug released was found to be 84.7±2.9% of AMOX and 67.5±2.8% of ranitidine in 72 hrs. The release of ranitidine from nanolipobeads formulation was slower as compared to PVA hydrogel nanoparticles. This could be due to the PE lipid layer fusion on PVA support which acted as a barrier for diffusion of drug from PVA hydrogel nanoparticles and affected the release rate (Table 5.2.10 and Fig. 5.2.15). From these findings it may be concluded that release of drugs from nanolipobeds occurred in sustained and controlled manner in pH 5 which is found at the site of residence of H. pylori.

Stability is a crucial aspect of lipoidal vesicular and particulate drug delivery systems since unstability is a major set back in their acceptance as pharmaceutical marketed preparation. Optimized double liposomes (OL2IL4) and nanolipobeads (LB3) formulations were subjected to stability studies by storage at 4±1°C and 28±1°C and the change in the vesicles size, shape, number of vesicles/mm³ and % residual drug content after storage for 15, 30, 45 and 60 day(s) time interval were determined.

In case of double liposomes formulation (OL2IL4), a small variation in mean vesicles diameter and number of vesicles/mm³ was observed when stored at 4±1°C. But storage at 28±1°C resulted in increased vesicles size from 791±5.4 nm to
830.6±4.9 nm with ellipsoidal shape and number of vesicles/mm$^3$ reduced from 34 ± 2 to 18 ± 2 after 60 days. This may be attributed to the fusion of vesicles at higher temperature therefore ellipsoidal shaped vesicles, resulting into unspecified structures and aggregated liposomes were seen on microscopic observation (Table 6.1 and 6.2 and shown in Fig 6.1 and 6.2).

In case of nanolipobeads (LB3) an average particle size 811.4±4.8 nm was observed when stored at 28±1°C for 60 days. This could be due to the fusion of external membrane and association of particles at higher temperature. Therefore aggregation or clump formation was observed at higher temperature (Table 6.4 and shown in Fig 6.5). These results suggested that average size and structural integrity of the double liposomes and nanolipobeads formulation was not changed when stored at 4±1°C and were more stable when stored in a refrigerator at 4±1°C.

The % residual drug content was determined after storing the optimized double liposomes (OL2IL4) and nanolipobeads (LB3) formulation at 4±1°C and 28±1°C. The samples for analysis were withdrawn after 15, 30, 45 and 60 days while considering initial drug content of both the drug(s) as 100%. The results of analysis showed that 92.2±2.2% of amoxicillin trihydrate and 94.4±1.6% of ranitidine remained in double liposomes after 60 days when stored at 4±1°C. However 81.4±2.3% of amoxicillin trihydrate and 86.2±2.1% of ranitidine remained after storage at 28±1°C for 60 days. This could be due to the disruption of vesicles at higher temperature and subsequent leakage of the drug(s) from the double liposomes (Table 6.3 and shown in Fig 6.3 and 6.4).

In case of nanolipobeads (LB3) the result of analysis showed that 94.3±0.2% of amoxicillin trihydrate and 95.4±0.8% of ranitidine remained when they were stored at 4±1°C for 60 days. However storage at 28±1°C for 60 days, the residual drug in nanolipobeads was 83.81±1.1% of amoxicillin trihydrate and 89.69±0.9% of ranitidine. This could be due to comparatively more leaching of the drug(s) from nanolipobeads at high temperature as compared to low temperature as recorded in Table 6.5 and shown in Fig 6.6 and 6.7. The results of stability study revealed that the double liposomes and nanolipobeads formulations when stored at 4±1°C were more...
stable than those stored at 28±1°C. Therefore this study suggested that these formulations should be stored at 4°C in a refrigerator.

Bacterial cell interaction study of optimized double liposomes (OL2IL4) and nanolipobeads (LB3) formulation was carried out with *H. pylori* strain (SKP 56) generously provided by Dept. of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India. The *H. pylori* colonies were found to be translucent, grey and with an average cell size of 2-4 µm, appeared as water droplets on petri dishes having solid agar media. Modified Gram’s staining showed the change in colour of solution from yellow to pink. The microscopic examination showed that the bacterium was spiral shaped and the identification of bacteria was confirmed by biological characterization studies viz. urease test, oxidase and catalase. All tests were positive which confirmed the identity and purity of the *H. pylori* culture.

