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Double liposomes mediated dual drug targeting for treatment of *Helicobacter pylori* infections

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In the present study the potential of phosphatidylethanolamine (PE) lipid anchored double liposomes (DL) to incorporate two drugs in a single system is exploited as a tool to augment the *H. pylori* eradication rate. Preparation of DL involves two steps, first formation of primary (inner) liposomes by thin film hydration method containing one drug, then addition of suspension of inner liposomes on thin film of lipid containing the other drug. The success of formation of DL was characterized by optical and transmission electron microscopy. Quantitation of DL-bacterial interaction was evaluated in terms of percent growth inhibition (%GI) on reference strain of *H. pylori* ATCC 26695. To confirm specific binding efficacy of DL to *H. pylori* PE surface receptor we performed an agglutination assay. Agglutination in DL treated *H. pylori* suspension suggested selectivity of DL towards the PE surface receptor of *H. pylori*. Monotherapy is generally not recommended for treatment of a *H. pylori* infection due to the danger of development of resistance and unacceptably low eradication rates. Therefore combination therapy with amoxicillin trihydrate (AMOX) as anti-*H. pylori* agent and ranitidine bismuth citrate (RBC) as antisecretory agent were selected for the study with an expectation that this dual-drug delivery approach will exert acceptable anti-*H. pylori* activity.

1. Introduction

More than two thirds of world's population is infected with *Helicobacter pylori*. This organism is recognised as the major etiological factor in chronic active type B gastritis, gastric ulcers, and gastric cancer (Kelly 1998; Parsonnet et al. 1991; Parsonnet et al. 1994; Wotherspoon et al. 1991). *H. pylori* is a gram negative micro aerophilic bacterium which was first isolated from a human gastric biopsy specimen in 1983 (Goodwin et al. 1989; Warren and Marshall 1983). Since *H. pylori* is responsible for large number of acute and chronic stomach and duodenal complications, it is important to understand the mode of its colonisation and persistence in the mucous layer of the human stomach. Motility is supposed to be necessary for both colonisation and persistence of the bacteria in gastric mucosa (Blaser 1994). Other virulence factors are urease, which neutralizes gastric acid (Labigne et al. 1991), and superoxide dismutase (Clyne and Drumm 1996). The pH in the gastric mucus layer is thought to vary between 4 and 6.5, with occasional acid shocks of pH < 2 ocurring when the mucus layer is damaged. Since *H. pylori* demonstrates optimal growth at neutral pH, survival and growth of *H. pylori* in the hostile environment of the stomach requires mechanisms to survive acid shocks and allow growth at mildly acidic pH (Scott et al. 2002; Sting et al. 2002). Resistance of *H. pylori* to acid shocks requires the production of ammonia by urease mediated degradation of urea (Sting et al. 2002; Sachs et al. 2002).

H. pylori adheres to the apical plasma membrane of surface epithelial cells in the antrum *in vivo* (Hessey et al. 1990). It has been shown that *H. pylori* binds to a glycerolipid species, a form of phosphatidylethanolamine, prefentially found in the antrum of human stomach (Lingwood et al. 1992). Therapy with antibiotics and antisecretory drugs usually achieves greater eradication rate than monotherapy (Lind et al. 1996). Single antibiotic regimens are ineffective in eradicating *H. pylori* infection and lead to microbial resistance. A proton pump inhibitor or H_2-receptor antoganist significantly enhances the effectiveness of *H. pylori* antibiotic regimens containing amoxicillin or clarithromycin (William 2006). The drugs selected for present study are amoxicillin (AMOX) and ranitidine bismuth citrate (RBC), a novel salt of ranitidine (cation) with bismuth and citrate (anion). It posseses both the anti-secretory activity of ranitidine and mucosal protective and anti- *Helicobacter pylori* effects of certain bismuth salts (Stables et al. 1993). It has been reported that RBC and AMOX combination leads to higher eradication rate than the two drugs alone (Kulkarni and Gupta 1999).

In a novel study in which mice were experimentally colonized with *H. pylori*, RBC was more effective in eliminating gastric mucosal infection than a ranitidine hydrochloride and bismuth citrate mixture which is almost totally in soluble (McColm et al. 1996). To achieve release of drug in a controlled manner, an entire novel concept of double liposomes is utilized, inner liposomes containing RBC and outer liposomes containing AMOX. Liposomes are lyotropic liquid crystals composed of relatively biocompatible and biodegradable materials, and consist of an aqueous core entrapped by one or more bilayers of natural and/or synthetic lipids. They are versatile drug carriers, which can be used to control retention of entrapped drugs in the presence of biological fluids, controlled vesicle residence in the systemic circulation or the compartments in the body and enhanced vesicle uptake by target cells (Gregoriadis and Florence 1993).

