**Research Paper**

**Liposomal Drug Delivery of Metronidazole for the Local Treatment of Vaginitis**

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**ABSTRACT:** The present investigation was aimed to formulate liposomes containing metronidazole for local therapy of vaginitis, capable to efficiently deliver entrapped drug during an extended period of time. Liposomes were prepared by simple thin film hydration technique using soya lecithin and cholesterol. Some preliminary trials and 3^2 factorial design were conducted to optimize the formulation. The drug to Soya lecithin to cholesterol ratio and volume of hydration media were chosen as independent variables. The percentage drug entrapment, particle size and drug release at 12 h were chosen as dependent variables. To achieve application viscosity and further improve the stability of liposomes, the prepared liposomes were incorporated in the bioadhesive carbopol 934P gel (1%), and the system were evaluated for the in vitro drug release and drug stability in phosphate-buffer pH 4.5 and simulated vaginal fluid (VFS) at 37±1°C. Stability study for liposome suspension and liposomal gel were carried out. All the performed experiments confirm the applicability of liposomes as a novel drug carrier system for the local treatment of bacterial vaginosis.

**KEYWORDS:** Metronidazole, liposome, mucoadhesive gel, vaginal fluid stimulant, thin film hydration.

**Introduction**

Metronidazole is classified therapeutically as an antibacterial and antiprotozoal agent, (Jack D. Sobel et al., 2006) indicated for the treatment of bacterial vaginosis (BV) (Jack D. Sobel et al., 2006, Hillier SL et al., 1993). For the treatment of vaginitis, local administration of metronidazole has been favoured due to numerous side effects, toxicity, and teratogenic potential of the systemically applied drugs (Hope MJ et al., 1993). Metronidazole vaginal gel is the intravaginal dosage form of the synthetic antibacterial agent, metronidazole USP at a concentration of 0.75% (Grewal MS. 1966). The limitation of local administration of metronidazole in vaginal therapy is the relatively short residence time of the drug at the site of application. To achieve desirable therapeutic effect, vaginal delivery systems for antimicrobial agents need to reside at the sites of infection for a prolonged period (Kukner S. et al., 1996, Fischbach F. et al., 1993). Hence, there is a need to develop effective drug delivery systems that should prolong the contact of the drug with vaginal mucosal surface and enable sustained release of incorporated drug. Liposomes have been widely used as drug carriers in topical treatments of diseases, especially in dermatology. They are capable of incorporating a variety of hydrophilic and hydrophobic drugs, to enhance the accumulation of drug at the administration site and to reduce side effects and incompatibilities (Hope MJ et al., 1993).

Since liposomes can provide sustained and/or controlled release of entrapped drug, they are considered for vaginal application, too (Jain SK. et al., 1997).

However, the major limitation of using liposomes topically and vaginally is the liquid nature of preparation. That can be overcome by their incorporation in an adequate vehicle where original structure of vesicles is preserved (Skalko N. et al., 1998). It has already been shown that liposomes are fairly compatible with gels made from polymers derived from cross linked poly (acrylic acid), such as Carpool® resins (Pavli Z. et al., 1999). Moreover, some Carpool® has proved excellent bioadhesive properties on the mucosal surface that would increase residence time in the vaginal cavity and at the same time increase absorption of the drug (Dittgen M. et al., 1997). Therefore, it seemed logical to choose gels prepared from Carbopol 934P as a vehicle for the incorporation of liposomes destined for vaginal delivery. A previous study has suggested application of liposomes containing antimicrobial drugs for the local therapy of vaginitis (Pavelic Z. et al., 1999), continuing that research, here, we report on the design and in vitro evaluation of a bioadhesive liposomal gel of metronidazole.

**Materials and Methods**

Metronidazole IP was received as a gift sample from Newcare Pharma Ltd., Mahesana, soya lecithin, cholesterol
and carbopol 934P were purchased from S.D. Fine Chem. Ltd., Mumbai. All other reagents used were of analytical reagent grade.

**Preparation of liposomes (Lasic DD. et al., 1998)**

Liposomes of metronidazole were prepared by the thin film hydration technique. Specified quantities of soya lecithin and cholesterol were dissolved in 8 ml of an equivolume solution of chloroform and methanol (1:1 v/v) at 60°C ± 2°C. Metronidazole was dissolved in 2ml of methanol. Then was added this drug solution to the Soya lecithin and cholesterol solution. This solution mixture was taken in 250 ml round bottom flask and was rotated in the rotary flash evaporator at 100 rpm for 10 minutes in thermostatically controlled water bath at 60°C under vacuum (250 mm of Hg). The organic solvent was evaporated to yield a thin, uniform and dry lipid film. The thin, dry lipid film formed was hydrated using 8 ml of phosphate buffer pH 7.4 and the flask was rotated once again same speed as before and at the 40°C using some glass beads (0.2 cm diameter), at the for 40 minutes. The liposomal suspension formed in flask, was transferred in to a suitable glass container.

