Controlled modulated drug delivery system is desired for minimizing local and systemic side effects, maximizing the bioavailability and therapeutic efficacy as well as economy of the treatment in terms of cost of therapy and drug utilization (1). Ionotropic gelation, one of the most effective methods of microencapsulation is used for controlled modulated drug delivery system in pharmaceuticals (2). Polysaccharides are polymers of simple sugar building blocks. Crosslinked polysaccharides are having many applications in pharmaceuticals (3). Gellan gum is an extracellular anionic, heteropolysaccharide produced by Sphingomonas elodea during aerobic fermentation. Gellan gum (GG) consists of a linear structure of repeating tetrasaccharide units of glucose, glucuronic acid and rhamnose (4). Gellan is having two forms as high acyl gellan and low acyl gellan. The native polysaccharide (high-acyl gellan) contains approximately 6% O-acetyl groups, which are lost during alkali treatment of commercial extraction yielding low-acyl GG. This commercial gellan cross-links more effectively with divalent cations than with monovalent cations (5). GG has wide variety of applications, mainly concentrated in ophthalmic drug delivery and oral sustained release preparations (6), moreover, this polymer has the desire suitability as a carrier for controlled colonic delivery (7).

5-Fluorouracil (5-FU) with the chemical name 2,4-dihydroxy-5-fluoropyrimidine is a cytotoxic agent, interferes with nucleic acid synthesis and inhibits DNA synthesis (8). The drug is widely used in the management of colorectal cancer. However, the major drawback of the drug is its incomplete and erratic oral bioavailability. The design of oral controlled release colorectal specific delivery of 5-FU with GG and GG in combination with ethyl cellulose might overcome the above problem and at the same time possibly will reduce the systemic side effects along with providing effective and safe therapy of colorectal cancer.

The purpose of this study was to formulate as well as evaluate calcium-zinc-gellan and calcium-zinc-gellan-ethyl cellulose controlled release microbeads.

Abstract: 5-Fluorouracil loaded calcium-zinc-gellan and calcium-zinc-gellan-ethyl cellulose microbeads were successfully prepared by simple ionotropic gelation and oil in water ionotropic gelation technique, respectively. Prepared microbeads were characterized by scanning electron microscopy, Fourier transform infrared spectroscopy and evaluated for particle size, drug content, encapsulation efficiency, drug release and cell cytotoxicity study. Microbeads formed were spherical with rough surface. As concentration of gellan and ethyl cellulose has increased encapsulation efficiency, particle size and sustained drug release effect also increased. The release of 5-fluorouracil from microbeads has followed Hixson Crowell model suggesting the mechanism of drug release as dissolution controlled. Cytotoxicity analysis on HT-29 human colon cancer cell lines indicated that 5-FU loaded gellan gum/gellan in combination with ethyl cellulose microbeads leads to sustained releases of drug and thus delayed apoptosis over a long period of time. The formulation with drug : gellan : ethyl cellulose ratio 2.5 : 7.5 : 1 was found to be more effectual in terms of sustained drug release activity in addition to anti-cancer activity.

Keywords: gellan gum, ethyl cellulose, microbeads, anticancer activity, 5-fluouracil

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microbeads of 5-fluorouracil formulated by simple ionotropic gelation technique and oil in water ionotropic gelation method, respectively, and moreover, to study and compare the cytotoxicity of free and encapsulated 5-FU using HT-29 human colon cancer cell lines.

**EXPERIMENTAL**

**Materials**

Deacetylated gellan gum (GG), Kelcogel®, was obtained as a gift sample from CP Kelco (Burzin and Leons, Pvt. Ltd., Mumbai, India). 5-FU was obtained as a gift sample from Fangge Pharmaceutical Co., Ltd. (Zhejiang, China). Ethyl cellulose (EC), dichloromethane, acetonitrile, zinc sulfate, calcium chloride, sodium hydroxide and potassium dihydrogen phosphate were purchased from E. Merck Ltd., (Mumbai, India). All the chemicals used were of analytical grade.

**METHODS**

Preparation of 5-FU loaded calcium-zinc-gellan and calcium-zinc-gellan-EC microbeads

5-FU loaded calcium-zinc-gellan and calcium-zinc-gellan-EC microbeads were prepared by the method of ionotropic gelation. The formulation codes with quantities are given in Table 1. For formulation F1 to F3, desired quantity of GG was dispersed in double-distilled water (25 mL) maintained at 55°C to which 5-FU (250 mg) was added to prepare GG 5-FU dispersion. Finally, the resultant dispersion was extruded dropwise into the counter ion solution containing a mixture of 3.5% calcium chloride and 3.5% of zinc sulfate (100 mL) using a 25 mL hypodermic syringe (22 G needle) with constant stirring (250 rpm) for 30 min. For formulation F4 and F5, an oil phase was prepared by dispersing the drug (250 mg) with desired quantity of EC in a mixed solvent system (7 mL) containing dichloromethane : acetonitrile in a ratio of 1 : 1 (v/v) by a magnetic stirrer. Water phase was prepared by dispersing the desired quantity of GG in double-distilled water (25 mL) maintained at 55°C. The oil phase was added in the form of a thin stream to the water phase while rotating at 750 rpm by an electrical stirrer to form oil in water type emulsion. Finally, the resultant emulsion was extruded dropwise into the counter ion solution following the same procedure as described for formulation F1 to F3. The obtained beads were separated by filtration, washed with double-distilled water and dried in an oven at 37°C for 24 h. All the formulations were prepared in triplicate to obtain desired quantity and also to check repeatability.

