5. MATERIALS AND METHODS

5.1. Materials

Table 6: List of materials used

<table>
<thead>
<tr>
<th>SL.NO</th>
<th>MATERIAL</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5-Fluorouracil</td>
<td>Hi-media, Mumbai</td>
</tr>
<tr>
<td>2</td>
<td>Chitosan</td>
<td>Sigma Aldrich, Mumbai</td>
</tr>
<tr>
<td>3</td>
<td>Metoprolol Tartrate</td>
<td>Astra Zeneca, India</td>
</tr>
<tr>
<td>4</td>
<td>Lutrol F68</td>
<td>BASF Pvt Ltd, Mumbai</td>
</tr>
<tr>
<td>5</td>
<td>Eudragit S100</td>
<td>Evonik Degussa, Mumbai</td>
</tr>
<tr>
<td>6</td>
<td>Eudragit®RL L100</td>
<td>Vikram Thermo, Gujarat</td>
</tr>
<tr>
<td>7</td>
<td>Tri ethyl citrate</td>
<td>Sigma Aldrich, Mumbai</td>
</tr>
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<td>8</td>
<td>Sodium hydroxide</td>
<td>Loba chemicals, Mumbai</td>
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<td>9</td>
<td>Potassium chloride</td>
<td>Loba chemicals, Mumbai</td>
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<td>10</td>
<td>Potassium dihydrogen phosphate</td>
<td>Loba chemicals, Mumbai</td>
</tr>
<tr>
<td>11</td>
<td>Hydrochloric acid</td>
<td>Loba chemicals, Mumbai</td>
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<tr>
<td>12</td>
<td>Sodium chloride</td>
<td>Loba chemicals, Mumbai</td>
</tr>
<tr>
<td>13</td>
<td>Purified pepsin</td>
<td>Loba chemicals, Mumbai</td>
</tr>
<tr>
<td>14</td>
<td>Potassium phosphate</td>
<td>Loba chemicals, Mumbai</td>
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<td>15</td>
<td>Brain heart infusion agar</td>
<td>Hi-media, Mumbai</td>
</tr>
<tr>
<td>16</td>
<td>Tryptone soy broth</td>
<td>Sigma Aldrich, Mumbai</td>
</tr>
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<td>17</td>
<td>Polyacrylamide slab gel</td>
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<td>18</td>
<td>Glyceryl monostearate</td>
<td>Sigma Aldrich, Mumbai</td>
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<td>19</td>
<td>Blood agar plates</td>
<td>Sigma Aldrich, Mumbai</td>
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<td>20</td>
<td><em>E. coli</em> strain (ATCC No, 35401, serotype O78:H11)</td>
<td>LGC Promochem Pvt Ltd, India.</td>
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</table>
### 5.2. Equipments

Table 7: List of equipments used

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>EQUIPMENT</th>
<th>MODEL/MANUFACTURER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Digital balance</td>
<td>Shinko Sansui, Japan</td>
</tr>
<tr>
<td>2</td>
<td>Electronic Digital balance</td>
<td>AW 120 – SHIMADZU, Japan</td>
</tr>
<tr>
<td>3</td>
<td>Dissolution apparatus (8 basket)</td>
<td>Electrolab, Mumbai</td>
</tr>
<tr>
<td>4</td>
<td>Digital pH meter</td>
<td>Systronics, Model No : 335</td>
</tr>
<tr>
<td>5</td>
<td>DSC</td>
<td>DuPont thermal analyzer 2010</td>
</tr>
<tr>
<td>6</td>
<td>FT-IR spectrophotometer</td>
<td>FT IR- 8400-S, Shimadzu, Japan</td>
</tr>
<tr>
<td>7</td>
<td>HPLC</td>
<td>LC2010AHT, Shimadzu, Japan</td>
</tr>
<tr>
<td>8</td>
<td>Magnetic stirrer</td>
<td>Remi motors, Mumbai</td>
</tr>
<tr>
<td>9</td>
<td>Scanning electron microscope</td>
<td>Joel- LV-5600, USA</td>
</tr>
<tr>
<td>10</td>
<td>UV-Visible Spectrophotometer</td>
<td>UV 1800 , Shimadzu Co, Japan</td>
</tr>
<tr>
<td>11</td>
<td>Cooling Centrifuge</td>
<td>REMI, Model No: 412-LAG</td>
</tr>
<tr>
<td>12</td>
<td>Vacuum filter</td>
<td>Crompton Greaves,11G6502</td>
</tr>
<tr>
<td>13</td>
<td>Lab stirrer</td>
<td>Remi motors, Mumbai</td>
</tr>
<tr>
<td>14</td>
<td>Spray drier</td>
<td>JISL, Mumbai</td>
</tr>
<tr>
<td>15</td>
<td>Polytron PT 1600E homogeniser</td>
<td>Kinematica, Switzerland</td>
</tr>
<tr>
<td>16</td>
<td>Freeze drier</td>
<td>Ilshin Lab Co. Korea</td>
</tr>
<tr>
<td>17</td>
<td>ELISA</td>
<td>Biotek Instruments, USA</td>
</tr>
<tr>
<td>18</td>
<td>Stability chambers</td>
<td>Thermolab, Mumbai</td>
</tr>
</tbody>
</table>
5.3. REAGENTS

1. Sodium hydroxide solution (0.2 M)

Accurately weighed 8 gm of sodium hydroxide in 1000 ml of distilled water.

2. Potassium dihydrogen phosphate (0.2 M)

Accurately weighed 27.218 gm of potassium dihydrogen phosphate in 1000 ml of distilled water.

3. Hydrochloric acid (0.1M)

8.5 ml of concentrated Hydrochloric acid was diluted with water to 1000 ml to give 0.1M HCl.

4. Hydrochloric acid (0.2 M)

17 ml of concentrated Hydrochloric acid was diluted with distilled water to 1000 ml to give 0.2 M HCl.

5. Potassium chloride (0.2 M)

Accurately weighed 14.91 gm of potassium chloride in 1000 ml of distilled water.

6. Hydrochloric acid buffer pH 1.2

50 ml of 0.2 M potassium chloride solution was taken in a 200 ml volumetric flask. To this 85 ml of 0.2 M HCl was added and the volume was made to 200 ml with distilled water.

