

DEVELOPMENT AND IN VITRO EVALUATION OF POLYSACCHARIDE-BASED SYSTEM FOR INTESTINAL DELIVERY OF AZATHIOPRINE FOR TREATMENT OF GASTRODUODENAL CROHN'S DISEASE, JEJUNOILEITIS AND ILEITIS

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ABSTRACT

Azathioprine is immunosuppressant drug used effectively for treatment of gastroduodenal Crohn's disease, jejunoileitis and ileitis. The systemic administration of azathioprine is characterized by its slow onset of action and causing serious adverse effects. The objective of the current work was to develop and evaluate azathioprine-loaded pectin beads for intestinal release of azathioprine (*i.e.* the inflammatory disease site) to overcome the characteristic drawbacks of the systemic administration of azathioprine. Two different preparation techniques were followed to develop azathioprine-loaded pectin beads by ionotropic gelation. Several characterizations were carried out for the

developed beads (*e.g.* size, morphological features, yield, drug loading capacity, drug entrapment efficiency and *in vitro* release) to select the better preparation technique, and further optimization studies were performed. Azathioprine-loaded pectin beads were successfully developed as spherical beads of 1.62 ± 0.04 mm diameter and high entrapment efficiency (*c.a.* 94%). The *in vitro* release profiles of the developed azathioprine-loaded pectin beads showed that most of the drug amounts released at intestinal simulating medium. The azathioprine-loaded pectin beads developed in the current study might have a promising potential for treatment of gastroduodenal Crohn's disease, jejunoileitis and ileitis effectively, more effectively and more safely than the systemic administration of azathioprine.

KEYWORDS: azathioprine, intestinal delivery, ileitis, jejunoileitis, pectin.

INTRODUCTION

Crohn's disease is an autoimmune chronic inflammatory disorder of the gastrointestinal tract. The exact cause of Crohn's disease is really unknown; however, the disease incidence is influenced by various risk factors such as genetic factor, environmental pollution and microbial infections.^[1-3] Crohn's disease has many subtypes that differ according to the site of inflammation. Gastroduodenal Crohn's disease, jejunoileitis and ileitis are types of Crohn's disease that affect the small intestine. Nausea, diarrhea, abdominal spasms, fatigue, appetite loss and weight loss are characteristic symptoms for these diseases.^[3-6] Azathioprine (AZA) is an immunosuppressant drug which is highly effective for treatment of gastroduodenal Crohn's disease, jejunoileitis and ileitis.^[3, 7-9] The systemic administration of AZA is characterized by its slow onset of action and by causing serious side effects such as profound leukopenia bone marrow depression, hepatitis, and increased risk of lymphoma.^[10-15]

Specific drug targeting to the site of inflammatory disease is a successful strategy to increase the concentration of the drug at the disease site and to enhance the therapeutic outcomes and minimize the systemic adverse effects, subsequently.^[16,17] Polysaccharides are highly abundant biodegradable polymers and characterized by being highly stable, safe and inexpensive. Polysaccharides are widely utilized to form drug carrier systems of pH-dependent drug release pattern.^[18,19]

Pectin (PCT) is non-toxic polysaccharide extracted from the plant cell walls and consists mainly of α -(1-4)-linked D-galacturonic acid residues interrupted by 1,2-linked L-rhamnose residues. The pK_a value of pectin is approximately 3.5 and the molecule is negatively charged at neutral pH. Therefore, pectin has limited solubility and swelling properties in gastric conditions, whereas pectin is highly soluble and has high swelling properties in small intestine conditions.^[18,20,21]

Based on pectin polymer characteristics, cross-linked pectin beads loaded with various drugs have been designed for specific drug release in the small intestine.^[22-24] Combinations of the cationic polysaccharide chitosan (CHT), with pectin have been evaluated as effective way to provide less porous cross-linked pectin matrix with reduced premature release of the entrapped drug at gastric simulating medium.^[25-27]

In the current work, AZA-loaded pectin beads strengthened with chitosan were formulated following two different preparation techniques. The formed beads were evaluated in terms of size, morphological features, yield, drug loading capacity, drug entrapment efficiency and *in vitro* release pattern in gastro-intestinal simulating media. Further optimization for the better preparation techniques was then carried to develop optimal AZA-loaded pectin beads that able to release azathioprine in high concentration specifically at the disease site and thus might accelerate the onset of drug action and reduce the drug adverse effects.