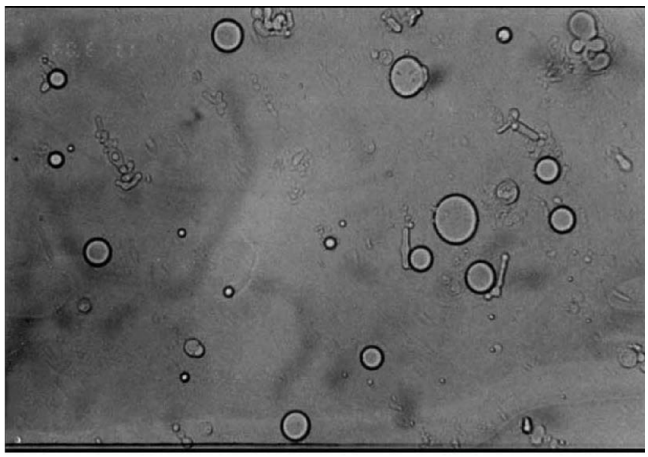
Liposomes are best suited for assessing their targetable properties because of ease of modifying their surface when compared

to other drug carriers such as nanoparticles (Grislain et al. 1983; Illum et al. 1983) and microemulsions (Hashida et al. 1977; Mizushima et al. 1982). Moreover liposomes offered a unique opportunity to deliver drugs into cells by fusion or endocytosis mechanism and practically any drug can be entrapped into liposome irrespective of its solubility.

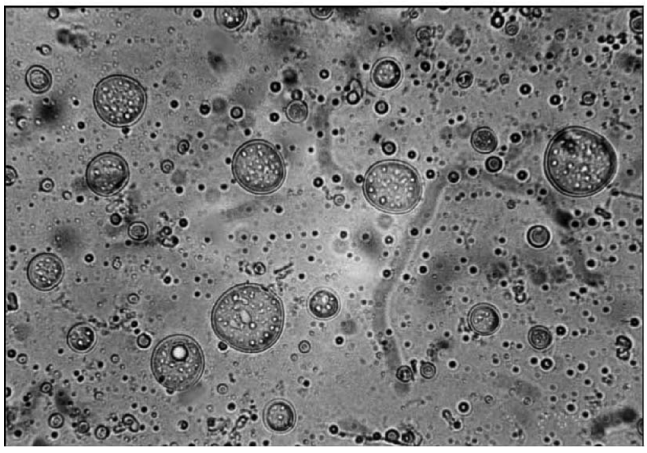
Liposomes have received much attention as potential drug carriers for the improvement of intestinal absorption of drugs when taken orally (Takeuchi et al. 1996; Rogers and Anderson 1998; Iwanaga et al. 1999; Freund, 2001) and for delivery of drugs into pathological sites such as tumors and inflammatory sites (Desormeaux and Bergeron 1998; Van slooten et al. 2000) by encapsulating drugs.

Conventional liposomes (unilamellar and multilamellar) have certain drawbacks like low entrapment efficiency, stability and release of drug after single breach in external membrane, have led to the new type of liposomal systems. The challenge has been successfully met in the form of Double Liposomes (DL). DL is a recently developed type of liposome, consisting of smaller liposomes enveloped in lipid bilayers (Kim et al. 1983; Talsma et al. 1987; Walker et al. 1997). The outer lipid layer of DL can protect inner liposomes against various enzymes (Katayama et al. 2002), therefore DL was thought to be more effective than ordinary liposomes. This concept was also supported by *in vitro* release characteristics i.e. DL formation inhibited the release of drugs encapsulated in inner liposomes (Katayama et al. 2002).

The aim of present study was to exploit the targetability potential of liposomes containing PE to surface PE receptors on *H. pylori*. Patel and Ryman 1976 first reported that the concentration of blood glucose of diabetic rats was lowered after oral administration of insulin encapsulated in liposomes. In the present study, DL were prepared by the glass beads method (Yamabe et al. 2003).