**Experimental design (Cochran et al., 1992)**

A 3² factorial design was employed to study the effect of independent variable, drug : Soya lecithin : cholesterol ratio (X1) and volume of aqueous phase in ml (X2) on dependent variable percent drug entrapment, percentage cumulative drug release at 12 hours (Y12) and particle size (µm). The experimental design is presented in Table 1. Formulations were characterized with respect to size distribution and average vesicle size, shape, % encapsulation efficiency and in vitro skin permeation.

**Microscopy**

All the batches of liposomes prepared were viewed under Leica DMIL inverted Fluorescence microscope to study that size and shape size of 100 liposomal vesicles from each batch at different location of slide and average size of liposomal vesicles determined. The results are given in Table 1 and photomicrograph is given in Fig. 1.

### Table 1 Full Factorial Experimental Design Layout

<table>
<thead>
<tr>
<th>Batch</th>
<th>Variable level</th>
<th>% Entrapment efficiency</th>
<th>Particle size (µm)</th>
<th>Y12</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>-1 -1</td>
<td>23.21</td>
<td>14.53</td>
<td>38.42</td>
</tr>
<tr>
<td>F2</td>
<td>-1 0</td>
<td>26.03</td>
<td>12.98</td>
<td>45.58</td>
</tr>
<tr>
<td>F3</td>
<td>-1 1</td>
<td>15.18</td>
<td>15.04</td>
<td>32.18</td>
</tr>
<tr>
<td>F4</td>
<td>0 -1</td>
<td>29.17</td>
<td>13.15</td>
<td>51.91</td>
</tr>
<tr>
<td>F5</td>
<td>0 0</td>
<td>36.45</td>
<td>11.77</td>
<td>62.64</td>
</tr>
<tr>
<td>F6</td>
<td>0 1</td>
<td>23.36</td>
<td>13.05</td>
<td>48.80</td>
</tr>
<tr>
<td>F7</td>
<td>1 -1</td>
<td>12.78</td>
<td>15.71</td>
<td>31.08</td>
</tr>
<tr>
<td>F8</td>
<td>1 0</td>
<td>29.12</td>
<td>12.37</td>
<td>36.76</td>
</tr>
<tr>
<td>F9</td>
<td>1 1</td>
<td>34.48</td>
<td>14.20</td>
<td>43.15</td>
</tr>
</tbody>
</table>

Y12 is drug release after 12 h, Translation of coded levels in actual units is as follows: coded level for X1, drug: soyalecithin: cholesterol (mg) are, -1 is 1:2:1, 0 is 1:4:2 and 1 is 1:6:3, while for X2, hydration volume (ml) are, -1 is 6, 0 is 8 and 1 is 10.

**Fig. 1** Photomicrograph of liposomes by light microscopy.
Encapsulation efficiency and % drug content (Betagiri GV. Et al., 1993, New RRC. 1990)
The liposomal suspension was centrifuged for 1 h. at 1500 rpm at 0°C for 15 min to allow the sedimentation of liposomes in the form of pellets. The supernant was collected and the pellets resuspended in fresh PBS. The definite amount of supernatant and sediment were diluted with 1ml of chloroform and 9 ml of methanol, and the absorbance was recorded by Shimadzu 1700 UV visible spectrophotometer at 311 nm and the amount of metronidazole in supernatant and sediment were determined. Percentage of drug entrapped was calculated by using the formula:

\[
\text{% Entrapment (% EE)} = \frac{\text{Amount of drug in sediment}}{\text{Total amount of drug added in sample}} \times 100
\]

The mean percentage of drug entrapment was measured after performing the experiment in triplicate. Percentage drug content was calculated by adding the amount of drug present in supernatant and amount of drug entrapped in vesicles.