MATERIALS AND METHODS

Materials

Genu pectin (type Im-104 as-fs) was obtained from cp kelco (atlanta, usa). Aza was provided by t3a pharmaceutical co. (assiut, egypt). Chitosan (100,000–300,000 kda) and sodium tripolyphosphate (stpp) were purchased from acros organics (geel, belgium). Zinc chloride, potassium dihydrogen phosphate were purchased from el-nasr pharmaceutical chemicals co. (cairo, egypt). All other chemicals were of pharmaceutical grades.

BEADS PREPARATION

1. Preparation of AZA-loaded chitosan microparticles dispersed in pectin beads (AZA-CHT MP @ PCT Beads)

For the preparation of AZA-loaded chitosan microparticles dispersed in pectin beads (A1), CHT solution (3%) was prepared by dissolving chitosan in acetate buffer (pH 5) under continuous stirring for 1 h. AZA was uniformly dispersed in CHT solution and homogenized for 30 min to prepare AZA dispersion (1.5%). AZA dispersion (1.2 g) was dropped through 0.19 mm inner diameter needle into 5 ml solution of PCT (5%) and STPP (2%) under high shear mechanical stirring (3000 rpm) for 15 min. Then, 5 g of the resultant mixture was dropped through 0.4 mm inner diameter needle into 35 ml ZnCl₂ solution (8%) under continuous stirring at 2500 rpm for 60 min. The formed beads were collected, washed three times with distilled water and dried at room temperature overnight.

1. Preparation of AZA-loaded pectin beads (AZA @ PCT beads)

For the preparation of AZA-loaded pectin beads (B1-B5), AZA was dispersed uniformly in PCT solution to prepare 1% AZA dispersion. Two grams of AZA dispersion was dropped into 13 mL solution of ZnCl₂ and chitosan through 0.4 mm inner diameter needle. The solution was stirred at 250 rpm for definite time using magnetic stirrer. The formed beads

were gathered by sieving, washed with distilled water triplicate and the dried at room temperature overnight. In case of B2 formulation, the collected beads were immersed into 10 ml STPP (2%) solution and stirred continuously for 30 min before the drying step. Different formulations were prepared at different compositions (*i.e.* PCT, CHT and ZnCl₂ concentrations) and under different preparation conditions (*i.e.* Cross-linking time and cross-linker solution temperature) were examined to select the optimal formulation (Table 1).

Table: 1 Compositions and preparation conditions of different AZA-loaded pectin beads.

Formulation	PCT (%)	CHT (%)	ZnCl ₂ (%)	STPP (%)	Cross-linking temperature (°C)
B1	4	1.1	1.7	0	25
B2	4	1.1	1.7	2	25
B3	8	2.2	3.4	0	25
B4	8	2.2	3.4	0	50
B5	8	0	3.4	0	25

Beads characterization.

1. Determination of Feret's diameter, circularity and roundness

For each formulation, sample of at least 20 beads was photographed using Canon® digital camera SX 230 HS (Japan). The digital images were analyzed using a computerized image analysis Imagej® software (Maryland, USA) to determine Feret's diameter, circularity and roundness of each bead. Equations (1) and (2) were applied to calculate circularity and roundness, respectively. The results of the calculated parameters were demonstrated as means and standard deviations of 20 beads.

$$Circularity = 4\pi \times \frac{(area)}{(perimeter)^2} \quad (1)$$

$$Roundness = \frac{4 \times (area)}{\pi \times (major\ axis)^2} \quad (2)$$

2. Determination of yield (%)

For calculating the yield of the formulated beads formulated *per* batch, the weight of the collected beads was determined and the total weight of input materials (*i.e.* drug, polymers and cross-linkers) was calculated. The yield percentage was calculated according to equation (3).

$$\text{Yield (\%)} = \frac{\text{Weight of dried beads}}{\text{Total weight of input materials}} \times 100 \quad (3)$$

3. Determination of drug loading capacity (%) and entrapment efficiency (%)

To determine the drug content in the Beads that containing chitosan (A1, B1-B4), the beads were grinded and 50 mg the powdered beads was placed into 100 mL volumetric flask. To the flask content, 50 ml 0.1 N HCl was added and the flask content was stirred at 1000 rpm 2h. After that, the volume of the flask (100 mL) was completed to with 0.2 N NaOH and the flask content was stirred again at 1000 rpm for additional 2h. The flask content was then filtered and the drug content was determined by UV-Vis spectrophotometer (Genway, England) at wavelength of 280 nm, with reference to the standard calibration curve (4 - 14 µg/ml) of AZA in 0.1 N NaOH solutions with the linearity range ($r^2 > 0.999$). The measured absorbance was in the range of 0.2–0.8. Drug loading capacity (DL) and entrapment efficiency (EE) were calculated based on equations (4) and (5), respectively.