(A)



(B)

Fig. 1: Photomicrograph of plain liposomes 65× (A) and double liposomes 45× (B)

2. Investigations, result and discussion

2.1. Process design and preparation of double liposomes

Different formulations containing different ratios of phosphatidylcholine, cholesterol and stearylamine were prepared. Process variables which were optimized during preperation of inner liposomes were; effect of phosphatidylcholine, cholesterol and stearylamine ratio on vesicle size and entrapment efficiency (EE), effect of sonication time on vesicle size, effect of hydration time on vesicle shape and effect of phosphatidylcholine, cholesterol and phosphatidylethanolamine ratio on vesicle size and shape. The final formulation was selected on the basis of best entrapment efficiency which was found to be maximum with PC:Ch:SA ratio of 7: 3: 0.1. Effect of PE was studied by using different molar concentrations with a fixed ratio of PC:Ch of 7: 3. Four formulations OL_1, OL_2, OL_3, OL_4, were prepared containing phosphatidylcholine, cholesterol and phosphatidylethanolamine in ratios of 7: 3: 0.05, 7: 3: 0.1, 7: 3: 0.5 and 7: 3: 1.0, respectively. The formulations were kept in pH 1.2 and 5.0 for 3 h. Final selection was made in terms of vesicle shape which was found to be spherical (PC: Ch: PE:: 7: 3: 0.1) as compared to other formulations (Fig. 1).

Formulations IL_2, IL_3 and IL_4 were having vesicle sizes in the range of 1–2 μm after sonication for 40 s. Hence these formulations were selected for the preparation of DL. The entrapment efficiency of formulations IL_2, IL_3 and IL_4 did not differ drastically but the maximum EE had IL_3 (33%). As there was no significant increase in EE beyond PC:Ch:SA (7:3:0.5) molar ratio (IL_3), this formulation was considered to be optimum and selected for further studies.

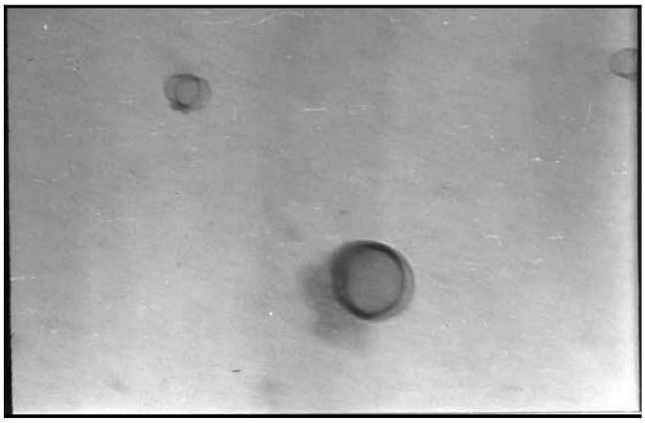
DL were prepared by the glass beads method although some other methods have been reported. Glass beads method provides simplicity, ease of preperation of formulation and easy availability of beads. Also, the results were reproducible, therefore the method was finalized.

The transmission electron micrograph (TEM) shows shape, size and surface characteristics of the double liposomes (DL, Fig. 2). The shape of DL was nearly spherical. Photographs shows clear view of liposomes entrapped within outer liposomes.

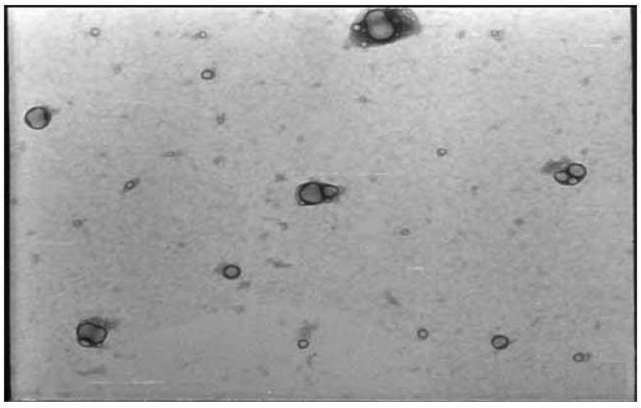
Vesicle size of inner liposomes ranged from 1.2–2.4 μm, whereas the vesicle size of double liposomes ranged from 6.7–8.2 μm. The vesicle size increased with increase in concentration of cholesterol. This may be due to a rigidizing and stabilising effect of cholesterol on the lipid film. The vesicles were subjected to sonication 40 W for different time intervals at. It was observed that the reduction in vesicle size depends not only on duration of sonication but also on initial vesicle size. The vesicles with an initial size of 4.2 μm were deformed within 40 s. Whereas those with an initial size of 7.4 μm, retained a size of 2.3 μm even after sonication for 40 s. It was realized that vesicles in the size range of 1–2.5 μm would be suitable for entrapment into outer liposomes. The hydration time had some influence on the final size and shape of the formulation. Hydration for 3 h produced perfectly spherical vesicles. Out of various compositions selected (PC:Ch:PE, 7:3:0.1) produces vesicles of optimum size range.