Kinetic of drug release from liposomes (New RRC. 1990)
A 10 cm long portion of the dialysis tubing was made into a dialysis sac by folding and tying up one end of the tubing with thread, taking care to ensure that there would be no leakage of the contents from inside the sac. The sac was then soaked overnight in pH 4.5 phosphate buffer. The wet sac was filled up with pH 4.5 phosphate buffer solution respectively and examined the leaks. The sac was then emptied and 1 ml of the liposomal suspension of optimized batch was accurately transferred into the sac. Diluted this suspension with pH 4.5 phosphate buffer or vaginal fluid simulant to make the volume 5 ml that becomes the donor compartment. The sac was once again examined for any leak and then suspended in a glass beaker containing 200 ml pH 4.5 phosphate buffer or vaginal fluid simulant, which acts as receptor compartment. The temperature of the contents of the beaker was maintained at the appropriate level required using the thermostatic controlled heater of the magnetic stirrer. The contents of the beaker was closed with aluminium foil to prevent any evaporative losses during the experimental run. The temperature of the system was monitored at a regular interval of time. At predetermined interval of time 5 ml aliquots were withdraw from the receptor compartment and subjected to analysis using Shimadzu 1700 UV- Visible Spectrophotometer. From this the percentage cumulative drug release was determined using calibration curve for Metronidazole in pH 4.5 phosphate buffer or vaginal fluid simulant (VFS). Fresh medium was used to replenish the receptor compartment. The cell was inverted and immersed slightly in 200.0 ml of pH 4.5 phosphate buffer or vaginal fluid simulant in a beaker at 37°C ± 1°C, and stirred at 100 rpm for 12 hours. Samples of 5.0 ml were withdrawn at certain intervals and assayed spectrophotometrically at 311 nm.

Storage stability studies (Betagiri GV. Et al., 1993)
In the present investigation both liposome suspension and liposomal gel were subjected to stability studies, in triplicate, at condition according to ICH guidelines i.e. 2-8°C with ambient humidity and at room temperature with ambient humidity. The prepared liposomal gels (3 gm) were taken in 10 ml amber color glass vials (USP Type 1 glass). The vials were then sealed using grey bromobutyl rubber stoppers and aluminium seals. For the condition of 2-8°C with ambient humidity, the vials were placed in refrigerator out side the ice chamber. At the intervals of 10, 20 and 30 days, liposomal gel samples were withdrawn from glass bottles placed at both the conditions and subjected for the analysis of the mean particle size, size distribution and drug retention efficiency of liposomes and pH of the gel formulations.

Preparation of topical dosage form (Pavellic Z. et al., 2001)
Liposomes containing Metronidazole (separated from the unentrapped drug) were mixed into the 1% Carbopol 934P gel with an electrical mixer (25 rpm, 2 min). The amount of liposomes of optimized batch (F5) was added to the gel, such that the prepared gel having 0.75% Metronidazole concentration. Control gels were made under the same conditions, instead of liposomes, and those samples contained free drug. Control experiments (Carbopol gels with free Metronidazole) were done simultaneously and under the same conditions.

The Physicochemical parameters of gel like pH, viscosity and content uniformity were measured.

In vitro diffusion study (Pavellic Z. et al., 2001)
The method is similar to liposome suspension but in place of liposome suspension, semisolid formulation (1 g) was taken on the cellophane membrane and tied securely to one end of the tube, the other end was kept open to ambient conditions. The cell was inverted and immersed slightly in 200.0 ml of pH 4.5 phosphate buffer or vaginal fluid simulant in a beaker at 37°C ± 1°C, and stirred at 100 rpm for 12 hours. Samples of 5.0 ml were withdrawn at certain intervals and assayed spectrophotometrically at 311 nm.
Percentage cumulative drug release of factorial design batches in VFS pH 4.5 shows that Metronidazole liposomes follows biphasic pattern, initial rapid release followed by sustain release. Batch F5 shows highest drug release at 62.82% when compared to other batches (Fig. 3).

The polynomial equation is generated by carrying out multiple linear regression analysis and F- statistics to identify statistically significant. 

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2
\]

where \(Y\) is the depended variable, \(b_0\) is the arithmetic mean response of the nine runs, \(b_i\) is the estimated coefficient for the factor \(X_i\). The main effects (\(X_1\) and \(X_2\)) represent the average results of changing one factor at a time from its low to high value. The interaction (\(X_1X_2\)) shows how the response changes when two factors are changed simultaneously. The polynomial terms (\(X_1^2\) and \(X_2^2\)) are included to investigate non-linearity.