To determine the drug content in the AZA @ PCT beads that not containing chitosan (B5), The same procedures were followed except that the flask was filled only with 0.1 N NaOH, instead of using 50 ml 0.1 N HCl followed by and 0.2 N NaOH.

$$\text{DL (\%)} = \frac{\text{Drug content in beads}}{\text{Weight of beads}} \times 100 \quad (4)$$

$$\text{EE (\%)} = \frac{\text{Actual drug content in beads}}{\text{Theoretical drug content in beads}} \times 100 \quad (5)$$

4. In vitro release studies

The drug release profiles from different formulated beads were studied in gastric simulating medium and small intestine simulating medium and compared with the free drug. Free drug or beads were placed within stainless-steel baskets, and the baskets were hanging in screw-capped vessels containing 100mL gastric simulating medium (*i.e.* 0.1 N HCl, pH 1.2). The vessels were incubated in shaking water bath at shaking rate of 50 rpm and temperature of $37.0 \pm 0.5^\circ\text{C}$. At specified time intervals (0.5, 1, 1.5 and 2 h), 3 ml aliquot samples were withdrawn from the vessel and an equal volume of fresh medium at $37.0 \pm 0.5^\circ\text{C}$ was replenished immediately. By the end of the two hours, the gastric simulating medium was taken out and replaced with 100 ml fresh intestinal simulating medium (*i.e.* phosphate buffer, pH 6.8) at $37.0 \pm 0.5^\circ\text{C}$. At specified time intervals (3, 4, 5 and 6 h), 3 ml aliquot samples

were withdrawn from the vessel and an equal volume of fresh medium at 37.0 ± 0.5 °C was replenished immediately. The collected samples were filtered and amounts of drug released were estimated by UV–Vis spectrophotometer (Genway, England) at 280 nm. The data were presented as mean \pm SD of at least triplicates.

Statistical analysis

All statistical analyses were performed using GraphPad Prism[®] version 5.00 for Windows (San Diego, California, USA). All experimental data were expressed as the mean \pm standard deviation (SD). For multiple comparisons between formulations in terms of diameter, roundness, circularity, and the amount of drug released, one-way analysis of variance (ANOVA) with Tukey post-hoc test was performed. For comparison between the release profiles of the different formulation, two-way analysis of variance (ANOVA) with Bonferroni post-hoc test was performed. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSIONS

Two preparation methods were followed to prepare AZA-loaded pectin beads to protect the drug against premature release in stomach and to deliver the drug in high concentrations at the intestinal region. The first preparation method based on the formation AZA-loaded CHT microparticles, under the influence of high shear stirring, which were cross-linked by the inotropic interaction between the cationic CHT polymer and the anionic STPP salt and PCT polymer.^[28, 29] Then, the formed mixture was dropped into $ZnCl_2$ solution as cross-linker for PCT (under mild stirring) to form composite PCT beads (A1). Accordingly, AZA was supposed to be doubly protected against rapid release in gastric simulating medium by enclosing internally within CHT microparticles and externally within PCT beads.^[25,30] The second preparation method based on the preparation of AZA-loaded beads of PCT that were cross-linked by $ZnCl_2$ and CHT (B1).^[31,32] When the formed AZA-loaded pectin beads (B2) were immersed in STPP solution (*i.e.* for further cross-linking of chitosan), the formed beads were destructed, indicating that STPP competed with PCT on the cationic charges of chitosan and thus the cross-linked pectin matrix was interrupted. This observation also confirmed that chitosan was involved in the structure of those AZA-loaded pectin beads. For comparing the two techniques, the formed beads were evaluated in terms of size, circularity, roundness, yield, DL, EE and drug release profiles from the beads in gastric and intestinal simulating media.

Morphologically, beads prepared by the first preparation method (A1) were significantly larger in size than the beads prepared by the second preparation method (B1). The larger size of A1 might be due to the increased polymers content within beads matrix. The beads prepared by the first technique were significantly less in sphericity as compared to those prepared by the second preparation method, as the presence of irregular shape chitosan microparticles within the beads matrix affected their sphericity (**Figure 1**) (**Table 2**). For illustration, the sphericity is evaluated in terms of circularity and roundness (*i.e.* In case of typical sphere the values of circularity and roundness equal one) [33, 34]. Both preparation methods formed beads in similar yield values, whereas DL and EE of the beads prepared by the first preparation method (A1) were less than those of the beads prepared by the second preparation method (B1) (**Table 2**).