7. pH 5.8 Phosphate buffer solution

50.0 ml of 0.2 M KH₂PO₄, 3.6 ml of 0.2 M NaOH were taken in a 200 ml volumetric flask and made up to the volume with distilled water and pH was adjusted if necessary.
Materials & Methods

8. **Saline phosphate buffer pH 6.8**

50ml of 0.2M potassium dihydrogen phosphate was taken in a 200 ml volumetric flask. To this 22.4ml of 0.2M NaOH was added and the volume was made up to 200ml with water.

9. **pH 7.4 Phosphate buffer solution**

50.0 ml of 0.2 M KH$_2$PO$_4$, 39.1 ml of 0.2 M NaOH were taken in a 200 ml volumetric flask and made up to the volume with distilled water and pH was adjusted if necessary.
5.4. Part A: METHODS

5.4.1. Analytical methods

5.4.1.1. UV spectra and standard plot of 5-fluorouracil in pH 1.2 HCl buffer.

5.4.1.2. UV spectra and standard plot of 5-fluorouracil in pH 7.4 Phosphate buffer solution

5.4.1.3. Assay validation

5.4.1.4. Standard plot of 5-fluorouracil in HPLC water (methanol: water) as a mobile phase.

5.4.2. Preparation of 5-fluorouracil loaded chitosan nanoparticles

5.4.3. Coating of 5-FU loaded chitosan nanoparticles

5.4.4. Characterization and evaluation of chitosan nanoparticles

5.4.4.1. Particle size and zeta potential of prepared nanoparticles

5.4.4.2. Scanning electron microscopy

5.4.4.3. Differential scanning calorimetry

5.4.4.4. Fourier transforms infrared radiation measurements

5.4.4.5. Molecular weight measurements by gel permeation chromatography

5.4.5. Evaluation of 5-FU loaded porous chitosan nanoparticles

5.4.5.1. Drug Content Uniformity

5.4.5.2. *In vitro* drug release studies

5.4.5.3. Stability studies of the optimized formulation

5.4.5.4. To carry out *in vivo* studies on albino rats for the optimized formulation
5.4.1.1. UV spectra and standard plot of 5-fluorouracil in pH 1.2 HCl buffer

**Stock solution:** 5-fluorouracil in pH 1.2 SGF (100 mg in 100 ml)

**Scanning:** A stock solution of 6μg/ml of 5-FU was prepared in pH 1.2 HCl buffer and scanned between 200-400 nm. The spectrum is reported in Figure 11. The absorption maximum was found to be 267 nm and it was used for further studies.

![Figure 11: UV Spectra of 5-fluorouracil in pH 1.2 HCl buffer.](image)

**Calibration curve of 5-Fluorouracil in pH 1.2 HCl buffer**

**Stock solution:** 100 mg of 5-fluorouracil was accurately weighed and transferred to a 100 ml volumetric flask. The drug was dissolved and diluted upto the mark with HCl buffer. This was further diluted to get a concentration of 100 μg/ml.

From the above stock solution, aliquots of 0.3, 0.6, 0.9, 1.2, and 1.5 ml were transferred to 10 ml volumetric flasks and made upto the mark with HCl buffer. The absorbance of these solutions was measured at 267 nm and a graph of concentration
versus absorbance was plotted. The standard plot of 5-fluorouracil in pH 1.2 HCl buffer is reported in Table 8 and the graph in Figure 12.

Table 8: Standard plot data of 5-fluorouracil in pH 1.2 HCl buffer

<table>
<thead>
<tr>
<th>Concentration in µg/ml</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.219±0.003</td>
</tr>
<tr>
<td>6</td>
<td>0.371±0.005</td>
</tr>
<tr>
<td>9</td>
<td>0.566±0.002</td>
</tr>
<tr>
<td>12</td>
<td>0.732±0.002</td>
</tr>
<tr>
<td>15</td>
<td>0.895±0.004</td>
</tr>
</tbody>
</table>

*Mean ± SD, n = 3

Figure 12: Standard plot of 5-fluorouracil in pH 1.2 HCl buffer.

5.4.1.2. UV spectra and standard plot of 5-fluorouracil in pH 7.4 phosphate buffer solution (PBS)

Stock solution: 5-fluorouracil in pH 7.4 PBS (100 mg in 100 ml)
Scanning: A stock solution of 9µg/ml of 5-FU was prepared in pH 7.4 PBS and scanned between 200-400 nm. The spectrum is reported in Figure 13. The absorption maximum was found to be 267 nm and it was used for further studies.

![Figure 13: UV spectra of 5-fluorouracil in pH 7.4 PBS.](image)

Standard plot of 5-fluorouracil in pH 7.4 PBS

Stock solution:

100 mg of 5-fluorouracil was accurately weighed and transferred in to a 100 ml volumetric flask. The drug was dissolved and diluted upto the mark with pH 7.4 SIF. This was further diluted to 100 µg/ml. From the above stock solution, aliquots of 0.3, 0.6, 0.9, 1.2, and 1.5 ml were transferred to 10 ml volumetric flasks and made upto the mark with SIF of pH 7.4. The absorbance of these solutions was measured at 267 nm and a graph of concentration versus absorbance was plotted. The calibration curve for 5-fluorouracil was plotted in pH 7.4 PBS and the standard plot obtained is reported in Table 9 and the graph in Figure 14.
Table 9: Standard plot data of 5-fluorouracil in pH 7.4 PBS

<table>
<thead>
<tr>
<th>Concentration in µg/ml</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.176±0.002</td>
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<tr>
<td>6</td>
<td>0.355±0.001</td>
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<tr>
<td>9</td>
<td>0.516±0.002</td>
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<td>12</td>
<td>0.607±0.004</td>
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<tr>
<td>15</td>
<td>0.793±0.001</td>
</tr>
</tbody>
</table>

*Mean ± SD, n = 3

Figure 14: Standard plot of 5-fluorouracil in pH 7.4 PBS.
5.4.1.3. Assay validation

Current assay was validated in compliance with current Food and Drug Administration (FDA) guidelines for method validation. Study includes a set of calibration and lower limit of quantification (LLOQ) samples performed on six separate occasions. The precision values obtained were calculated by one-way analysis of variance (ANOVA) for each test concentration, using the run-day as the classification variable.

Precision, accuracy and limit of quantitation.

Trial injections (n=6) were carried out in a single day to assess the intra-day coefficient of variation (precision). Inter-day coefficient of variation was analyzed in the same way. Precaution had been taken and it was kept in check that before each sample run, the syringe was thoroughly rinsed and the injector loop was back-flushed with mobile phase at a flow rate of 1.0 ml/min. In terms of accuracy, it was expressed as the ratio of the compound added to that measured (mean value/nominal value) *100. The limit of detection (LOD) was obtained as the concentration that gives the smallest measurable peak. LLOQ was taken as the lowest concentration in the standard calibration curve. The LLOQ was 100 ng/mL and the LOD of 5-FU was 10 ng/mL.