2.2. In vitro drug release

show the AMOX release and AMOX + RBC release profile from the inner liposomes and DL of the optimized formulation.



(A)



(B)

Fig. 2: Transmission electron photograph of plain liposomes 8600× (A) and double liposomes 6400× (B)

The maximum amount of RBC released over the period of 12 h was 29.3 ± 1.1% from inner liposomes for formulation IL_3 (PC:Ch:SA, 7:3:0.1) and AMOX + RBC release for DL was 32.6 + 1.5 and 20.3 + 2.8, respectively.

Formulations finalized were IL_3 in case of inner liposomes and OL_2 in case of DL, hence the final release of AMOX and RBC was studied from DL containing both the drugs. Results show that drug release from inner liposomes was subsequently retarded when entrapped in DL. This suggests that the ability of DL to retard drug release from inner liposomes can be exploited as sustained release of drug thereby releasing drug for a longer duration of time as plain liposomes do.

2.3. In vitro growth inhibition studies

Minimum inhibitory concentration (MIC) of Amox for a reference strain of *H. pylori* ATCC 26695 was determined using an Epsylometer-test strip (containing AMOX from 0.16 to 256 μl) and it was found to be 0.016 μg/ml.

Selected formulation OL_2IL_3 was tested by the disk inhibition test for its capacity to inhibit *H. pylori* growth *in vitro*. The inhibition zone of 32 mm clearly establishes the fact that the formulation releases RBC and AMOX in sufficient concentrations to inhibit the growth of *H. pylori*.

The DL-cell line interaction and adsorption to the bacterial cell surface could be exploited for effective localization and targeting of DL to a pre-selected bacterial cell line. The method selected for the present study was turbidometry based on optical density measurements. Quantification of DL-bacterial interaction was approached in terms of percentage growth inhibition (% GI). One of the major advantages of culture is that it allows sensitive testing of *H. pylori* to the agents used in treatment. The results

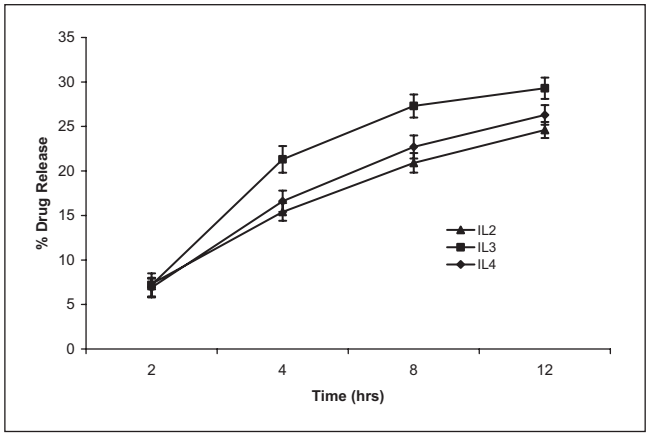


Fig. 3: RBC release from inner liposomes

of DL-bacterial interaction study could be arranged in an order of % GI performance:

OL_2IL_3 > AM-RB > AM > Placebo I

The effect of the selected formulation bearing both RBC and AMOX was investigated and compared with pure AMOX and AMOX+RBC solutions. The maximum GI of AMOX and AMOX+ RBC was found to be 27.27% and 72.72%, respectively. Drug solutions of AMOX and RBC were used in concentrations equivalent to MIC i.e. 2.0 μg/ml and 212.5 μg/ml, respectively. While maximum GI of formulation was 86.75%, bearing a concentration of antimicrobial drugs equivalent to MIC. The results revealed a GI of DL superopr tp an equal amount of pure drug solution by almost 14%. It could be assumed that the affinity of DL towards PE specific surface receptors of *H. pylori* might be a reason for the better results. PE-*H.pylori* interaction could lead to an increase in drug concentration as well as a higher therapeutic index at the *H. pylori* surface. Fig. 3, Fig. 4

2.4. Agglutination assay

Clumps of *H. pylori* were found in the double liposome treated suspension slides showing agglutination while *H. pylori* suspension without double liposome did not show any agglutination (Fig. 5). This agglutination ability of DL towards *H. pylori* might be due to the retention of PE specific receptor on *H. pylori* surface. Agglutination assay further confirmed a previous report that PE is more likely to function as a receptor for *H. pylori* related adhesion (Lingwood et al. 1992). It also stresses the fact that drug targeting could be achieved with DL having