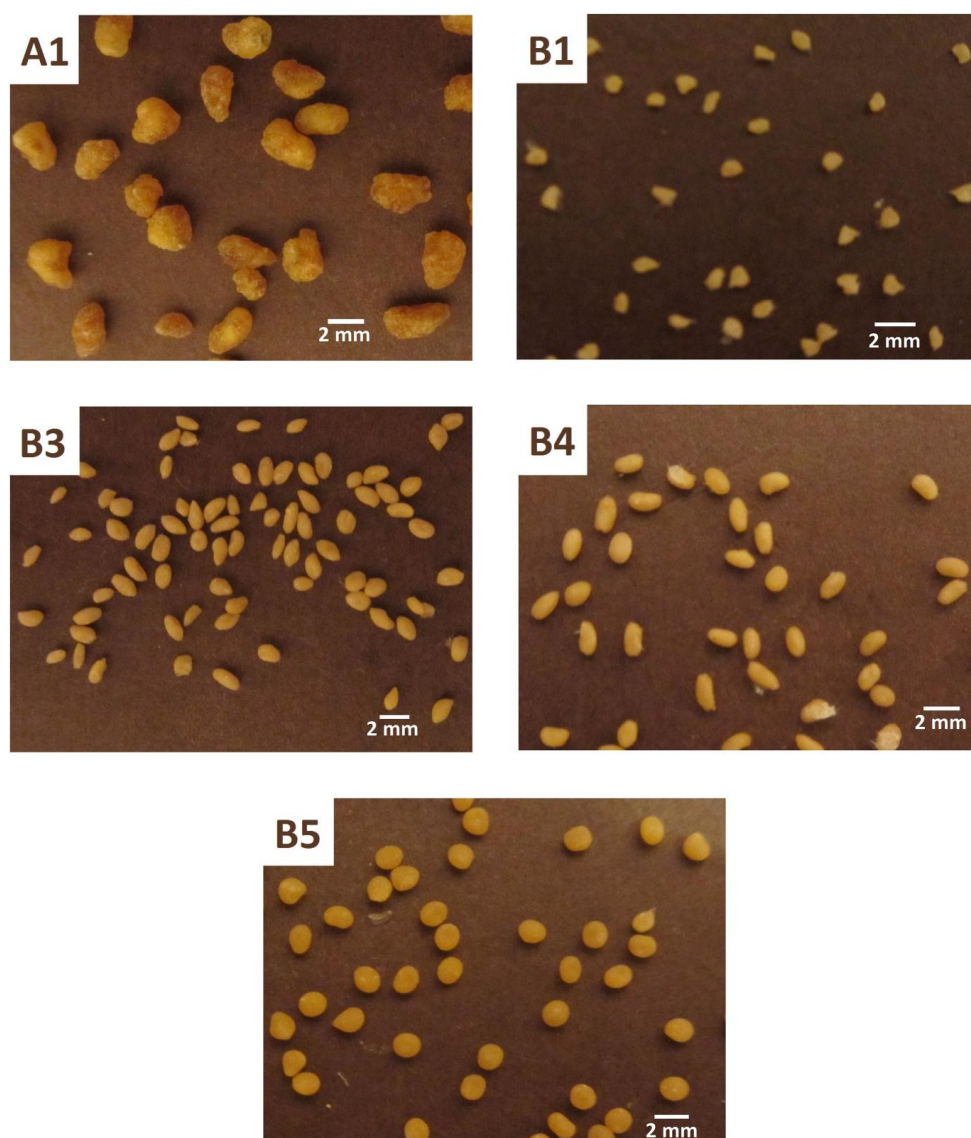


Figure 1: Photographic images for different AZA-loaded pectin beads

Table 2: Average Feret's diameter, circularity and roundness, yield, drug loading capacity (DL) and entrapment efficiency (EE) of the different AZA-loaded pectin beads.

Formulation	Feret's diameter (mm) \pm SD	Circularity \pm SD	Roundness \pm SD	Yield (%)	DL (%)	EE (%)
A1	3.09 \pm 0.45	0.86 \pm 0.05	0.69 \pm 0.13	21.29	1.42	57.13
B1	1.05 \pm 0.09	0.91 \pm 0.04	0.76 \pm 0.11	23.32	16.49	92.03
B2	---- ^a	----	----	----	----	----
B3	1.49 \pm 0.12	0.87 \pm 0.05	0.61 \pm 0.10	24.15	8.57	96.92
B4	1.48 \pm 0.08	0.89 \pm 0.04	0.61 \pm 0.08	21.89	7.8	79.99
B5	1.62 \pm 0.04	0.97 \pm 0.01	0.86 \pm 0.07	23.27	7.47	94.62

^a: No beads were formed.