Pharmacokinetic parameters

The pharmacokinetic parameters were calculated by using Quick calc. software. The maximum concentration of drug in plasma (Cmax), the time taken to reach maximum concentration of drug in plasma (tmax) and the time taken for the first appearance of 5-FU in the plasma (Tlag time) were obtained as directly measured values from the plasma – concentration versus time profile. The AUC0→∞, Ka, Ke,
Materials & Methods

total clearance (ClT) and elimination half life (t\textsubscript{1/2}) were calculated with the help of Quick calc. software. Pharmacokinetic parameters were statistically analyzed for significant differences using two-tailed unpaired t-test. A p < 0.001 was considered to be significant.

5.4.1.4. Standard plot of 5-fluourouracil in HPLC\textsuperscript{227}

Chromatographic conditions

A sensitive, reproducible, selective and accurate high performance liquid chromatographic (HPLC) method for quantitative determination of 5-FU in plasma and drug present in the contents. Separation was achieved by isocratic elution with a mobile phase methanol: water (10: 90 v/v) with pH adjusted to pH 3.2 (perchloric acid) delivered at a flow rate of 1.0 ml/min at ambient temperatures through a genesis C\textsubscript{18} column. Quantification was achieved by the measurement of peak-height ratios and concentration (ng/ml). The retention time of 5-FU was found to be 4.3 ± 0.2 min. Retention time of thymine was found to be 8.7 ± 0.1 min. For plasma analysis 0.5 ml of samples were taken and 5 ml of acetonitrile was added. Samples were further centrifuged at 5000 rpm for 10 min and supernatant was filtered through a 0.2 µm membrane filter and injected into the column. The limit of detection (LOD) was obtained as the concentration that gives the smallest measurable peak of 5-FU and was found to be 10 ng/mL.
Method of preparation of stock solution

Stock solutions of 5-FU were prepared by dissolving 10 mg of 5-FU in 100 ml of HPLC grade water to 100 µg/ml. A stock solution of thymine as an internal standard was also prepared by dissolving 10 mg of thymine in 100 ml of HPLC grade water. Standards were prepared with the following concentrations of 0, 30, 100, 150, 200, 300, 350 and 400 ng/ml. Plasma samples (500 µl) were spiked with thymine (25 µl/ml). The detector was adjusted to the absorbance of 267 nm. The standard graph was plotted against concentration in µg/ml and peak area ratio and the calibration curve obtained is reported in Table 10 and graph in Figure 15. A typical chromatogram of 5-FU with standard thymine using HPLC is shown in Figure 16.

Table 10: Standard plot data of 5-fluorouracil using HPLC water

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Peak Area Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>3.0012</td>
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<tr>
<td>100</td>
<td>10.1232</td>
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<td>150</td>
<td>14.2367</td>
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<td>200</td>
<td>19.7345</td>
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<td>350</td>
<td>34.8762</td>
</tr>
<tr>
<td>400</td>
<td>39.8765</td>
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</table>
Figure 15: Standard plot of 5-fluourourcil using HPLC grade water.

Figure 16: HPLC chromatogram of 5-FU with internal standard thymine.
5.4.2. **Preparation of 5 FU loaded chitosan nanoparticles**

Firstly experimental trails have been done in order to optimize the various parameters that might affect the nanoparticle morphology and size distribution. Effect of molecular weight, effect of polymer concentration, effect of inlet temperature and effect of storage time on nanoparticle morphology and size distribution has been determined. Gel permeation chromatography (GPC) analysis was also carried out in order to establish and verify if the conditions employed during the spray drying process caused any chitosan polymer degradation.

Chitosan (low molecular weight) in different polymer concentrations (0.5, 1 and 1.5 %) in aqueous acetic acid (1% w/v) was prepared by continuous magnetic stirring for 24 h in order to ensure complete solubilization of chitosan in the aqueous acidic solution. To the prepared chitosan solution, 5-FU was added under stirring to get the final spray drying solution. 3% Lutrol F68 was added to the above solution as stabilizer. The above solution was kept for homogenization at 25000 rpm for 2 h (Polytron PT 1600E). Final resulting solution was spray dried by Mini spray dryer (JISL, Mumbai) operating in the closed mode, using the inert loop and nitrogen as the drying gas with other standard operating conditions (inlet temperature: 120 °C; Outlet temperature: 80-90 °C; Aspirator rate: 45-55% & Feed inlet rate: 0.5 ml/min, at 0 day storage). Each formulation was carried out in triplicate, n=3.

5.4.3. **Coating of 5-FU loaded chitosan nanoparticles**

Coating of NPs was done according to the method adopted by Balmuralidhara V et al., with slight modifications. The enteric coating solution of Eudragit S100 (3 % w/v) was prepared in acetone. Triethylcitrate (TEC) 6 % w/v was used as
plasticizer for the coating dispersion, Tween 80 as an emulsifier and glyceryl monostearate (GMS) was used as a glidant for the coating dispersion.

Firstly, dispersion of TEC, Tween 80 and GMS was made by mixing them in aqueous solution for 30 min on magnetic stirrer to ensure complete homogeneity of solution. This dispersion was added to the Eudragit dispersion under high mixing to obtain a homogenous coating dispersion. Solution was stirred for 3 h with a magnetic stirrer to ensure complete solubilisation of polymer in the medium. Nanoparticle batches were added to the coating solution and was spray dried using a spray drier (JISL Spray Drier, Mumbai, India). Process conditions were set as follows for the enteric coating of nanoparticles: 85-90°C, aspirator 45%, spray flow rate 300 ml/min with a nozzle diameter of 2 mm. Coated NPs were collected into final bottom vessel of spray drier. The coated NP batches were dried in an oven at 35 °C for one day and stored in air-tight container.

5.4.4. Characterization and evaluation of chitosan nanoparticles

5.4.4.1 Particle size and zeta potential of prepared nanoparticles

Size and zeta potential of 5-FU loaded NPs was measured by photon correlation spectroscopy (PCS) using Zetasizer Nano ZS (Malvern Instruments, UK). The particle size analysis was performed at a scattering angle of 90°C at room temperature. The concentration of the particles was adjusted to an appropriate value by pure water filtered through a 0.22 μm membrane. The average diameter of the three parallel measurements was taken and expressed as mean ± standard deviation.