Minimum inhibitory concentrations of amoxicillin trihydrate and ranitidine were determined using disk diffusion assay and epsylometer strip test (E test). In disk inhibition assay, the zone of inhibition of *H. pylori* was 32 mm with amoxicillin trihydrate while it was 21 mm with ranitidine. The results clearly indicated that the strain of *H. pylori* (SKP-56) was sensitive to AMOX and ranitidine.

E test is a single reliable and cost effective method used to determine the *in vitro* susceptibility of *H. pylori* to antimicrobial agents. An elliptical zone of inhibition was formed in the E test. The minimum inhibitory concentration (MIC) was found to be 0.016 µg / mL of AMOX and 15 µg / mL of ranitidine.

Binding specificity and targeting propensity of double liposomes (OL2IL4) and nanolipobeads (LB3) formulation toward *H. pylori* was studied with the help of agglutination assay. In Fig 7.3 (a) phosphatidylethanolamine shows little or spotty agglutination. Mild agglutination of *H. pylori* smear was seen with the double liposomes formulation as shown in Fig 7.3 (b). Slide treated with nanolipobeads suspension showed highest agglutination reaction when incubated with *H. pylori* after Gram’s staining as shown in Fig 7.3 (c). Agglutination assay further confirmed the binding specificity and targeting propensity of PE towards receptor on *H. pylori*. This study also revealed the fact that drug targeting could be achieved by double liposomes.
and nanolipobeads formulation because of their specificity towards the PE specific receptor on the bacterial surface glycocolyx and eliciting plug and seal effect.

The turbidometric method was selected for % growth inhibition study of *H. pylori* which was based on the optical density measurement at 660 nm compared to the test organism as a blank. The % growth inhibition by the combination of plain drugs solution, double liposomes (OL2IL4) and nanolipobeads (LB3) was found to be 48.95 %, 73.21 % and 80.23 % respectively after 4 days. The study result suggested that double liposomes and nanolipobeads formulation showed better antimicrobial activity as compared to plain drug solutions. The targeting of double liposomes and nanolipobeads formulation towards PE specific surface receptor on *H. pylori* might be influential in pursuing better results. PE- *H. pylori* interaction could lead to increase in drug concentration at the surface of *H. pylori*. The study result also revealed that the nanolipobeads formulation entrapping AMOX was more effective antibacterial agent against *H. pylori* infection as compared to double liposomes AMOX bearing which might be due to higher mechanical strength of the formulation.

*In situ* adherence assay is useful for *in vitro* identification and characterization of bacterial surface adhesion receptors present on *H. pylori*. The receptors for phosphatidylethanolamine (PE) on the surface of cells of *H. pylori* were selected for the evaluation of the binding propensity of double liposomes and nanolipobeads formulation towards *H. pylori*. The protocol reported by Flalk *et al.*, (1993) was used for *in situ* adherence assay.

Biopsies an important model to study pathogenesis of microorganism and only non diseased tissue samples of human gastric antrum cells were used for the study. The ability of double liposomes (OL2IL4) and nanolipobeads (LB3) formulation to block binding capacity of FITC labeled bacteria to gastric antrum tissue sac was studied by fluorescence microscopy. It may be clearly seen from photomicrograph Fig.7.5 (a) that the gastric cell binding capacity was more when FITC loaded bacterial suspension was incubated alone without prior incubation with drug(s) formulation with tissue sac. Fig 7.5 (b) and Fig 7.5 (c) are showing less adherence of *H. pylori* to the gastric mucosa preincubated with double liposomes (OL2IL4) and nanolipobeads (LB3) formulations. This less adherence may be due to decrease in cell
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viability and binding of double liposomes and nanolipobeads to PE receptors present on the surface of *H. pylori* due to “plug and seal” effect exerted by double liposomes and nanolipobeads formulation which do not allow the bacteria to bind with the gastric antrum mucosal cells.

The in vivo studies of selected formulation on albino rats were performed according to protocol approved by Institutional animal ethical committee of Dr. H. S. Gour Central University, Sagar (M.P.). The study of serum level in animals showed that in the rats of group I which were administrated combination of plain drug(s) solution, the pharmacokinetic parameters of ranitidine were found to be $C_{\text{max}}$ 1.34±0.05 μg/mL, $t_{\text{max}}$ 1.5 hr, AUC 6.115 μg. hr / mL and elimination half life ($t_{1/2}$) 1.26 hr. The pharmacokinetic parameters of amoxicillin trihydrate were found to be $C_{\text{max}}$ 9.1±1.1 μg /mL, $t_{\text{max}}$ 1 hr, AUC 3.150 μg. hr / mL and elimination half life ($t_{1/2}$) 1.55 hr. (Table 8.1.5 and Fig 8.1.2).