The release profile of the free drug showed that most of the drug amount (*i.e. ca.* 85 \pm 0.7%) was dissolved during the first 30 min in the gastric simulating medium and the drug was completely dissolved before medium replacement, confirming the reported increased AZA solubility in the diluted solution of mineral acids (**Figure 2**).^[35] Generally, the overall amount of drug released from beads prepared by the first preparation method was reduced significantly as compared to the free drug. However, by the end of the first two hours in gastric simulating medium, the amount of drug released was 76.1 \pm 2.2% and less 24 % of the loaded drug was reached the intestinal simulating medium. On the other hand, the amount of drug released from the beads prepared by the second preparation method (B1) was significantly slower than that of both the free drug and the beads prepared by the first preparation method. Only less than 50% of the drug was dissolved in the acidic medium and the rest of drug was released rapidly in the intestinal simulating medium.

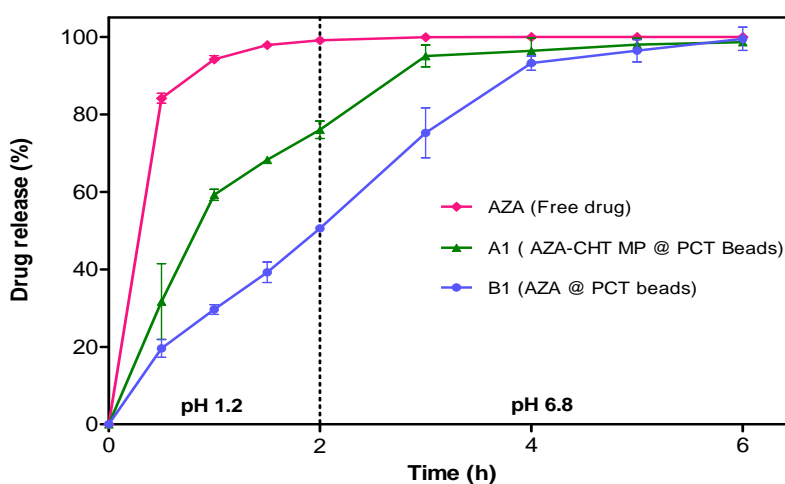


Figure 2: Release profiles of AZA-loaded pectin beads prepared by two different preparation methods, compared to the free drug, using 0.1 N HCl at pH 1.2 for 2 h and then phosphate buffer pH 6.8 for 4 h. Data are shown as mean \pm SD.

B1 beads (AZA @ PCT beads) had superior morphological characters, higher DL and EE, and were more efficient for achieving the aimed controlled release profile than A1 (AZA-CHT MP @ PCT Beads). In addition, the second preparation method is simpler than the first one. Hence, the second preparation method was selected as optimal method and included in the further optimization studies.

1. Effect of pectin concentration

Beads prepared of 8% pectin concentration (B3) were significantly larger in size and less in sphericity as compared to the beads prepared of 4% pectin concentration (B1), due to the increased polymers content within B3 beads (**Figure 1**) (**Table 2**).^[36] Yield of both formulations were similar. DL of the beads prepared at higher pectin concentration (8%) was about half that of the beads prepared of lower pectin concentration, as the amount of polymers and cross-linker used for B3 preparation were twice those used for B1 preparation (*i.e.* relative to the drug content). EE of the beads prepared of 8% pectin solution was higher than that of the beads prepared at 4% pectin, indicating the probability of formation of denser bead wall that prevent the drug escape from the beads matrix during processing and washing steps (**Table 2**).^[37] The amount of drug released in the gastric simulating medium from the beads prepared at higher pectin concentration ($39 \pm 1.1\%$) was significantly less than those released from the beads prepared of lower concentration ($50.6 \pm 0.5\%$) confirming the incidence of denser cross-linking of pectin due to the higher polymers content (**Figure 3**)^[34, 37] Therefore, using higher pectin concentration (B3) was more appropriate for the desired goal than lower pectin concentration.

