Development and evaluation of porous tablets of sodium alginate for treatment of Enterotoxigenic Escherichia coli infection

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Received 28 May 2012; revised 24 October 2012; accepted 08 January 2013

Enterotoxigenic Escherichia coli (ETEC) infections result in large mortality rate and usually a frequent cause of diarrhea in infants. To prevent enterotoxigenic Escherichia coli infections animal needs an active mucosal immunity at the moment of weaning. In the present study, F4 loaded porous sodium alginate tablets were prepared by direct compression technique for oral vaccination using ammonium carbonate as a pore former. In order to prevent the release the antigen in upper GI tract and to release it at target site nanoparticles were coated with Eudragit S100 which will protect the antigen against the detrimental effects in the gastrointestinal tract. Tablets were evaluated for pharmaco-technical properties and results were found to be satisfactory. SEM studies were conducted in order to show the porous surface of tablets. Ammonium carbonate as a pore forming agent proves to be promising and was successful in creating pores on surface of tablets through which F4 was loaded in tablets. SEM had given a clear picture showing major and minor pore with different pore size. It was found that an increase in pore forming agent leads to decrease in hardness and disintegration time of porous tablets. Mucosal immune response study revealed that, immune response was elicited and animals vaccinated with porous tablets group shows a significant reduction in excretion of F4+ E coli. Studies indicate that a solid vaccine formulation will be more efficient and these systems can contribute to the development of oral vaccines in veterinary as well as in human medicines.

Keywords: Porous tablets, Enterotoxigenic Escherichia coli, vaccination

Introduction

Oral route has been the most popular and successfully used for sustained delivery of drugs because of convenience and ease of administration, greater flexibility in dosage form design and ease of production and low cost of such a system. The growing interest in controlled drug delivery release is because of its benefits like increased patient compliance due to reduced frequency of administration and less undesirable side effects. Considerable attention had been laid on the development of porous materials as a carrier for drug delivery. Porous dosage form as a drug delivery adjuvant offers advantages such as stable uniform porous structure, high surface area, tunable pore size with narrow distribution and well defined surface properties 1. Mesoporous, microporous and nanoporous carriers for drug delivery are major fields of research for current researchers. It was reported that drug release was more reproducible and predictable in porous type of dosage forms. However liquid penetration in these types of porous dosage forms depends on bulk property of liquid and surface property of porous medium, Displacement of the liquid depends on pore size, surface tension of liquid and contact between surface of the adsorbent and liquid medium 2,3. Drug release from these dosage forms may get completed in few minutes and can be prolonged upto several hours to days depending on surface properties and absorption from the porous media 4. Alginate is linear, naturally occurring polysaccharide extracted from brown sea algae. It contains D-mannuronic acid (M) and L-guluronic acid (G) acids which are arranged in homopolymeric MM or GG blocks separated by blocks with an altering sequence, MG blocks. Hydration of alginate with media leads to the formation of gelatinous mass which act as retardant material for the drug to diffuse out. Matrix tablets of sodium alginate by direct compression have been prepared 5-7.

The presence of pores in ceramic foams offers the possibility to use these porous ceramics as carriers for local and controlled delivery of drugs. This application is useful in treatment of many skeletal diseases. The incorporation of antibiotics, chemotherapeutica, NSAIDs

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and growth factor in porous bone scaffolds is already been established 8-13. Ammonium carbonate (AC) had been already used as a pore forming to produce porous microparticles of bendroflumethazide by spray drying 14. Acute infectious diarrhoea is the second most common cause of deaths in children’s in developing countries. The pathogenic E Coli strain has been reported to be associated with gastroenteric diseases which cause significant losses in neonatal animals 15. Vaccination of sow during pregnancy leads to secretion of antigen specific antibodies in colostrums and milk, which provides immunity to the suckling piglets against infections. After being weaned, however the piglets are deprived of the passive protection and can become susceptible to ETEC infection. At this particular moment, an active immunisation is needed for protection. An immune mucosal response is desirable in order to eradicate the E. coli infections. Some of ETEC strain bears F4 fimbriae, a surface antigen which enables the microorganism to adhere to the receptors in brush borders of villous enterocytes and subsequently colonizing the small intestine from which the infection spreads to proximal part of intestine. Newly weaned piglets can be orally immunised with detached F4 fimbriae 16-19.