5.4.4.2 Scanning electron microscopy

The morphology of the nanoparticles was examined using a scanning electron microscope (Joel- LV-5600, USA) at the required magnification at room temperature.
For this purpose, obtained nanoparticles were resuspended in ultrapure water and centrifuged at 27,000×g for 20 min at 4 °C. Then, supernatants were rejected and the pellets were mounted on a glass plate adhered with a double-sided adhesive tape onto metal stubs and dried under hot flow air. Finally, the particles were coated with a thin layer of 12 nm of gold using an Emitech K550 sputtering device (Emitech, UK). The photographs were observed for morphological characteristics. The photographs were observed to visualize the surface morphology of nanoparticles.

5.4.4.3. Differential scanning calorimetry

DSC is a technique in which the difference in heat flow between the sample and a reference is recorded versus temperature. DSC thermal analytical profile of a pure chemical represents its product identity. By comparing the DSC curves of a pure drug sample with that of the formulation, the presence of an impurity can be detected in a formulation. The scanning temperature for reference pure drug and formulation are the same when dynamic measurements are performed, and hence the required heat energy for chemical transformation is directly recorded on a heat flow versus temperature graph. The energy is measured as Joules per kilocalorie. All dynamic DSC studies were carried out on Dupont thermal analyzer with 2010 DSC module. The instrument was calibrated using high purity indium metal as standard. The dynamic scans were taken in nitrogen atmosphere at the heating rate of 5 °C/min.

5.4.4.4. Fourier transform infrared radiation measurements

FT-IR analysis was carried out for pure drug and for formulation using KBr pellet method on FTIR spectrophotometer type Shimadzu model 8033, USA in order to ascertain compatibility between drug and polymer used.
5.4.4.5. Molecular weight measurements by gel permeation chromatography

Molecular mass measurements were carried out using size exclusion chromatography (GPC) (Pump Waters model 501; injector Waters model U6K, USA). Commercially available Bio-Gel TSK columns (ToyoSoda, Japan) were used. A Knauer HPLC pump Type 64.00 and Rheodyne injector were used, with a refractive index detector Model ERC 7512 (Erma CR. Inc., Japan).

5.4.5. Evaluation of metoprolol tartrate loaded porous chitosan nanoparticles

5.4.5.1. Drug Content Uniformity

For drug content uniformity, NPs (100 mg) were crushed and then transferred into a 250-mL volumetric flask. The volume was adjusted with pH 7.4 phosphate buffer and kept on a rotary shaker for 24 hr in order to completely extract the drug. The mixture was filtered, and the drug was assayed spectrophotometrically at 267 nm at Shimadzu UV-1800, (Japan).

5.4.5.2. In vitro drug release studies

In vitro drug release studies were performed by using a USP dissolution rate apparatus (apparatus 1, 100 rpm, 37± 0.5 °C) in pH 1.2 SIF (simulated intestinal fluid, 900 mL) for 2 h as the average gastric emptying time. Then, the dissolution medium was replaced with a pH 7.4 phosphate buffer (900 mL) for rest of the dissolution studies till complete drug release was obtained. 5-FU loaded nanoparticles equivalent to 100 mg of drug were placed into a dialysis bag (pore size: 50,000 Da). The amount of 5-FU released from the NP at different time intervals was determined spectrophotometrically at 267 nm (Shimadzu UV-1208, Japan). All experiments were done in triplicate.
5.4.5.3. Stability Studies

The 5-FU3 NPs formulation was separated into three portions. One portion was kept at room temperature, the second at 45°C and the third at 4°C for 6 months. Samples were withdrawn on monthly intervals and checked for changes in particle size and drug content.

5.4.5.4. In-vivo studies

The project proposal has been cleared and approved by Institutional animal ethical committee, J.S.S. College of Pharmacy, Mysore.

The 5-FU3 was selected in order to study in vivo performance of the NP preparation, on the basis of in-vitro release studies.

Latin cross over design method was employed for the in vivo studies to be carried out. Adult albino rats of similar weight (150-200 gm) were selected, kept in well-spaced ventilated cages, and the rats were kept on fast for a day. The animals were divided into two groups of 6 animals each. The first group received the plain drug suspension of 5-FU, which were prepared using 1% gum acacia (dose calculated in relation to body weight of the animal). The second group was given the NP formulation. The doses were given orally with the help of cannula, and blood samples were collected from the neck vein of the rats at 0, 0.3, 1, 2, 3, 4, 6, 8, 12 upto 20 h. Blood samples were further centrifuged at 5000 rpm for 30 min (REMI International, Mumbai, India) in order to separate plasma to carry out assay of 5-FU. Plasma samples were immediately stored at −20 °C until further analysis.
5.5. **Part B:**

5.5.1. **Analytical methods**

5.5.1.1. UV spectra of Metoprolol tartrate and standard plot in HCl buffer pH 1.2, phosphate buffer pH 7.4 and phosphate buffer pH 6.8.

5.5.2. **Preparation of Spray dried chitosan nanoparticles**

5.5.2.1. Preparation of porous chitosan nanoparticles.

5.5.2.2. Coating of 5-FU loaded chitosan nanoparticles

5.5.3. **Characterization of porous chitosan nanoparticles**

5.5.3.1. Micromeritic properties like density, angle of repose and porosity.

5.5.3.2. Particle size and zeta potential of prepared nanoparticles

5.5.3.3. Scanning electron microscopy (SEM)

5.5.3.4. Transmission electron microscopic (TEM)

5.5.3.5. Fourier transforms infrared radiation measurements (FTIR)

5.5.3.6. Differential scanning calorimetry (DSC)

5.5.3.7. X-Ray Powder Diffraction (XRD)

5.5.3.8. Molecular weight measurements by gel permeation chromatography (GPC studies)

5.5.4. **Evaluation of metoprolol tartrate loaded porous chitosan nanoparticles**

5.5.4.1. Drug Loading Of Metoprolol tartrate (MT) in Porous NPs batches

5.5.4.2. Drug Content Uniformity

5.5.4.3. *In vitro* drug release studies

5.5.4.4. Stability studies of the optimized formulation.
5.5.1. ANALYTICAL METHODS

In the present study, the UV spectrophotometric method of analysis of Metoprolol tartarate was employed.

5.5.1.1. Determination of $\lambda_{\text{max}}$ of Metaprolol tartarate in HCl buffer pH 1.2, phosphate buffer pH 6.8 and phosphate buffer pH 7.4.