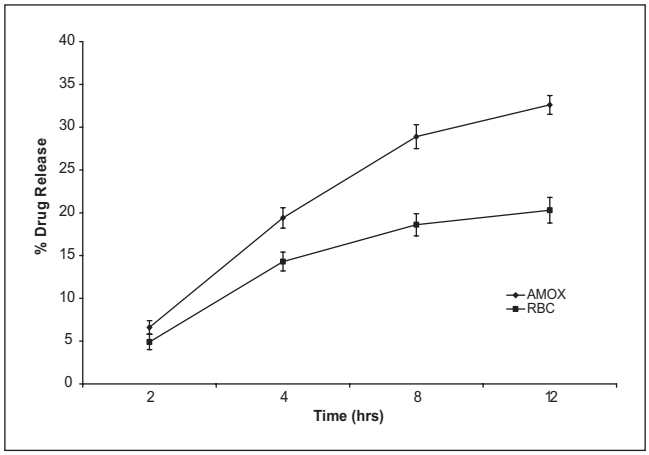
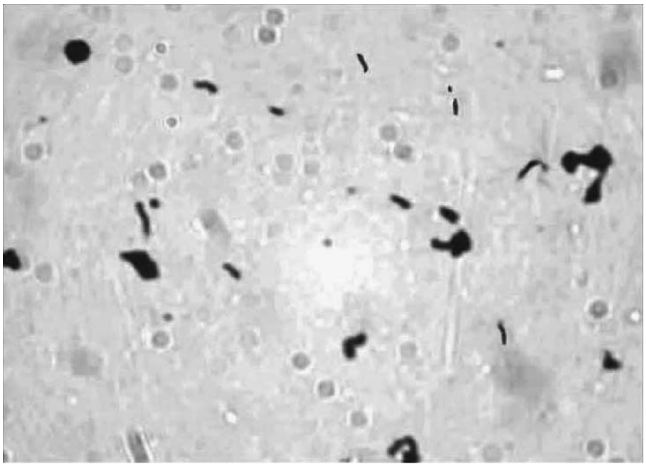
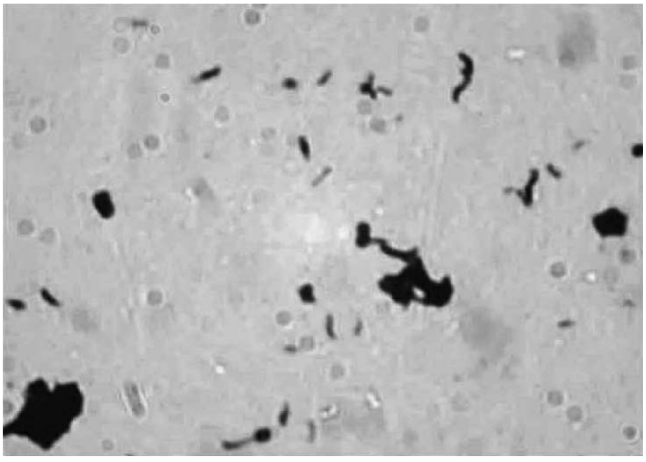


Fig. 4: AMOX and RBC release from DL



(A)



(B)

Fig. 5: Gram stained *Helicobacter pylori* smear with (B) and without (A) double liposomes

specificity towards the PE specific receptor on the bacterial surface glycocalyx.

The present work is the first evidence demonstrating the potential of DL as dual-drug delivery via targeted approach, i.e. AMOX and RBC bearing DL for eradication of *H. pylori* via PE-*H. pylori* interaction. Significant work has been done on the same line at our research laboratory (Umameshwari et al. 2003). The results obtained from all the studies performed suggested that the developed system DL might be successfully used for the effective treatment of *H. pylori*. The system could not only curtails or alleviates the shortcomings of conventional drug delivery system (CDDS), but can plug and seal the PE surface receptor of *H. pylori*. It could also impart superior targetability and protect the drugs from harsh gastric environments.

This approach may also lead to increase in patient compliance as combination therapy is given through a single dosage form.

3. Experimental

3.1. Materials

Lipids such as phosphatidylcholine (PC), phosphatidyle thanolamine (PE), cholesterol and stearylamine (SA) were purchased from Sigma chemical company (St. Louis), USA. Glass beads (0.5–1.5 mm) were obtained as courtesy from the Dept. of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India. Ranitidine bismuth citrate and amoxicillin were obtained as gift samples from Glaxo Smith Kline, Florida, Australia and Alkem lab. ltd. Mumbai, India, respectively. All other chemicals were of analytical grade.