**Determination of yield, encapsulation efficiency and particle size**

Beads prepared were weighed after drying and percent yield was calculated using the following formula.

\[
\text{Percentage yield} = \frac{(\text{actual weight} \times 100)}{(\text{theoretical weight})}
\]

For determination of encapsulation efficiency, drug loaded microbeads equivalent to 50 mg of 5-FU were added to 50 mL of 6.8 pH phosphate buffer. The resulting mixture was kept shaking on a mechanical shaker for 24 h. Then, the solution was filtered through membrane filter (0.45 µm pore size) and 1 mL of this solution was appropriately diluted using 6.8 pH phosphate buffer and analyzed spectrophotometrically at 266.5 nm using UV–Vis spectrophotometer (Systronic 2101). Encapsulation efficiency was calculated by the following formula.

\[
\text{Encapsulation efficiency} = \frac{(\text{estimated drug content} \times 100)}{(\text{theoretical drug content})} \times 100
\]

<table>
<thead>
<tr>
<th>Formulation codes</th>
<th>GG (mg)</th>
<th>EC (mg)</th>
<th>Yield (%) (n = 3)</th>
<th>EE (%) (n = 3)</th>
<th>MPS (µm) (n = 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>250</td>
<td>0</td>
<td>92.80 ± 1.6</td>
<td>29.28 ± 1.0</td>
<td>474.4 ± 3.1</td>
</tr>
<tr>
<td>F2</td>
<td>500</td>
<td>0</td>
<td>80.94 ± 1.4</td>
<td>33.67 ± 1.8</td>
<td>498.3 ± 2.5</td>
</tr>
<tr>
<td>F3</td>
<td>750</td>
<td>0</td>
<td>85.00 ± 1.6</td>
<td>43.26 ± 1.3</td>
<td>530.2 ± 1.9</td>
</tr>
<tr>
<td>F4</td>
<td>750</td>
<td>100</td>
<td>85.10 ± 1.9</td>
<td>50.07 ± 1.9</td>
<td>561.5 ± 2.2</td>
</tr>
<tr>
<td>F5</td>
<td>750</td>
<td>150</td>
<td>82.15 ± 1.8</td>
<td>55.64 ± 1.1</td>
<td>564.2 ± 2.7</td>
</tr>
</tbody>
</table>

GG - gellan gum, EC - ethyl cellulose, MPS - mean particle size, EE - encapsulation efficiency. Each observation is the mean ± SD of three observations.
Particle size of the prepared microbeads was determined by randomly counting average diameter of 100 particles with optical microscope using stage micrometer and eyepiece scale.

**Scanning electron microscopy (SEM)**

Scanning electron microscope (Joel JSM-5200) was used to characterize surface topography of prepared microspheres. The microbeads were placed on a metallic support with a thin adhesive tape and were coated with gold under vacuum (Fine coat, ion sputter JFC-1110) to render them electron conductive. The surface was scanned and photomicrographs were taken by 20 kV accelerating voltage.

**Fourier transform infrared spectroscopy (FTIR)**

FTIR spectra were recorded for pure drug, blank microbeads and drug loaded microspheres using FTIR (JASCO 410). Samples were prepared in KBr disks (2 mg sample in 200 mg KBr). The scanning range was 1000-4000 cm\(^{-1}\) and the resolution was 2 cm\(^{-1}\).

**In-vitro drug release studies**

The release studies of prepared beads were carried out in the USP-I (basket) dissolution apparatus (Labindia-2000, Mumbai, India) using 500 mL of 6.8 pH phosphate buffer at 37 ± 5°C and 100 rpm. Beads equivalent to 100 mg of 5-FU were taken and 2 mL samples were withdrawn at 0, 1, 2, 3, 4, 5, 6 and 12 h time intervals and the same volume was replaced in the dissolution medium by 6.8 pH phosphate buffer. After appropriate dilutions the samples were analyzed by UV-VIS spectrophotometer (Systronic, 2101) at 266.5 nm. In vitro release profile was analyzed by various kinetic models (Zero order, first order, Higuchi, Hixon Crowell) in order to find out the mechanism of drug release from beads.