In second group of rats which were administrated double liposomes (OL2IL4) formulation, the pharmacokinetic parameters of ranitidine were found to be $C_{\text{max}}$ 1.56 ± 0.05 μg/mL, $t_{\text{max}}$ 2.5 hr, AUC 7.580 μg. hr / mL and elimination half-life ($t_{1/2}$) 2.02 hr. the pharmacokinetic parameters of amoxicillin trihydrate were found to be $C_{\text{max}}$ 9.2±0.6. μg/ mL, $t_{\text{max}}$ 1.5 hr, AUC 3.180 μg hr/mL and elimination half life ($t_{1/2}$) 1.64 hr. (Table 8.1.5 and Fig 8.1.3).

In third group of rats which were administrated nanolipobeads (LB3) formulation, the pharmacokinetic parameters of ranitidine were found to be $C_{\text{max}}$ 1.79±0.8. μg / mL, $t_{\text{max}}$ 2.5 hr, AUC 9.483 μg hr/L and elimination half-life ($t_{1/2}$) 5.02 hr. the pharmacokinetic parameters of AMOX were found to be $C_{\text{max}}$ 9.3±0.8 μg/mL after 1.5 hr ($t_{\text{max}}$), AUC 3.210 μg hr/ mL and elimination half-life ($t_{1/2}$) 5.02 hr (Table 8.1.5 and Fig 8.1.4). The *in vivo* study showed that the optimized formulations OL2IL4 and LB3 released the antibiotic trihydrate slowly and in sustained manner which is desirable for the absorption of amoxicillin from upper part of the gastrointestinal tract in order to maintain the minimum effective concentration of amoxicillin for a long period of time i.e. about 6 hr.
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The antisecretory and ulcer protective activity of double liposomes (OL2IL4) and nanolipobeads (LB3) formulation was studied in absolute alcohol induced ulcerative rats and the volume of gastric juice, free acidity, total acidity and ulcer index were determined. In the animals of group I acute ulcers were produced on oral administration of absolute alcohol (2 mL/kg b.w.). The animals were found to have 163.4 ±1.3 meq/L of total acidity, 70.1±0.9 meq /L of free acidity and 3.01 ± 0.25 ulcer index.

In the animals of group II which were administered 10 ml of plain drug(s) solution 30 min prior oral dose of absolute alcohol (2 mL/kg p.o), 34.09 % reduction in the volume of gastric juice was observed. The total acidity was reduced from 163.4 ±1.3 to 101.3±1.9 meq /L. The free acidity was found to be 39.5±0.6 meq /L and ulcer index reduced from 3.01 ± 0.25 to 1.94 ± 0.19.

In the rats of group III which were administered 10 ml suspension of double liposomes (OL2IL4) (equivalent to approx. 10 mg/kg b.w. ranitidine and 5 mg/kg b.w. amoxicillin trihydrate p.o) 30 min prior to oral dose of absolute alcohol (2 mL / kg p.o) the volume of gastric juice was reduced 56.81 %. The total acidity was reduced from 163.4 ±1.3 to 58.9±1.3 meq /L. The free acidity was measured to be 19.4±0.2 meq /L and ulcer index reduced from 3.01 ± 0.25 to 1.94 ± 0.19.

The rats of group IV which were administered 10 mL suspension of nanolipobeads (LB3) formulation (equivalent to approx. 10 mg/kg b.w. ranitidine and 5mg/kg b.w. amoxicillin trihydrate p.o) 30 min prior to oral dose of absolute alcohol (2 mL /kg p.o) showed a reduction in volume of gastric juice by 72.72 %. The total acidity was reduced from 163.4 ±1.3 to 54.7±1.8 meq/L. The free acidity was found to be 16.4±0.6 meq /L and ulcer index reduced from 3.01 ± 0.25 to 0.31 ± 0.09. The observations are recorded in Table 8.2.1 and shown in Fig 8.2.1. Therefore it is clear from the results of this study that the formulation OL2IL4 and LB3 protected the gastric mucosa of rats from absolute alcohol induced ulceration as shown in the animals which were not treated with the drug(s) formulation (negative control group).