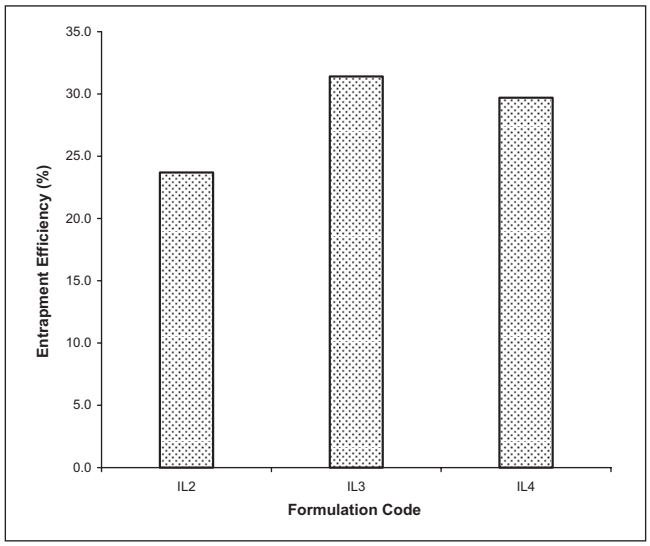


Fig. 6: Entrapment efficiency of selected formulations

3.2. Preparation of inner liposomes using thin film hydration method

The proposed system was based on multi vesicular vesicles prepared by thin film hydration technique, followed by sonication at lower frequency. Five different formulations i.e, IL_1, IL_2, IL_3, IL_4, IL_5, were prepared containing phosphatidylcholine, cholesterol and stearylamine in ratios of 9:1:0.01, 8:2:0.05, 7:3:0.1, 6:4:0.5 and 5:5:1 respectively. Posphatidylcholine, cholesterol and stearylamine were dissolved in chloroform contained in a round bottomed flask. The organic solvent was evaporated by rotating the flask to form a thin film on the walls of round bottom flask. The dried lipid film was hydrated with acetate buffer (pH 5.0) solution containing different amounts of RBC (10, 15, 20 mg). The resulting liposomal suspension was kept for hydration at room temperature for 3 h to allow swelling and rigidization of vesicles. Liposomal suspension was washed with acetate buffer (pH 5.0) by alternate centrifugation (10,000 rpm for 15 min) and resuspension cycle to remove free RBC. The liposomal suspension thus obtained was subjected to sonication (probe sonicator, Imeco, India) to get inner liposomes containing RBC. The effect of phosphatidylethanolamine was studied by using its different molar concentration with ratio of phosphatidylcholine and cholesterol (7:3) (Fig. 6, Fig. 7).

3.3. Preparation of double liposomes (DL)

PC, Ch and PE were dissolved in chloroform in a molar ratio of 7:3:0.1. AMOX was dissolved in minimum quantity of ethanol (90% solution) and mixed with the above solution. The resultant solution was transferred onto glass beads contained in a round bottom flask (25 g). The organic solvents were evaporated at room temperature to form a thin lipid film containing AMOX over glass beads. This film was hydrated with the suspension of inner liposomes, with gentle shaking for 10 min. The DL, thus obtained were separated by aspiratory filtration.

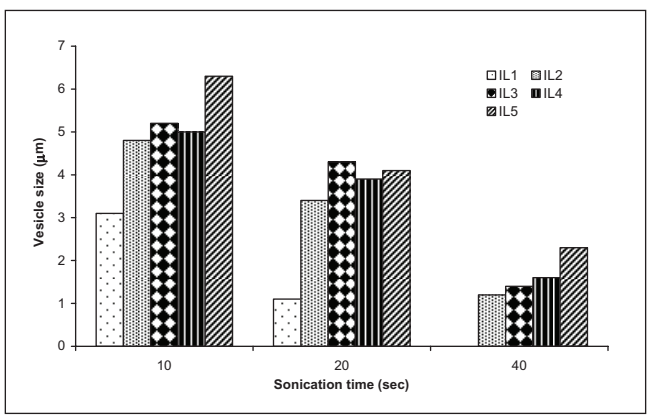


Fig. 7: Effect of sonication time on vesicle size of various formulations

3.4. Charecterization of DL

3.4.1. Microscopic observation

The prepared inner liposomes and DL were charecterized for their shape and vesicle type by optical and transmission electron microscopy. The liposomal suspensions with different formulation codes were mounted on a glass slide and photomicrographs were taken by Optical (Leica Microscope). For TEM studies grids were prepared using 2% phosphotungustic acid solution and photographs were taken at different magnifications (Fig. 2).