Scanning: From the stock solution, 50 $\mu$g / ml solution of Metoprolol tartrate was prepared and scanned between 200-400nm. The spectrum for MT in HCl buffer pH 1.2, phosphate buffer pH 6.8 and phosphate buffer pH 7.4 is reported in Figure 17, Figure 18 and Figure 19 respectively. The $\lambda_{\text{max}}$ of drug was found to be 275 nm was selected and used for further studies.

Figure 17: UV spectra of Metoprolol tartarate in HCl buffer pH 1.2.
Figure 18: UV spectra of Metoprolol tartrate in Phosphate buffer pH 6.8.

Figure 19: UV spectra of Metoprolol tartrate in Phosphate buffer pH 7.4.
Preparation of standard stock solution

Solution A (1 mg/ml) – 100 mg of Metoprolol tartarate was accurately weighed, transferred to a 100ml volumetric flask. The drug was then dissolved and diluted up to the mark with 0.1 N HCl.

From this solution A, aliquots of 5, 10, 15, 20, 25 ml were transferred to 100ml volumetric flask and diluted to 100 ml to contain 50, 100, 150, 200, 250 µg/ml of Metoprolol tartrate respectively. The absorbance was measured at 275 nm and a graph of concentration versus absorbance was plotted. The standard plot of Metoprolol tartarate in 1.2 pH HCl buffer is shown in Table 11 and Figure 20.

In the similar pattern, the standard plots for Metoprolol tartarate were plotted in phosphate buffer pH 6.8 and phosphate buffer of pH 7.4. The calibration curves data obtained were shown in Table 12 and Table 13 and corresponding figures have been shown in Figure 21 and Figure 22 respectively.
Table 11: Standard plot data of Metoprolol tartarate in 0.1 N HCl

<table>
<thead>
<tr>
<th>SL.NO</th>
<th>Concentration (µg/ ml)</th>
<th>Absorbance Mean ± S.D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>0.1712±0.001</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>0.3011±0.009</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>0.4253±0.004</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>0.5905±0.004</td>
</tr>
<tr>
<td>6</td>
<td>250</td>
<td>0.7391±0.013</td>
</tr>
</tbody>
</table>

* Standard deviation n=3

Figure 20: Standard plot of Metoprolol tartarate in 0.1 N HCl.

\[ y = 0.0029x \]
\[ R^2 = 0.9991 \]
Table 12: Standard plot data of Metoprolol tartarate in 6.8 pH buffer.

<table>
<thead>
<tr>
<th>SL.NO</th>
<th>Concentration (µg/ ml)</th>
<th>Absorbance Mean ± S.D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>0.1412±0.001</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>0.2263±0.000</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>0.3306±0.000</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>0.4315±0.001</td>
</tr>
<tr>
<td>6</td>
<td>250</td>
<td>0.735±0.001</td>
</tr>
</tbody>
</table>

* Standard deviation n=3

Figure 21: Standard plot of Metoprolol tartarate in 6.8 pH buffer.

\[ y = 0.0027x \]
\[ R^2 = 0.9999 \]
Table 13: Standard plot data of Metoprolol tartarate in 7.4 pH buffer.

<table>
<thead>
<tr>
<th>SL.NO</th>
<th>Concentration (µg/ ml)</th>
<th>Absorbance Mean ± S.D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>0.1670±0.001</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>0.2993±0.000</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>0.4362±0.000</td>
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<tr>
<td>5</td>
<td>200</td>
<td>0.6112±0.001</td>
</tr>
<tr>
<td>6</td>
<td>250</td>
<td>0.7355±0.001</td>
</tr>
</tbody>
</table>

* Standard deviation n=3

Figure 22: Standard plot of Metoprolol tartarate in 7.4 pH buffer.
5.5.2.1 Preparation of porous chitosan nanoparticles

Chitosan solution in aqueous acetic acid was prepared with continuous magnetic stirring. To the prepared chitosan solution, ammonium carbonate (pore forming agent) was added under stirring to get the final spray drying solution. Polyvinyl alcohol (PVA) in varying concentrations was added prior to spray drying solution as a emulsion stabilizer. Prepared solution was spray dried with a Mini spray drier (JISL, Mumbai) operating in the closed mode, using the inert loop and nitrogen as the drying gas with other standard operating conditions (inlet temperature: 120 °C; Outlet temperature: 80-90 °C; Aspirator rate: 45-55% & Feed inlet rate: 0.5 ml/min, at 0 day storage). Obtained NPs batches were sealed tight and kept in air tight container for further analysis.

Each formulation was carried out in triplicate as shown in Table 14.

Table 14: Formulation chart of prepared NPs formulations.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Polymer : Pore Forming agent Ratio</th>
<th>Acetic acid (% w/v)</th>
<th>PVA (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH1</td>
<td>1: 0.15</td>
<td>2 %</td>
<td>1 %</td>
</tr>
<tr>
<td>CH2</td>
<td>1: 0.45</td>
<td>2 %</td>
<td>1 %</td>
</tr>
<tr>
<td>CH3</td>
<td>1: 0.75</td>
<td>2 %</td>
<td>1 %</td>
</tr>
<tr>
<td>CH4</td>
<td>1: 0.15</td>
<td>2 %</td>
<td>2 %</td>
</tr>
<tr>
<td>CH5</td>
<td>1.5: 0.45</td>
<td>2 %</td>
<td>2 %</td>
</tr>
<tr>
<td>CH6</td>
<td>1.5: 0.75</td>
<td>2 %</td>
<td>2 %</td>
</tr>
<tr>
<td>CH7</td>
<td>2: 0.15</td>
<td>2 %</td>
<td>4 %</td>
</tr>
<tr>
<td>CH8</td>
<td>2: 0.45</td>
<td>2 %</td>
<td>4 %</td>
</tr>
<tr>
<td>CH9</td>
<td>2: 0.75</td>
<td>2 %</td>
<td>4 %</td>
</tr>
</tbody>
</table>
5.5.2.2. Coating of Porous Nanoparticles

As reported in previous part of the study.

5.5.3. Characterization of porous chitosan nanoparticles

5.5.3.1. Micromeric properties

5.5.3.1.1. Density measurement:

Both poured (or fluff) bulk (Do) and tapped bulk densities (DF) were determined, according to the method reported, whereby a quantity of powder bed from each formula, previously lightly shaken to break any agglomerates formed, was introduced into a 10-mL measuring cylinder. After the initial volume was observed, the cylinder was allowed to fall under its own weight onto a hard surface from the height of 2.5 cm at 2-second intervals. The tapping was continued until no further change in the volume was noted.