The method described by Ishak et al. (2007) was used for the % bacterial recovery and % clearance rate of H. pylori by infecting the animal with H. pylori. The
advantages of this method are the avoidance of error which may occur due to variation in the sample site because in this method the whole of the stomach is used to determine the bacterial cell count.

The % bacterial recovery and % clearance rate of *H. pylori* from infected animals after oral administration of combination of plain drug(s) solution, double liposomes (OL2IL4) and nanolipobeads (LB3) formulation were studied. The control group of rats received only physiological saline in which the mean bacterial count (Log CFU) was found to be 9.64 ± 0.35. The mean bacterial count (Log CFU) after oral administration of combination of plain drug(s) solution was found to be 5.83 ± 0.23 may be due to nonavailability of 100 % drug to the bacterium, short residence time of drugs solution in the stomach and low concentration of drugs reaching the *H. pylori* under the gastric mucus layer.

The mean bacterial count after oral administration of double liposomes (OL2IL4) formulation was found to be 3.72 ± 0.58 and in case of nanolipobeads (LB3) formulation no bacterial count was recorded. These observations revealed that nanolipobeads formulation achieved better clearance rate of *H. pylori* as compared to double liposomes formulation and combination of plain drug(s) solution at the same dose level in albino rats (Table 8.3.1).

The histopathological photograph (Fig. 8.3.1 c) of gastric mucosa of *H. pylori* infected rats after receiving combination of plain drug(s) solution shows moderate infection with a few populations of *H. pylori*. Fig 8.3.1(d) the histopathological photograph of gastric mucosa of *H. pylori* infected rat after receiving double liposomes (OL2IL4) formulation at the same dose level shows mild infection with a very little population of *H. pylori*. The Fig. 8.3.1(e) *H. pylori* infected gastric mucosa of rats after receiving nanolipobeads (LB3) formulation at the same dose level shows total clearance of *H. pylori* infection from the gastric mucosa. Therefore from the histopathological examination of *H. pylori* infected rats it is clear that animal groups III and IV receiving drugs in the form of double liposomes and nanolipobeads formulation respectively at the same doses were better cleared off *H. pylori* infection than the animals treated with the combination of plain drug(s) solution at same dose.
level. These results also showed that the drug encapsulated nanolipobeads formulation provided two times antibacterial activity against *H. pylori* as compared to double liposomes formulation and three times greater antibacterial activity against *H. pylori* than combination of plain drug(s) solution. This enhanced activity of drugs may be attributed to the targeting potential of double liposomes and nanolipobeads toward the PE receptors on surface of *H. pylori*. Thus the developed formulations were found to be more effective than the conventional dosage forms available for the treatment of *H. pylori* eradication.

**CONCLUSION**

The results obtained from all the experiments performed as a part of research work suggested that the developed double liposomes and nanolipobeads formulations may be successfully used for the treatment of peptic ulcer associated with *H. pylori*. These drug delivery systems could not only curtail or alleviates the shortcomings of conventional dosage forms but also exert a “plug and seal effect” which could also impart superior targetability to drug carriers and deliver the drugs in the vicinity of *H. pylori*.

In conclusion double liposomes and nanolipobeads based targeted dual drug delivery system would more effectively cleared off *H. pylori* from GIT than previously developed conventional dosage form. This will also increase the patient compliance. Therefore it may be concluded that development of double liposomes and nanolipobeads entrapping drug combinations have great potential as drug delivery system for eradication of *H. pylori*.

**FUTURE DIRECTIONS OF INVESTIGATION**

It is clear from these investigations that double liposomes and nanolipobeads formulation possess a number of advantages and potential applications as drug delivery system. However the following aspects require more investigations before recommending to these formulations for their clinical investigations:-

- The supported bilayer in nanolipobeads formulation needs to be further examined using other instrumental techniques like X-ray diffraction, DSC etc.
• The relationship between lipid composition and membrane properties including stability, permeability, biocompatibility, and sensitivity to chemical and biological stimulation should be investigated in a systematic manner.

• The work should be further elaborated for determining the targeting potential of vesicular double liposomes and particulate nanolipobeads by radiolabeling assay and microliter binding assay using human stomach cell lines.