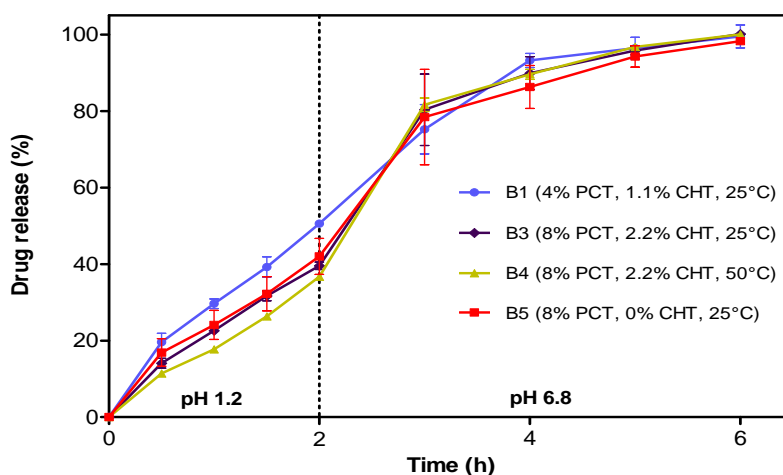


Figure. 3: Release profiles of different formulations of AZA-loaded pectin beads prepared at different pectin (PCT) concentrations, chitosan (CHT) concentrations and

cross-linking temperatures. The release media were using 0.1 N HCl at pH 1.2 for 2 h and then phosphate buffer pH 6.8 for 4 h. Data are shown as mean \pm SD.

2. Effect of cross-linking temperature

The morphological characters of the beads prepared at cross-linking solution of 50 °C temperature (B4) were not different significantly from those of the beads prepared using cross-linking solution at ambient temperature (B3) (**Figure 1**) (**Table 2**). Yield, DL and EE of the beads prepared at higher cross-linking temperature (B4) were less than those of the beads prepared at ambient temperature (B3). This result might be due to the increased drug and pectin solubility in the cross-linking solution of higher temperature and increased beads components loss during preparation step, subsequently (**Table 2**). The release profiles of the drug from the beads prepared at different cross-linking temperatures were similar, indicating that the cross-linking temperature had no influence on the density of pectin cross-linking reaction (**Figure 3**). Accordingly, preparing AZA-loaded pectin beads (B3) at room temperature were preferred and selected as optimal preparation temperature.

3. Effect of chitosan concentration

Morphologically, beads (B5) prepared at cross-linking solution of ZnCl₂ only (*i.e.* not containing chitosan) were larger in size and more spherical in shape as compared to the beads (B3) prepared at cross-linking solution of ZnCl₂ and chitosan (**Figure 1**) (**Table 2**). Both formulations were similar regarding yield, DL and EE, revealing that chitosan polymer was included effectively within the structure of B5 beads matrix (**Table 2**). The release profiles of AZA from both formulations in the gastric and intestinal simulating media were identical; the beads were observed visually to be swelled slightly in the gastric simulating medium and low amounts of AZA were released slowly, whereas the beads swelled rapidly in the intestinal simulating medium and most of drug amounts were released within the first hour in the medium and released totally by the end of the study (**Figure 3**). Therefore, on the contrary to the reported effective role of chitosan coat as drug release retardant^[38], it could be concluded that chitosan had minimal effect on the drug release from the beads, which means that the controlled release profile of the beads based mainly on the interaction between pectin and ZnCl₂. Therefore, preparation of AZA-loaded pectin beads (B5) of high pectin concentration (8%) at cross-linking solution of only ZnCl₂ (*i.e.* without consuming additional chitosan amounts) at ambient temperature was considered as optimal preparation conditions.

CONCLUSION

Azathioprine-loaded pectin beads were developed by ionotropic gelation using a simple preparation technique. The developed beads were spherical in shape and their entrapment efficiency was high. The *in vitro* release profiles of the developed azathioprine-loaded pectin beads showed that most of the loaded drug amounts might be released specifically at the disease site, and thus the beads might have a promising potential for enhanced management of gastroduodenal Crohn's disease, jejunoileitis and ileitis and minimized adverse effects.