3.4.2. Determination of size and size distribution

Vesicle size distribution of various formulations prepared durin optimization was studied by dilution of sample (2 ml) with acetate buffer and analysed using particle size analyser (Cilas, France)

3.4.3. Encapsulation efficiency (%EE)

The percentage of drug entrapment was determined by taking 1.0 ml of liposomal suspension and separating the unentrapped drug via centrifugation at 10,000 rpm for 15 min. Pellets thus obtained were suspended in 10 ml of acetate buffer (pH 5.0) and the process was repeated 2–3 times to remove free drug as completely as possible. Triton-X-100 solution (0.1 % v/v) was used for the lysis of the liposomal membrane. The suitably diluted samples were analyzed for percent drug entrapment at 314 nm using a Shimadzu 1601 UV/Visible spectrophotometer against acetate buffer solution as blank. The Encapsulation efficiency was calculated using following equation (1)

$$\text{EE}\ (\%) = \frac{\text{Amount of RBC encapsulated in liposomes}}{\text{Amount of RBC used}} \times 100 \quad (1)$$

Similarly, the above procedures were performed for DL, and encapsulation efficiency (%) was worked out according to following equation (2)

$$\text{EE}\ (\%) = \frac{\text{Amount of AMOX encapsulated in DL}}{\text{Amount of AMOX used}} \times 100 \quad (2)$$

3.4.4. Study of in vitro release

The various vesicular formulations were taken in a dialysis tube (Sigma chemicals, USA). Both ends of the dialysis tube were tied with a thread. The assembly was hanged in a beaker containing 200 ml of acetate buffer (pH 5.0). The beaker was placed over a magnetic stirrer. The temperature of the assembly was maintained at $37 \pm 1\,^{\circ}C$ throughout the study. Samples were withdrawn at specific time intervals and an equal volume of fresh media was added to replace the withdrawn sample. After appropriate dilutions, the samples were measured at 314 nm against blank using Shimadzu 1601 UV/Visible spectrophotometer. Release pattern of RBC and AMOX from inner liposomes and DL is shown in Table 2 and 3.

A literature survey revealed that no method for simultaneous estimation of AMOX and RBC has been reported elsewhere. The present work is the first report, which illustrates a simple, accurate, economical and reproducible procedure for simultaneous spectrophotometric estimation of both the drugs. For simultaneous spectroscopic measurement, stock solutions of both the drugs were prepared by accurately weighing 10 mg each of AMOX and RBC into 10 ml of acetate buffer and diluted separately to 100 ml with same to obtain final concentrations of 100 μg/ml. The solutions were further diluted to give a concentration range of 5–50 μg/ml of each drug. Overlaind spectra of both the drug solutions were scanned (Fig. 8) and it was observed that AMOX and RBC showed maximum absorbance at 228 nm and 314 nm, respectively. It was observed that RBC and AMOX showed almost zero absorbance at 228.0 and 314 nm, respectively. Thus, these two wavelengths were employed for the estimation of AMOX and RBC without any interference.

The calibration curves of each drug were plotted at both the μ_{max} i.e., at 228 and 314 nm, in a concentration range of 5–50 μg/ml. Absorptivity of both the drugs were calculated, which showed good linearity. Three mixed standard solutions with concentrations 10, 20, 30 μg/ml of AMOX and 15, 25, 35 μg/ml of RBC were prepared in acetate buffer. All these standard mixtures were then scanned at 228 and 313.38 nm λ_{max} and absorbance were recorded. The spectral data from these scans were used to determine the concentration of two drugs in sample solutions.

Simultaneous release of RBC and AMOX from DL were determined by the following same procedure, concentration of RBC and AMOX were calculated by the simultaneous equation method (Dangi et al. 2005).