A \(3^2\) factorial design was employed to study the effect of independent variable, drug : soya lecithin : cholesterol ratio (\(X_1\)) and volume of aqueous phase in ml (\(X_2\)) on dependent variable percent drug entrapment, percentage cumulative drug release at 12 hours (\(Y_{12}\)) and particle size (\(\mu m\)). The polynomial equation was generated for percentage entrapment efficiency as, 

\[
Y = 34.59 + 1.888X_1 + 1.415X_2 + 7.275X_1X_2 - 7.088X_1^2 - 7.398X_2^2 \quad (R^2=0.869, F= 4.012, DF= 8)
\]

for \(Y_{12}\) is 

\[
Y = 59.385 - 0.865X_1 + 0.453X_2 + 4.577X_1X_2 - 16.588X_1^2 - 7.403X_2^2 \quad (R^2=0.919, F= 6.107, DF= 8)
\]

and for mean particle size is 

\[
Y= 11.385 -0.045X_1 - 0.183X_2 - 1.90X_1X_2 - 0.505X_1^2 + 1.481X_2^2 \quad (R^2=0.956, F= 13.14, DF= 8)
\]

The polynomial equation can be used to draw conclusions after considering the magnitude of the coefficient and the mathematical sign it carries, i.e. positive or negative. The data demonstrate that both \(X_1\) and \(X_2\) affect the percentage entrapment efficiency, mean particle size and in vitro drug release (\(Y_{12}\)). Counter plots were generated based on above equations. The shaded area in the Fig. 4 demonstrated the optimize area of the individual dependent variables to get desired sustained release profile.
Drug release kinetic study for optimized liposome batch (F5) was carried out in phosphate buffer pH 4.5 and in VFS to mimic the physiological condition of vagina. Drug release from liposome compare with plain drug release (Fig. 5). The mean flux value for liposome and plain drug in VFS is 37.54 and 149.34 respectively while in phosphate buffer pH 4.5 is 39.88 and 155.12 respectively which indicate that liposome provide sustained release of entrapped drug. So, liposomes containing metronidazole with drug: soyalecithin: cholesterol weight ratio 1:4:2 and volume of hydration media 8 ml gave highest entrapment efficiency with smallest particle size and 62.82% drug release after 12h.

Carbopol 934P 1% mucoadhesive gel use as a vehicle for liposomes of metronidazole that provide proper residence time to drug incorporated in liposomes because gel was adhere to the mucus membrane of vagina. The pH of prepared gels (liposomal gel and control gel) was 4.5. Viscosity of liposomal gel and control gel were 10,309 cPs and 10,276 cPs respectively. Content uniformity of liposomal gel and control gel were 99.2% and 99.8% respectively.

The cumulative percent drug release from prepared liposomal gel and control gel after 12 hrs in VFS is 50.17 and 81.05, respectively while in phosphate buffer pH 4.5 is 53.46 and 84.77, respectively. The mean flux value for liposomal gel and control gel after in VFS is 27.25 and 45.15, respectively while in phosphate buffer pH 4.5 is 29.37, and 51.35, respectively. Comparative dissolution profile of both gels in VFS shown in Fig. 6 indicates that drug release from liposome gel was slower than control gel.

In the present investigation both liposome suspension and liposomal gel were subjected to stability studies, in triplicate, at condition according to ICH guidelines. The mean particle size after 30 days for liposome suspension, at 2-8°C and room temperature were 12.95µm and 14.77µm respectively. The mean particle size after 30 days for liposomal gel, at 2-8°C and room temperature were 11.98µm and 13.18µm respectively. The result shows that both preparation were more stable at 2-8°C than room temperature and liposomes were more stable in gel formulation than in suspension form. The Percentage of drug retention after 30 days for liposome suspension, at 2-8°C and room temperature were 34.81 and 30.83 respectively. The Percentage of drug retention after 30 days for liposomal gel, at 2-8°C and room temperature were 35.98 and 33.11 respectively. The results of drug retention studies show higher drug leakage at higher temperature. This may be due to the higher fluidity of lipid bilayers at higher temperature, resulting in higher drug leakage. Drug leakage from liposomes was very low when, dispersed in gel than liposomal suspension. There was no change in pH of liposomal gel during 30 days storage at 2-8°C and at room temperature.

Incorporation of liposomes in bioadhesive Carbopol gel preserved the original size distributions of incorporated liposomes, improved their stability and enabled a sustained release of the drug, confirming the applicability of liposomes containing Metronidazole as a novel delivery system with reduced dosing interval for effective and convenient local treatment of bacterial vaginosis.
Fig. 5 In vitro release of metronidazole in VFS

Fig. 6 Drug release from different formulations in VFS

Fig. 7 Size distribution of liposomes incorporated in gel for stability study
Fig. 8 Percentage of drug retention in liposomal suspensions and gels after storing at different temperatures for a period of 30 days

Where, S1- liposome suspension stored at 2-8°C, S2- liposomal gel stored at 2-8°C, S3- liposome suspension stored at R.T., S4- liposomal gel stored at R.T.

Acknowledgements
The authors wish to thank Newcare Pharma Ltd. Mahesana for providing the free gift sample of Metronidazole. Helpful discussion with Mr. Jayesh Hadia is also gratefully acknowledged.

References