5.5.3.1.2. Angle of Repose

Static angle of repose was determined according to the fixed funnel and free standing cone method, where by accurately weighed powder bed (3 g) was carefully poured through the funnel with its tip at 2-cm height, H, until the apex of the conical heap so formed just reached the tip of the funnel. The mean diameter, 2R, of the base for the powder cone was measured and the angle of repose (θ) was calculated using the equation,

\[ \tan \theta = \frac{H}{R} \] (eq. 1).

Angle of repose represents whether the given sample was free flowing or not. The relationship between angle of repose and flowability is shown in Table 15.
5.5.3.1.3. Porosity: Porosity (€) of the powder is defined as the ratios of the void volume to the bulk volume of the packing of particles and is expressed in percentage.

\[ \epsilon = \frac{V_b - V_p}{V_b} = 1 - \frac{V_p}{V_b} \]

Where, \( V_p \) is true volume of particle
\( V_b \) is bulk volume.

5.5.3.2. Measurement of particle size and zeta potential of prepared nanoparticles.
As described earlier in above part of study. The diameter was averaged from three parallel measurements and expressed as mean ± standard deviation.

5.5.3.3. Scanning electron microscopic (SEM)
As described earlier in above part of study.

5.5.3.4. Transmission electron microscopic (TEM) study
TEM was used to observe the morphology of MT loaded NPs. Sample was first diluted and a drop was placed onto a carbon coated 400 mesh copper grid and dried in an oven at 40\(^\circ\)C for 15 min. The images were taken using a Hitachi Ultra-thin film evaluation system (HD-2300A) in Phase contrast, Z contrast, Secondary Electron (SE) modes.
5.5.3.5. Differential scanning calorimetry (DSC):

As described earlier in above part of study.

5.5.3.6. Fourier Transform Infrared Radiation Measurements (FT-IR)

As described earlier in above part of study.

5.5.3.7. X-Ray Powder Diffraction

X-ray powder diffraction patterns were recorded at room temperature with a D8 Advance wide-angle diffractometer in the range of 5–40° of 2θ. Tablets for IR analysis were made with KBr and analyzed with an IR Perkin-Elmer model 1420, in the range from 4000 to 600 cm⁻¹.

5.5.3.8. Molecular weight measurements by Gel Permeation chromatography (GPC)

As described earlier in above part of study.

5.5.4. Evaluation of metoprolol tartrate loaded porous chitosan nanoparticles

5.5.4.1. Drug Loading Of Metoprolol Tartrate (MT) in Porous NPs batches

Loading of MT was done through incubating NPs batches in 3% w/v drug solution under mild agitation at room temperature for 30 min. The loading capacities of the uncoated particles were calculated by an indirect way, quantifying the amount of MT that remained in solution.

The drug loading capacity (LC) was calculated from the following equations

\[
\text{LC (\\%, w/w)} = \frac{\text{(total amount of MT - amount of MT remained in solution)}}{\text{weight of the particles}} \times 100.
\]

After particular period of time NPs was collected by filtration and were dried at room temperature.
5.5.4.2. **Drug Content Uniformity**

In the case of drug content uniformity test NPs (100 mg) were crushed and then transferred into a 250-ml volumetric flask. The volume was adjusted with pH 7.4 phosphate buffer and kept on rotary shaker for 24 h in order to completely extract the drug. The mixture was filtered, and the drug was assayed spectrophotometrically at 275 nm at Shimadzu UV-1800.

5.5.4.3. **In-vitro drug release studies**

*In vitro* drug-release studies was performed by using a USP dissolution rate apparatus (apparatus 1, 100 rpm, 37± 0.5°C) in pH 1.2 hydrochloric acid buffer (900 ml) for 2 h as the average gastric emptying time. Then, the dissolution medium was replaced with a pH 7.4 phosphate buffer (900 ml) for the rest of the dissolution studies till complete drug release were obtained. MT loaded NPs equivalent to 100 mg of drug were placed into a dialysis bag (pore size: 50,000 Da). The amount of MT released from the NPs at different time intervals was determined spectrophotometrically at 275 nm (Shimadzu UV-1208). All experiments were done in triplicate.

5.5.4.4. **Mathematical model fitting of obtained drug release data**

The release data was fitted into various mathematical models using PCP.Disso-V2.08 software to know which mathematical model will best fit the obtained release profile. The parameters like ‘n’ the time exponent ‘k’ the release rate constant and ‘R’ the regression co-efficient were determined to know the release mechanisms. The various models studied were:

- First order
- Zero order
- Peppas model
- Higuchi model
Peppas model fitting

The data obtained from in vitro release studies was fit into Peppas model \(^{233}\). Koresmeyer-Peppas equation:

\[
\frac{M_t}{M_\infty} = a t^n
\]

\[
\log \left( \frac{M_t}{M_\infty} \right) = \log a + n \log t
\]

- \( M_t \) = Amount of drugs released at time \( t \)
- \( M_\infty \) = Total amount of drug loaded
- \( n \) = Release exponent
- \( a \) = Geometric and structural characteristics of dosages form

The value of ‘\( n \)’ determined from Korsmeyer-Peppas equation if found to be below 0.45, it indicates that the drug release from the formulation follows Fickian diffusion, if ‘\( n \)’ value is between 0.5-0.85, it indicates Non-Fickian diffusion or anomalous mechanism (relaxation controlled) and if ‘\( n \)’ value is above 0.89, it indicates Super case II transport.