The cumulative amount of drug released from the formulations were calculated using the following equation (3)

$$\text{Cumulative amount released}\ (\%) = \frac{M_t}{M_{actual}} \times 100 \quad (3)$$

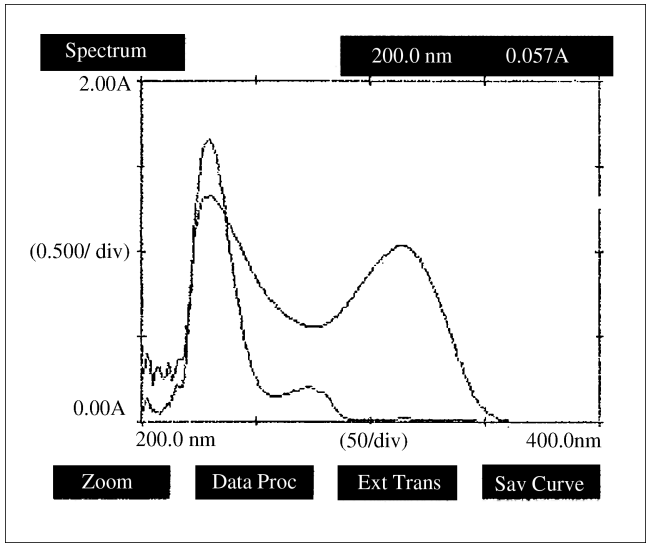


Fig. 8: Overlain spectra of AMOX and RBC

where M_t is the amount of drug released from the formulations at time t and M_{actual} is the actual amount of drug loaded in formulations at the time of preparation.

To study the accuracy, reproducibility and precision of the proposed method, recovery studies were carried out by taking standard mixture solution of both AMOX and RBC and absorbance was determined at 228 and 313.38 nm, respectively.

3.4.5. In vitro growth inhibition studies

The *in vitro* growth inhibition studies were performed on developed system using the reference strain of *H. pylori* ATCC 26695. Antibiotic susceptibility testing and disc inhibition assay were performed to evaluate the ability of formulations to inhibit *H. pylori in vitro*. Minimum inhibitory concentration of *H. pylori* strain for amoxicillin was determined using the Epsylometer-test strip (Mishra et al. 2002). Sensitivity of double liposome bearing RBC and AMOX was tested by disk inhibition assay for their capacity to inhibit *H. pylori* growth *in vitro*. The effect of double liposome on *H. pylori* growth was monitored and estimated by measuring the diameter of the zone corresponding to the area of *H. pylori* growth inhibition.

The stock culture was grown on Brucella chocolate agar containing antibiotic supplement (vancomycin 6 mg/ml, amphotericin-B 2 mg and polymyxin B 2500 IU/ml). The medium was prepared using 7% sheep blood as per the standard method (Mishra et al. 2002). Plates were incubated at $37\,^{\circ}C$ microaerobically by candle jar technique. *E. coli* growth was used as to maintain microaerobic condition and a sterile cotton wool soaked in sterile distilled water was also put to provide high humidity condition. Plates were harvested under sterile condition for 72 h, into brucella chocolate agar and finally the isolated colonies of *H. pylori* were subcultured in liquid broth. In liquid medium appearance of turbidity was taken as an indication of growth.

To study the effect of formulations on *H. pylori* growth, 10 ml suspension of *H. pylori* brucella broth (10^9 cfu/ml) were taken in culture flask. Various double liposomes formulations were added to the tubes and all the tubes were incubated at $37\,^{\circ}C$ in microaerophilic atmosphere.

The effect of following formulations on bacterial growth was investigated.

1- Plain drug (AMOX) = AM
2- Combination of drugs (AMOX + RBC) = AM + RB
3- DL bearing AMOX and RBC = OL_2IL_3
4- Drug free DL = Placebo I (Control I)

The growth inhibition was calculated for each time interval in terms of optical density (OD) at 600 nm using a Shimadzu-1601 UV/visible spectrophotometer. The method selected for the proposed study was turbidimetry based on optical density measurements (Umamaheshwari et al. 2003).

The percentage growth inhibition (% GI) was calculated using following equation (4)

$$\%\text{GI} = \frac{\text{OD of test organism at a particular time interval - OD of test mixture at same time interval}}{\text{OD of test organism at a particular time interval}} \times 100 \quad (4)$$

3.4.6. Agglutination assay

Binding of *H. pylori* to phosphatidylethanolamine (PE) anchored double liposomes was assayed by a agglutination assay. suspension of *H. pylori* was

prepared in normal saline and turbidity was adjusted to McFarland Standard 1 (McFarland Standard 1 (3.0×10^8 cfu/ml). Bare DL (8 ml suspension) was added to *H. pylori* suspension. *H. pylori* suspension without DL was taken as negative control. The tubes were kept for 30 min at 37 °C. Smears were prepared and stained by modified Gram's stain. Slides were observed at different magnifications (Fig. 1).

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