REFERENCES

1. M. Orholm, P. Munkholm, E. Langholz, O.H. Nielsen, V. Binder, Familial occurrence of inflammatory bowel disease, *New England journal of medicine*, 1991; 324(2): 84-88.
2. A. Dignass, J.O. Lindsay, A. Sturm, A. Windsor, J.-F. Colombel, M. Allez, G. D'Haens, A. D'Hoore, G. Mantzaris, G. Novacek, T. Öresland, W. Reinisch, M. Sans, E. Stange, S. Vermeire, S. Travis, G. Van Assche, Second European evidence-based consensus on the diagnosis and management of ulcerative colitis Part 2: Current management, *Journal of Crohn's and Colitis*, 2012; 6(10): 991-1030.
3. T.M. Bayless, S.B. Hanauer, *Advanced Therapy of Inflammatory Bowel Disease, Volume 2: IBD and Crohn's Disease*, Pmph-USA, 2014.
4. R.A. van Hogezaand, A.M.C. Witte, R.A. Veenendaal, M.J. Wagtmans, C.B. Lamers, Proximal Crohn's disease: review of the clinicopathologic features and therapy, *Inflammatory bowel diseases*, 2001; 7(4): 328-337.
5. M. Freeman, S.R. Cho, Nongranulomatous ulcerative jejunoileitis, *Am J Gastroenterol*, 1984; 79(6): 446-9.
6. W. Miehsler, A. Püspök, G. Oberhuber, H. Vogelsang, Impact of different therapeutic regimens on the outcome of patients with Crohn's disease of the upper gastrointestinal tract, *Inflammatory bowel diseases*, 2001; 7(2): 99-105.
7. R. Enns, T. Lay, R. Bridges, Use of azathioprine for nongranulomatous ulcerative jejunoileitis, *Canadian journal of gastroenterology*, 1997; 11(6): 503-6.
8. G. D'Haens, K. Geboes, E. Ponette, F. Penninckx, P. Rutgeerts, Healing of severe recurrent ileitis with azathioprine therapy in patients with Crohn's disease, *Gastroenterology*, 1997; 112(5): 1475-1481.
9. T. Higuero, C. Merle, G. Thiéfin, S. Coussinet, D. Jolly, M.-D. Diebold, P. Zeitoun, G. Cadiot, Jejunoileal Crohn's disease: a case-control study, *Gastroenterologie clinique et biologique*, 2004; 28(2): 160-166.

10. U. Mahadevan, W.J. Tremaine, T. Johnson, M.G. Pike, D.C. Mays, J.J. Lipsky, W.J. Sandborn, Intravenous azathioprine in severe ulcerative colitis: a pilot study, *The American Journal of Gastroenterology*, 2000; 95(12): 3463-3468.
11. C. Mowat, A. Cole, A. Windsor, T. Ahmad, I. Arnott, R. Driscoll, S. Mitton, T. Orchard, M. Rutter, L. Younge, Guidelines for the management of inflammatory bowel disease in adults, *Gut*, 2011; 60(5): 571-607.
12. O. Pozler, J. Chládek, J. Malý, M. Hroch, P. Dědek, M. Beránek, P. Krásničanová, Steady-state of azathioprine during initiation treatment of pediatric inflammatory bowel disease, *Journal of Crohn's and Colitis*, 2010; 4(6): 623-628.
13. I.D.R. Arnott, D. Watts, J. Satsangi, Azathioprine and anti-TNF α therapies in Crohn's disease: a review of pharmacology, clinical efficacy and safety, *Pharmacological Research*, 2003; 47(1): 1-10.
14. M. Camus, P. Seksik, A. Bourrier, I. Nion-Larmurier, H. Sokol, P. Baumer, L. Beaugerie, J. Cosnes, Long-term Outcome of Patients With Crohn's Disease Who Respond to Azathioprine, *Clinical Gastroenterology and Hepatology*, 2013; 11(4): 389-394.
15. L. Langmead, D.S. Rampton, Colitis, Ulcerative, in: J. Editor-in-Chief: Leonard (Ed.), *Encyclopedia of Gastroenterology*, Elsevier, New York, 2004; 385-399.
16. U. Klotz, M. Schwab, Topical delivery of therapeutic agents in the treatment of inflammatory bowel disease, *Advanced Drug Delivery Reviews*, 2005; 57(2): 267-279.
17. D. Pertuit, B. Moulari, T. Betz, A. Nadaradjane, D. Neumann, L. Ismaïli, B. Refouvet, Y. Pellequer, A. Lamprecht, 5-amino salicylic acid bound nanoparticles for the therapy of inflammatory bowel disease, *Journal of Controlled Release*, 2007; 123(3): 211-218.
18. V.R. Sinha, R. Kumria, Polysaccharides in colon-specific drug delivery, *International Journal of Pharmaceutics*, 2001; 224(1-2): 19-38.
19. T. Kean, M. Thanou, Biodegradation, biodistribution and toxicity of chitosan, *Advanced Drug Delivery Reviews*, 2010; 62(1): 3-11.
20. F. Maestrelli, M. Cirri, G. Corti, N. Mennini, P. Mura, Development of enteric-coated calcium pectinate microspheres intended for colonic drug delivery, *European journal of pharmaceutics and biopharmaceutics*, 2008; 69(2): 508-518.
21. A. Noreen, Z.-i.-H. Nazli, J. Akram, I. Rasul, A. Mansha, N. Yaqoob, R. Iqbal, S. Tabasum, M. Zuber, K.M. Zia, Pectins functionalized biomaterials; a new viable approach for biomedical applications: A review, *International Journal of Biological Macromolecules*, 2017; 101: 254-272.