**Cell cytotoxicity study**

The HT-29 human colon cancer cell lines were purchased from National Cell Lines Facility, Pune and cultured in DMEM (Dulbecco’s Modified Eagles Medium) supplemented with 10% heat inactivated fetal calf serum at 37°C, 5% CO\(_2\) and 80% humidity. The raw cells were washed with sterile DMEM twice and cultured in plates at a density 5 × 10\(^4\) cells/well.

Cell viability was determined by using the 3-(4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based cytotoxicity assay (9). The cells were stimulated with free 5-FU (100 mM), entrapped 5-FU formulations containing drug equivalent to 100 mM. After 24, 48 and 72 h, the cells were washed and treated with MTT. Plates were incubated in dark for 4 h, and the absorbance was measured at 570 nm using UV-visible spectrophotometer.

To determine the cell viability, percent viability was calculated as:

\[
\text{Percent viability} = \left( \frac{\text{absorbance of drug-treated sample}}{\text{control absorbance}} \right) \times 100
\]

**RESULTS AND DISCUSSION**

Preparation of 5-FU loaded calcium-zinc-gellan and calcium-zinc-gellan-EC microbeads

During ionotropic gelation of gellan, the ion interaction between the negatively charged gellan polymer and the positively charged divalent calcium and zinc was responsible for formation of beads. Commercially available GG is insoluble in cold water and also the low level of divalent ions are present in it, which inhibited the hydration, so during dropwise addition of the GG dispersion in a solution of divalent cations the dispersion was pre-heated to 55°C for complete hydration (10).

Evaluation of 5-FU loaded calcium-zinc-gellan and calcium-zinc-gellan-EC microbeads

All evaluation parameters of drug loaded microbeads were given in Table 1. The yields of beads for all formulations were greater than 80%. An increase in polymer amount increased drug encapsulation efficiency. This might be due to the fact that at higher polymer concentration higher will be the probability of drug surrounded by polymer, which acts as a barrier to prevent diffusion of drug into the external medium and also an increase in gel...
strength with GG concentration. The mean particle size of the microbeads was increased with an increase in GG and EC concentration, may be due to increasing the polymer concentration which produced a significant increase in the viscosity, thus leading to an increase of the droplet size and finally a higher microbead size (11).

SEM study has shown that the resulting microbeads were spherical in nature with rough surface (Fig. 1) The FTIR spectra of 5-FU has shown prominent peaks at 3131 cm\(^{-1}\) for NH\(_2\), 1658.48 cm\(^{-1}\) for C=O and 1246.75 cm\(^{-1}\) for carbon and halide bond. Drug loaded microbeads exhibited peaks within the same region confirming the stable nature of drug during encapsulation process.

It was found that the drug release rate was decreased with increasing polymer concentration in the order of F3 < F2 < F1 (Fig. 2). At higher concentration of GG, crosslinking increases, and free volume of matrix was less, which leads to decreases...
in early transport of drug through calcium-zinc-gellan beads (10). Sustaining action of delivery system was further amplified with insertion of EC and amount of EC in microbeads that is F5 > F4 > F3 (Fig. 2). This may be due to inclusion of hydrophobic polymer in the GG that increased the sustaining action. It was found that amongst all formulations the highest correlation value (0.996 ± 0.003) was observed with the Hixon Crowell model suggesting the mechanism of drug release to be dissolution controlled.

Cell toxicity study was carried out with all formulations excluding F1 because of its low sustained release characteristics. The results are presented in Figure 3. The findings show that both free and entrapped 5-FU produced cytotoxicity. In case of free 5-FU, the cytotoxicity effect was prominent and % viability decreased from 0 to 89.20 to 16% within a period of 0 to 24 to 72 h. In formulations the decrease in % viability was not immediate but gradual and continuous depending on polymer : drug ratio and the nature of polymer used. In case of formulations F2 and F3, the formulation with higher gellan amount (F3) aids in slow release of drugs from microbeads and hence gradual cytotoxic effect. Likewise, amount of hydrophobic polymer EC within GG further decreases the release of drugs from microspheres and consequently slows the cytotoxicity activity that is F5 < F4.

From cell toxicity study it is seen that formulation F2 and F4 have shown the highest activity against the cancerous cells but the latter has sustained drug release most effectively for 12 h. It was concluded that formulation F4 may be considered to be more effective in terms of sustained drug release activity as well as anti-cancerous deed.

CONCLUSION

5-FU loaded calcium-zinc-gellan microbeads with varying concentrations of EC were found to be spherical with rough surface. Increased proportion of GG and EC has increased encapsulation efficiency, particle size and sustained drug release effect of 5-FU. All formulations showed cytotoxicity. Formulation with drug : gellan : ethyl cellulose ratio of 2.5 : 7.5 : 1 which sustained the release for 12 h had a gradual and prolonged cytotoxic effect on HT-29 human colon cancer cell lines. This formulation affords a platform to develop an oral controlled release drug delivery system of 5-FU that not only improves the patient condition but also reduced systematic toxicity and thus reduced side-effects of chemotherapy.

REFERENCES


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