Oral solid dosage forms can be an attractive carrier to produce a mucosal immunity. Solid vaccine formulations can be mixed with creep food or can be given in a suspension form can be appropriate for successful immune response. In order to reach the target site, solid formulation can be enteric coated that will help to protect the antigen against possible detrimental effects of acids, bile enzymes present in stomach and duodenum. In the present study, F4 fimbriae loaded porous alginate tablets were developed against ETEC infection using AC as a pore former. Practicability of F4 fimbriae loaded porous alginate tablets for oral vaccination against ETEC was investigated. Immune response upon oral vaccinations was observed by determining F4 specific IgG serum antibodies that had been produced and protection was examined by oral challenge with F4+ETEC.

Materials and methods

Materials

E. coli strain (ATCC No, 35401, serotype O78:H11) was purchased from LGC PROMOCHEM Pvt Ltd, Banglore. Sodium alginate, Ammonium Carbonate and directly compressible microcrystalline cellulose (MCC), was procured from Loba Chemie Pvt Ltd. Mumbai. Eudragit S100 was received as a gift sample from Vikram Thermo Pvt Ltd, India. All other reagents were of analytical grade.

Methods

Preparation of Porous Tablets

Porous tablets were prepared by direct compression method. All the ingredients were passed through # 44-mesh separately. Then the ingredients were weighed and mixed in geometrical order and compressed into tablets of 200 mg using 10 mm round flat punches on 10-station rotary tablet machine (Rimek Mini Press, Mumbai). Ammonium carbonate (AC) previously sieved was used as pore forming agent. Tablets are washed with water twice for 10 sec for aqueous extraction of AC from the tablets in order create pores.

Bacterial inoculums

E. coli strain (ATCC No, 35401, serotype O78:H11) was cultured for 24 h 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).

Purification of F4 fimbriae

Briefly, the bacteria were cultured in tryptone soy broth (Sigma Aldrich, Mumbai) at 37°C for 15 h, 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. The purity was assessed by electrophoresis on a sodium dodecyl sulfate–10% polyacrylamide slab gel. Protein bands were visualized by staining with Coomassie brilliant blue G by standard procedures 21.

Loading of F4 in Porous tablets

Loading of F4 in porous nanoparticles were done by imbibing method. Tablets were loaded with F4 by incubating a solution of F4 (2.5 mg/ml) with porous tablets under mild agitation at room temperature. Loading was done in order to have 2 mg of isolated F4 fimbriae in a tablet.

Coating of Porous tablets

Enteric coating of all tablets formulations prepared bearing F4 was done by spray coating in a conventional coating pan. In brief coating solution was prepared by mixing Eudragit S100 with acetone for 1h using stirrer. After an hour, Tri ethyl citrate (TEC) 2% was added
and stirring was continued for 30 min. The tablets were coated in a conventional pan with 20 RPM, at a coating solution spray rate of 2ml/ min and inlet temperature of 60°C. Coating of tablets was continued till the weight gained was 2% per average weight of the tablet. The coated tablets were dried in an oven at 35°C for one day and stored in air-tight container.

**Evaluation of Pre-compression parameters of powders blends and Post compression parameters of prepared porous tablets**

Pre compression parameters of powder blends and other physical parameters like weight variation, hardness, and friability, disintegration time were investigated. 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. Hausner found that the ratio DF / Do was related to inter-particle friction and, as such, could be used to predict powder flow properties.

In order to determine the uniformity in weight of tablets, 20 tablets of each formulation were randomly collected and weighed using class A weight balance and their percentage variation was determined. Hardness of tablets was also determined using Erweka Hardness Tester. The tablets of each formulation were also subjected to friability testing employing Pharma Test Friabulator. The acceptable limit of weight loss was not more than 1.00%.