Crank’s equation (modified Peppas equation)

\[
\frac{M_t}{M_\infty} = a t^{1/2}
\]

If diffusion is the main drug release mechanism, a graphic representing the drug amount released, versus the square root of time should originate a straight line \(^{234}\). Higuchi plots \(^{235}\)

\[
M = K \sqrt{t}
\]

Where, \( M_\infty \) = Amount of drug dissolved in
- \( M_\infty \) = Amount of drug dissolved in
- \( t \) = Release time
5.5.4.5. Stability studies of the formulations

The physical stability of the nanosuspensions was evaluated after storage for 6 months. NP formulation was separated into three portions. One portion was kept at room temperature, the second at 45°C and the third at 4°C for 6 months. Samples were withdrawn on monthly intervals and checked for changes in particle size and drug content.
5.6 Part C

5.6.1. Preparation of porous chitosan nanoparticles by spray drying

5.6.2. Bacterial inoculum

5.6.3. Purification of F4 fimbriae

5.6.4. Freeze-drying of the F4 solution

5.6.5. Loading of F4 in Porous Chitosan Nanoparticles

5.6.6. Enteric coating of the F4 loaded nanoparticles

5.6.7. Characterization and evaluation of porous chitosan nanoparticles.

5.6.7.1. Measurement of particle size and zeta potential of prepared nanoparticles
5.6.7.2. Micromeritic properties
5.6.7.3. Porosity
5.6.7.4. Scanning electron microscopic (SEM)
5.6.7.5. Transmission electron microscopic (TEM) study
5.6.7.6. Estimation of nanoparticles mucoadhesive strength
5.6.7.7. F4 biological activity
5.6.7.8. Distribution of F4 in NPS
5.6.7.9. In Vitro release
5.6.7.10. Experimental animals
5.6.7.11. Faecal excretion of F4†ETEC
5.6.7.12. In vitro villous adhesion assay for F4 characterization

5.6.8. Stability studies

5.6.9. Statistical Analysis
5.6.1. Preparation of Porous nanoparticles by Spray drying

Chitosan solution in aqueous acetic acid was prepared with continuous magnetic stirring for 24 h. To the prepared chitosan solution, ammonium carbonate (pore forming agent) was added under stirring to get the final spray drying solution. Polyvinyl alcohol (PVA) in varying concentrations was added prior to spray drying solution as an emulsion stabilizer and solution was further homogenized for 30 min at 30,000 rpm. Solutions thus prepared was spray dried with a Mini spray dryer (JISL, Mumbai) operating in the closed mode, using the inert loop and nitrogen as the drying gas with other standard operating conditions as optimized in earlier part of our research work (inlet temperature: 120 °C; Outlet temperature: 80-90 °C; Aspirator rate: 45-55% & Feed inlet rate: 0.5 ml/min, at 0 day storage). Particles obtained after spray drying process were further freeze dried. Each formulation was carried out in triplicate as shown in Table 16.

Table 16: Formulation chart of prepared porous nanoparticles.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Polymer : Pore Forming agent Ratio</th>
<th>Acetic acid (% w/v)</th>
<th>PVA (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH1</td>
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<td>2 %</td>
<td>1 %</td>
</tr>
<tr>
<td>CH2</td>
<td>1: 0.75</td>
<td>2 %</td>
<td>1 %</td>
</tr>
<tr>
<td>CH3</td>
<td>1.5: 0.15</td>
<td>2 %</td>
<td>2 %</td>
</tr>
<tr>
<td>CH4</td>
<td>1.5: 0.75</td>
<td>2 %</td>
<td>2 %</td>
</tr>
<tr>
<td>CH5</td>
<td>2: 0.15</td>
<td>2 %</td>
<td>4 %</td>
</tr>
<tr>
<td>CH6</td>
<td>2: 0.75</td>
<td>2 %</td>
<td>4 %</td>
</tr>
</tbody>
</table>
5.6.2. Bacterial inoculum

*E. coli* strain (ATCC No, 35401, serotype O78:H11) was cultured for 24 hr on brain heart infusion agar plates (Himedia, Mumbai), and bacteria were collected by washing the agar plates with phosphate-buffered saline (PBS; pH 7.4). Bacteria were further suspended in PBS and the concentration of bacteria in the suspension was determined by measuring the optical density at 660 nm (OD$_{660}$) of the collected bacterial suspension. An OD of 1 equals 106 bacteria/ml, as determined by counting Colony forming Unit (CFU). The concentration of the suspension was adjusted to 106 bacteria per ml.

5.6.3. Purification of F4 fimbriae

Briefly, the bacteria were cultured in tryptone soy broth (Sigma Aldrich, Mumbai) at 37°C for 15 hr, then subjected for centrifugation, and washed in phosphate-buffered saline (PBS; pH 7.4). Secondly, the F4 fimbriae were isolated by homogenization of the bacterial suspension, Fimbriae that were solubilized in the supernatant, were precipitated with 40% ammonium sulfate, and the fimbriae slug was dissolved and dialyzed overnight against ultrapure water. The purity was assessed by electrophoresis on a sodium dodecyl sulfate–10% polyacrylamide slab gel. The samples were heated at 95°C for 5 min in the presence of sample buffer (62.5 mM Tris-HCl, 2% [w/v] SDS, 5% [v/v] β-mercaptoethanol, 10% [v/v] glycerol [pH 6.8]) before electrophoresis. Protein bands were visualized by staining with Coomassie brilliant blue G and bands were observed with the ImageMaster. F4ac fimbriae were identified in Western blot technique with F4ac- specific MAb. Samples were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (0.45-lm pore size) with a trans-blot semi-dry transfer cell (Bio-Rad, Richmond, California).
Receptor activity was detected by incubating the membranes with biotinylated K88ab fimbriae (0.6 µg ml in PBS-Tween containing 2% BSA). After 1 hr at room temperature, membranes were washed with PBS-Tween (three washes of 5 min each) and then once with PBS. Bound biotinylated K88ab fimbriae were detected by incubating the filter with horseradish peroxidase-streptavidin (1:2,000 dilution in PBS-Tween, containing 2% BSA) for 1 hr at room temperature.

5.6.4. Freeze-drying of the F4 solution

A PBS solution of F4 (2.5 mg/ml) was filled in vials. The samples were frozen to -50°C over 2 hr at 1000 mbar. The primary drying (12 h) was performed at -20°C and 0.6–1 mbar and the secondary drying (8 h) at 10°C and 0.1–0.2 mbar. After freeze drying, the vials were closed under vacuum. Samples were kept on ice bars until further analysis.

5.6.5. Loading of F4 in Porous Chitosan Nanoparticles

Loading of F4 in porous chitosan nanoparticles was done by imbibing method. Firstly, suspension of the freeze-dried nanoparticles (1 gm) in phosphate buffer (pH 7.4) was placed in an ultrasound bath for 10 min in order to disaggregate the particles. Secondly nanoparticles were loaded with F4 by incubating a solution of F4 (10mg/100ml) with chitosan nanoparticles under mild agitation at room temperature for 15 min. After a particular period of time solution was centrifuged to obtain the F4 loaded NPs and was freeze dried. After drying 500 mg of dried NPs were kept in 100 ml of phosphate buffer (pH 7.4) for 24 hr and was analyzed by subjecting it to indirect ELISA using the F4 fimbrial solution as standard to determine the concentration of F4 in NPs.
5.6.6. Enteric coating of the F4 loaded nanoparticles

Prepared NP formulations were coated by Eudragit L 100 (3 % w/v aqueous solution) as they dissolve above pH 6. It has been already reported that pH 6.3 was the pH measured at the target site (the beginning of the jejunum) of suckling piglets\(^{239}\). Rest of procedure is as same as carried in earlier part of research study.