22. T. Wong, H. Lee, L. Chan, P. Heng, Drug release properties of pectinate microspheres prepared by emulsification method, *International journal of pharmaceutics*, 2002; 242(1): 233-237.
23. S. Das, K.Y. Ng, Resveratrol-loaded calcium-pectinate beads: Effects of formulation parameters on drug release and bead characteristics, *Journal of pharmaceutical sciences*, 2010; 99(2): 840-860.
24. S.S. Badve, P. Sher, A. Korde, A.P. Pawar, Development of hollow/porous calcium pectinate beads for floating-pulsatile drug delivery, *European journal of pharmaceutics and biopharmaceutics*, 2007; 65(1): 85-93.
25. C.-Y. Yu, B.-C. Yin, W. Zhang, S.-X. Cheng, X.-Z. Zhang, R.-X. Zhuo, Composite microparticle drug delivery systems based on chitosan, alginate and pectin with improved pH-sensitive drug release property, *Colloids and Surfaces B: Biointerfaces*, 2009; 68(2): 245-249.
26. P.-H. Chen, T.-Y. Kuo, J.-Y. Kuo, Y.-P. Tseng, D.-M. Wang, J.-Y. Lai, H.-J. Hsieh, Novel chitosan–pectin composite membranes with enhanced strength, hydrophilicity and controllable disintegration, *Carbohydrate Polymers*, 2010; 82(4): 1236-1242.
27. K.L.B. Chang, J. Lin, Swelling behavior and the release of protein from chitosan–pectin composite particles, *Carbohydrate Polymers*, 2000; 43(2): 163-169.
28. M. Marudova, A.J. MacDougall, S.G. Ring, Pectin–chitosan interactions and gel formation, *Carbohydrate Research*, 2004; 339(11): 1933-1939.
29. X. Shu, K. Zhu, A novel approach to prepare tripolyphosphate/chitosan complex beads for controlled release drug delivery, *International Journal of Pharmaceutics*, 2000; 201(1): 51-58.
30. H. Andishmand, M. Tabibiazar, M.A. Mohammadifar, H. Hamishehkar, Pectin-zinc-chitosan-polyethylene glycol colloidal nano-suspension as a food grade carrier for colon targeted delivery of resveratrol, *International Journal of Biological Macromolecules*, 2017; 97: 16-22.
31. S. Das, A. Chaudhury, K.-Y. Ng, Preparation and evaluation of zinc–pectin–chitosan composite particles for drug delivery to the colon: Role of chitosan in modifying in vitro and in vivo drug release, *International Journal of Pharmaceutics*, 2011; 406(1–2): 11-20.
32. H. Jonassen, A. Treves, A.-L. Kjøniksen, G. Smistad, M. Hiorth, Preparation of Ionically Cross-Linked Pectin Nanoparticles in the Presence of Chlorides of Divalent and Monovalent Cations, *Biomacromolecules*, 2013; 14(10): 3523-3531.

33. S. Eun Shim, S. Yang, S. Choe, Mechanism of the formation of stable microspheres by precipitation copolymerization of styrene and divinylbenzene, *Journal of Polymer Science Part A: Polymer Chemistry*, 2004; 42(16): 3967-3974.
34. H.H. Gadalla, G.M. Soliman, F.A. Mohammed, A.M. El-Sayed, Development and in vitro/in vivo evaluation of Zn-pectinate microparticles reinforced with chitosan for the colonic delivery of progesterone, *Drug delivery*, 2016; 23(7): 2541-2554.
35. W.P. Wilson, S.A. Benezra, Azathioprine, In: K. Florey (Ed.), *Analytical Profiles of Drug Substances*, Academic Press, New York, 1981; 29-53.
36. S. Jose, M. Prema, A. Chacko, A.C. Thomas, E. Souto, Colon specific chitosan microspheres for chronotherapy of chronic stable angina, *Colloids and surfaces B: Biointerfaces*, 2011; 83(2): 277-283.
37. M. Nand Singh, H. KS Yadav, M. Ram, H. G Shivakumar, Freeze dried chitosan/poly-(glutamic acid) microparticles for intestinal delivery of lansoprazole, *Current drug delivery*, 2012; 9(1): 95-104.
38. A.J. Ribeiro, C. Silva, D. Ferreira, F. Veiga, Chitosan-reinforced alginate microspheres obtained through the emulsification/internal gelation technique, *European Journal of Pharmaceutical Sciences*, 2005; 25(1): 31-40.