**Scanning electron microscopic (SEM)**

SEM photographs were taken with a scanning electron microscope Model Joel- LV-5600, USA, at the required magnification at room temperature. The photographs were observed to visualize the porous structure of the tablets.

**Determination of F4 fimbriae concentration in porous tablets**

F4 fimbriae concentration in tablets was determined as given by Snoeck et al. with slight modifications. Firstly, F4 loaded tablet was crushed and added to 15 ml of buffer pH 7.4 (PBS) followed by gentle shaking at room temperature for 2 hrs. After required shaking the suspension was filtered through whatman filter paper (Sigma Aldrich, Mumbai). Filtered solution was subjected to indirect ELISA using the F4 fimbrial solution as standard to determine the concentration of F4 in tablet.

**Experimental animals**

All the experimental procedures and animal management procedures were done 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 study were housed in 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 with minor modifications. At the age of 7 days, F4 fimbriae dose with F4 loaded tablets. Tablet was orally administered with 10 ml of PBS. Other group of animals was not immunized and was taken as control group. Tablet group received a booster immunization at during 3 consecutive days at age of 21. Finally at 31st day all animals were infected with virulent F4 + ETEC strain. At last all piglets were orally infected with F4 + ETEC 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.

**Faecal excretion of F4 + ETEC**

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 h and was quantified using dot blotting.

**Roentgenography studies**

In vivo performance for the prepared dosage form was evaluated by Roentgenography. Rabbit was selected as suitable animal model. The protocol for in vivo roentgenographic study was approved by the Institutional Animal Ethics Committee of JSS College of Pharmacy of JSS University, Mysore. The optimized formulation was used for the study in which the F4 was replaced by Barium sulphate. These formulations were made to
swallow by the rabbit and X-rays were taken at various time intervals.

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.

Result and discussion
Pre-compression parameters of powder blends and Post compression parameters of prepared porous tablets

The values of pre-compression parameters of powder blends evaluated were within prescribed limits and indicated good free flowing property as shown in Table 1. Bulk density varies between 0.43 ± 0.027 to 0.59 ±0.087 g/cc for all batches of formulations prepared. Angle of repose and Carr’s index are within limits ensuring good flow properties of pre compression powder blends indicating its acceptability for direct compression of tablets. Post compression parameter of porous tablets was evaluated and it was found that hardness of all prepared tablets varies between 6.4 ± 0.02 to 3.9 ± 0.06 Kg/cm². Friability varies between 0.001 ± 0.034 to 0.069 ± 0.12 % (USP limit < 1 %). Disintegration time varies between 88 min to 27 min for the prepared formulations.

Effect of the level of pore forming agent on Hardness of Porous tablets
Hardness of all prepared porous tablets varies between 6.5 ± 0.01 to 5.1 ± 0.02 Kg/cm². Effect of pore forming agent on various tablets hardness was shown in Fig. 1 (a). It is found that as we increase the amount of pore forming agent in tablets preparations, hardness goes on decreasing. Formulations A1, B1 and C1 are composed of fixed amount of SA with varying concentrations (increasing) of AC (pore forming agent). These formulations show maximum hardness but in decreasing order. A1 shows maximum hardness of 6.5 ±