5.6.7. Characterization and evaluation of porous chitosan nanoparticles.

5.6.7.1. Measurement of particle size and zeta potential of prepared nanoparticles.

As described earlier in above part of study.

5.6.7.2. Micromeritic properties

Both poured (or fluff) bulk (Do) and tapped bulk densities (DF) was determined using a 10-mL graduated cylinder using a bulk density apparatus, Angle of Repose was calculated from fixed funnel method and porosity was computed and was expressed in percentage\(^{230}\).

5.6.7.3. Porosity

As described earlier in above part of study.

5.6.7.4. Scanning electron microscopic (SEM)

As described earlier in above part of study.

5.6.7.5. Transmission electron microscopic (TEM) study

As described earlier in above part of study.

5.6.7.6. Estimation of nanoparticles mucoadhesive strength

The method\(^{240}\) described in the literature to estimate the number of mucoadhesive microspheres was used with slight modifications. The F4 loaded
chitosan NPs were immersed in a 50 mL glass beaker at 37 °C containing a phosphate buffer solution (pH 6.8) for 5 min in such a way that the solution just covered the nanoparticles. After nanoparticle wetting, a round fresh intestinal mucosa was placed on nanoparticle surface so as to cover all the nanoparticles and remained for 5 min in contact with the nanoparticles.

The intestinal mucosa with the attached NPs was removed and the remaining nanoparticles on the glass beaker were dried at 60 °C till constant weight. The percent of adhered nanoparticles (AN) was estimated using the following equation:

$$AN(\%) = \frac{Wo - Wr}{Wo} \times 100$$

Where, Wo is the initial weight of nanoparticles and Wr the remained unattached weight of nanoparticles.

5.6.7.7. F4 biological activity

In order to investigate if F4 fimbriae retained their activity during the manufacturing process, 500 mg freeze-dried NPs (n=3) were soaked in 100 ml PBS. NPs were further subjected to centrifugation at 2500g, the supernatant was collected and stored at -20°C until analysis. The amount of biologically active F4 was determined via ELISA.

5.6.7.8. Distribution of F4 in NPS

To visualize the F4 fimbriae, the prepared NPs batches were incubated with anti-F4 rabbit antiserum overnight. The NPs were examined using a fluorescence microscope.
5.6.7.9. *In Vitro* F4 release

*In vitro* drug release studies was performed by using a USP dissolution rate apparatus (apparatus 1, 100 rpm, 37± 0.5 °C) in pH 1.2 hydrochloric acid buffer (900 mL) for 2 h as the average gastric emptying time. Then, the dissolution medium was replaced with a pH 5.8 for 3 hr and subsequently to pH 6.8 for rest of the dissolution studies till complete release was obtained. F4 loaded NPs were placed into a dialysis bag (pore size: 50,000 Da). The amount of F4 released from the NPs at different time intervals was determined by ELISA. All experiments were done in triplicate.

5.6.7.10. Experimental animals

All the experimental procedures and animal management procedures were carried out in accordance with the requirements of the animal care and ethical committee of the JSS College of Pharmacy, JSS University, Mysore, India. 18 suckling piglets which were seronegative for antibodies against F4 were included in the study which were housed in a conventional farm in the breeding house of JSS Medical college at 30°C ± 5 °C together with sow, which were negative for F4 as determined by ELISA. At the age of 27th day, piglets were weaned and were brought in 1 isolation unit 24±2°C with food and water ad libitum.

Procedure

Immunization procedure was done in accordance with Snoeck et al., with minor modifications. At the age of 7 days, 2 mg of F4 was given through syringe to 6 animals. F4 was given with 6 ml of PBS (solution form) on 3 successive days. 6 animals received the same F4 fimbriae dose with F4 loaded NPs (NPs containing 2 mg of F4). NPs were orally administered with 10 ml of PBS. The other groups of animals were not immunized and were taken as the control group. Both solution and NP vaccinated procedure was restricted to 1 litter.
Both the vaccinated group (oral and NPs groups) received a booster immunization on 3 consecutive days at age of 21. Finally at 31st day all animals were infected with virulent F4+E. coli strain. At last, all piglets were orally infected with F4+E. coli strain. Blood was sampled weekly from the jugular vein for determining total antibody titer in serum. Serum was collected and inactivated at 55°C and was treated with kaolin to decrease the background reading in ELISA.

5.6.7.11. Faecal excretion of F4+E. coli

Faecal samples were collected to determine the F4+E. coli excretion from the infected piglets. Samples were analyzed after making a suspension of faecal matter in PBS. 50 ml of faecal sample suitably diluted was subjected on blood agar plates (Sigma Aldrich, Mumbai) at 37°C for 24 hr and was quantified using dot blotting as described by Van den Broeck 242.

5.6.7.12. In vitro villous adhesion assay for F4 characterization

Presence of F4 receptors is a pre requisite for F4+E. coli infection. In order to determine the presence of F4 on the small intestinal villous enterocytes, an in vitro villous adhesion assay was performed at the end of 47th day. In brief, 20 cm long intestinal segment was completely excised at the time of slaughter. Excised intestinal segment was washed thrice with PBS. Further villi were scraped from the surface of intestinal segment. Isolated villi were washed with Kreb-Henseleit buffer. Lastly, E. coli were added to the villi. Adhesion of bacteria will give an indication of either negative, weak or strong positive for F4+E. coli infection. Adhesion of more than 25 bacteria per 250 μm villous length was noted as strong adhesion, less than 25 bacteria
per 250 μm villous length was indicated as weak adhesion. Villi were examined by phase contrast microscopy at a magnification of 600x.

5.6.8. Stability studies

Optimized formulation was subjected for stability studies. It was reported that if the drug products are intended to be stored in freeze condition only storage conditions of –20 ± 5 °C are recommended for 6 months. Samples were withdrawn on monthly intervals and checked for changes in particle size, zeta potential and *in vitro* release study.

5.6.9. Statistical Analysis

Statistical analysis was done using software package SPSS version 10.0. Differences in antibody serum titers between different groups at various time points were tested for statistical significant differences by one-way ANOVA. Statistical significance was defined as P<0.05.