<table>
<thead>
<tr>
<th>S. No</th>
<th>Formulation Code</th>
<th>Bulk Density (g/cc)</th>
<th>Tapped Density (g/cc)</th>
<th>Angle of Repose ±SD, n=3</th>
<th>Carr’s index (%) ±SD, n=3</th>
<th>Hausner’s Ratio ±SD, n=3</th>
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<tbody>
<tr>
<td>1</td>
<td>A1</td>
<td>0.43 ±0.027</td>
<td>0.65 ±0.02</td>
<td>23.13 ±1.86</td>
<td>14.17 ±1.73</td>
<td>1.29 ±0.07</td>
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<tr>
<td>2</td>
<td>A2</td>
<td>0.47±0.046</td>
<td>0.71 ±0.04</td>
<td>22.16 ±2.56</td>
<td>15.23 ±1.25</td>
<td>1.25 ±0.08</td>
</tr>
<tr>
<td>3</td>
<td>A3</td>
<td>0.56 ±0.016</td>
<td>0.73 ±0.16</td>
<td>22.29 ±1.37</td>
<td>16.33 ±1.32</td>
<td>1.29 ±0.03</td>
</tr>
<tr>
<td>4</td>
<td>B1</td>
<td>0.49 ±0.026</td>
<td>0.68 ±0.23</td>
<td>26.18 ±1.81</td>
<td>16.21 ±1.59</td>
<td>1.18 ±0.05</td>
</tr>
<tr>
<td>5</td>
<td>B2</td>
<td>0.53 ±0.062</td>
<td>0.69 ±0.11</td>
<td>23.31 ±1.53</td>
<td>17.22 ±1.65</td>
<td>1.17 ±0.07</td>
</tr>
<tr>
<td>6</td>
<td>B3</td>
<td>0.59 ±0.087</td>
<td>0.71 ±0.19</td>
<td>24.21 ±2.76</td>
<td>17.03 ±1.47</td>
<td>1.29 ±0.05</td>
</tr>
<tr>
<td>7</td>
<td>C1</td>
<td>0.43 ±0.061</td>
<td>0.72 ±0.22</td>
<td>21.20 ±1.16</td>
<td>17.22 ±1.35</td>
<td>1.21 ±0.09</td>
</tr>
<tr>
<td>8</td>
<td>C2</td>
<td>0.48 ±0.049</td>
<td>0.76 ±0.17</td>
<td>26.17 ±2.06</td>
<td>18.65 ±1.18</td>
<td>1.29±0.07</td>
</tr>
<tr>
<td>9</td>
<td>C3</td>
<td>0.54 ±0.017</td>
<td>0.79 ±0.26</td>
<td>27.23 ±1.17</td>
<td>18.23 ±1.56</td>
<td>1.27 ±0.07</td>
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</table>
0.01 Kg/cm², B1 showing 6.2 ± 0.01 Kg/cm² and C1 showing 5.8 ± 0.01 Kg/cm². All batches show same type of results. In case of formulation A2, B2 and C2 having same amount of SA but with varying concentrations (increasing) of AC (pore forming agent) the same results came into finding. Hence it is clearly indicated that as we increase the amount of pore forming agent there will be more porous structure of tablets available thereby increasing the porosity of tablets thus decrease in hardness of tablets came into existence.

Effect of the level of pore forming agent on Disintegration time of Porous tablets

Disintegration time (DT) is another parameter by which the porous structure of tablets can be defined. DT varies between 88 min to 27 min for prepared batches of tablets in pH 7.4. DT was first carried out in pH 1.2 to for 1 h to observe any cracking, deformity of tablets or any disruption of coating. It was found that there was no cracking or any other changes in physical appearance of tablets in pH 1.2. More the amount of pore forming agent presents in tablets less the time taken by the tablets to disintegrate. As we increase the amount of pore forming agent there will be more porous structure of tablets available thereby increasing the porosity of tablets, thus disintegration media will easily ingress into the tablets and helps the tablet to disintegrate in less time. Whereas in case of tablets having less amount of pore forming agent there will be not enough porosity of tablets structure in order for media to easily penetrate the tablet surface for disintegration. Effect of pore forming agent on disintegration time of porous tablets was shown in Fig. 1 (b).

As we increase the amount of pore forming agent in tablets preparations DT of porous tablets goes on decreasing. Formulations A1, B1 and C1 are composed of fixed amount of SA with varying concentrations (increasing) of AC (pore forming agent). These formulations show maximum DT but in decreasing order. A1 shows maximum DT of 88 min, B1 showing 81 min and C1 showing 38 min. All batches show same type of results. Hence it is clearly indicated that as we increase the amount of pore forming agent, there will be decrease in DT of porous tablets. It is clearly shown from the SEM image that pores are successfully created in the tablets by dipping the tablets in aqueous solution (by aqueous extraction of pore forming agent) through which AC had been leached out and further drug can be loaded in the porous tablets. SEM had given a clear picture showing major and minor pores with different pore size. C3 formulation was used for loading F4 and for immune studies because of its less disintegration time as it will easily get disintegrated and can releases F4 faster in order to have desirable immune response.

Protein Concentration & Purification of F4 fimbriae

A total of 15 mg of F4 was collected from 85 plates of culture. Mixture of proteins was visualized when purity was assessed by electrophoresis on a sodium dodecyl sulfate–12% polyacrylamide slab gel. Protein bands were visualized by staining with Coomassie brilliant blue G by standard procedures with molecular weights of 45, 26, 18.8 and 14.3 kDa.

In vivo experimental observation: oral immunization of piglets with oral F4 fimbriae and F4 loaded NPs

Test groups (formulation C3) were orally vaccinated at day 7, 8, 9, 21, 22, 23 days. Control was not immunized. At the end of 31st day, all animals were inoculated with virulent F4+ ETEC strain. F4 specific antibody response was observed till day 47. From the antibody titre (Fig. 2) it was shown that primary vaccination increases the F4
specific antibody in test groups but not in control groups as it has not been immunized. Following a second booster vaccination, it was observed that there has been considerable increase in F4 specific antibody in test group. After the challenge immunization, test group shows F4 specific antibody response, whereas in case of control group antibody titre after challenge immunization was found to be prominent. It was observed in the later stage antibody titre goes on increasing in control group whereas in case of test group the antibody remains stabilized. At the age of 21 day, there has been considerable increase in F4 specific IgG in test group. After challenge vaccination only the control group has shown the antibody titers. It was found that F4 specific IgG titer has been decreased in case of test group (p<0.05) indicating protection. On the whole, results completely revealed F4 loaded porous tablets were able to induce an immune response.

Faecal excretion of F4+ETEC

Faecal excretion studies revealed that all groups excreted hemolytic F4+ ETEC after challenge vaccination was done as shown in Fig. 3. It was found that immune response was evoked in the tablet group. Studies revealed that there was no excretion of F4+ ETEC after 6 dpc in case of NPs group compared to other groups which excrete F4+ ETEC after 6 dpc. This is considerably beneficial in order to protect the piglets from E. Coli infections. Studies clearly proved that enteric coated solid porous tablets prevent the colonization of small intestine by F4+ETEC and can be a promising tool which will protect the antigen from acidic pH and thus deprive its ability to evoke a premature immune response and will target the antigen to the proper site thus producing a desired immune response.

Roentgenography studies

Rabbit were selected to carry out roentgenography studies as pH of GIT of rabbits was similar to that of human. Also there is similarity in stomach transit time to that of humans. X-ray studies were carried out in rabbit at different time intervals at 2nd, 7th and 16th hour as shown in Fig. 4. It was observed that, optimized formulation was intact in stomach (2nd hr). A small change in shape of tablet was found in small intestine (7th hr), but a remarkable disruption in tablet shape was shown in the colon (16th hr). This can clearly prove that as pores formed in the tablets dissolution media will ingress in the tablet and tablet integrity will lose releasing the antigen for desired response.

Conclusion

Porous tablets of sodium alginate were obtained by direct compression using ammonium carbonate as pore forming agent. Pre compression parameters for the powders blends to make the porous tablets were evaluated and were found to be within prescribed limits, indicating good free flowing property. Considerable effect of pore forming agent on hardness, disintegration time was found. It has been found that an increase in concentration of pore forming agent leads to decrease in hardness and disintegration. SEM confirmed the formation of open pores with different pore size which make the media to easily go inside the porous structure of tablets, making the porous tablets as a good candidate for drug delivery. Immunization experiment revealed that that F4 fimbriae present in porous tablets were able to evoke an immune response upon oral administration in piglets. A significant reduction in the excretion of F4+ E Coli confirms the protection by tablet group against ETEC.
infection. So the above results completely suggest the use of porous tablets as a vaccine adjuvant in treating the ETEC infection.

References