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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

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ABSTRACT

Zinc–pectin–chitosan composite microparticles were designed and developed as colon-specific carrier. Resveratrol was used as model drug due to its potential activity on colon diseases. Formulations were produced by varying different formulation parameters (cross-linking pH, chitosan concentration, cross-linking time, molecular weight of chitosan, and drug concentration). Single-step formulation technique was compared with multi-step technique. Effect of these parameters was investigated on shape, size, weight, weight loss (WL), moisture content (MC), encapsulation efficiency (EE), drug loading (L), and drug release pattern of the microparticles. The formulation conditions were optimized from the drug release study. *In vivo* pharmacokinetics of the zinc-pectinate particles was compared with the zinc–pectin–chitosan composite particles in rats. Formulations were spherical with 920.48–1107.56 μm size, 21.19–24.27 mg weight of 50 particles, 89.83–94.34% WL, 8.31–13.25% MC, 96.95–98.85% EE, and 17.82–48.31% L. Formulation parameters showed significant influence on drug release pattern from the formulations. Formulation prepared at pH 1.5, 1% chitosan, 120 min cross-linking time, and pectin:drug at 3:1 ratio demonstrated colon-specific drug release. Microparticles were stable at 4 °C and room temperature. Pharmacokinetic study indicated *in vivo* colon-specific drug release from the zinc–pectin–chitosan composite particles only.

1. Introduction

Over the past few years, colon-specific drug delivery systems have attracted significant attention of the researchers. The advantages of colonic drug delivery have been well-documented (Yang et al., 2002; Chourasia and Jain, 2003; Liu et al., 2003). These systems are useful not only for local treatment of colon diseases, but also for the systemic therapy by both conventional and labile molecules (Yang et al., 2002; Liu et al., 2003). Various forms of drug carriers have been utilized for the effective colonic delivery, such as tablets, capsules, pellets, beads, microspheres, micro- and nano-particles. However, multiple-unit systems proved to be better than single-unit systems due to their several advantages (Rodriguez et al., 1998; Maestrelli et al., 2008a). In addition, colonic microflora-activated systems, where drug release is triggered by the enzymatic breakdown of the carrier by specific colonic bacteria, have emerged as the most effective colon-specific drug delivery systems (Liu et al., 2003; Basit, 2005). Pectin is an example of such polymers, which is selectively degraded by the pectinolytic enzymes of colonic microflora (Friend, 2005). However, solubility of pectin in upper GI fluids makes it unsuitable as colon-specific carrier. Hence, divalent cations, such as Ca^{2+} and Zn^{2+}, have been used to produce a stronger and more water resistant gel (Sriamornsak, 1999; El-Gibaly, 2002; Atyabi et al., 2005; Chambin et al., 2006; Dupuis et al., 2006; Maestrelli et al., 2008a, 2008b).

We have earlier developed multi-particulate calcium-pectinate (Das and Ng, 2010c) and zinc-pectinate (Das et al., 2010b) formulations by cross-linking pectin chains with Ca^{2+} and Zn^{2+}, respectively. The *in vitro* drug release study in simulated intestinal fluid (SIF) demonstrated better delayed drug release profile from the Zn-pectinate formulation than Ca-pectinate formulation (Das et al., 2010b). However, the formulations were unable to

Abbreviations: GI tract, gastro-intestinal tract; Ca, calcium; Ca^{2+}, calcium cation; Zn, zinc; Zn^{2+}, zinc cation; Ca-pectinate, calcium pectinate; Zn-pectinate, zinc pectinate; LMW, low molecular weight; MMW, medium molecular weight; DI, deionized; EE, encapsulation efficiency; L, drug loading; MC, moisture content; WL, weight loss during drying; ER, elongation ratio; RT, room temperature; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SCF, simulated colonic fluid; P:D, pectin:drug; NUS, National University of Singapore; HPLC, high performance liquid chromatography; AUC, area under the curve; C_{max}, maximum plasma concentration; T_{max}, time require to reach C_{max}.

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show colon-specific drug release when drug release study was performed in the simulated GI conditions (i.e., 0–2 h in simulated gastric fluid (SGF), 2–5 h in SIF, and 5–8 h in simulated colonic fluid (SCF)). Rapid drug release was observed in SIF following their exposure to SGF (Das and Ng, 2010a). Similar observation of enhanced drug release after consecutive exposure to acidic (i.e., SGF) and basic (i.e., SIF) environment has been reported by other researchers (Atyabi et al., 2005). To improve colon-specificity, glutaraldehyde and polyethyleneimine was used as hardening agents (Das and Ng, 2010a, 2010b; Das et al., 2010a). Although colon-specific drug release was observed from these formulations, toxicity is the major concern with glutaraldehyde and polyethyleneimine (Khandare et al., 2010; Ahishali et al., 2009).

To overcome these drawbacks, combination of pectin with a biocompatible second polymer has been employed. This second polymer would interact with pectin to form a complex structure that would be stable until its arrival to the colon, followed by rapid degradation by the colonic enzymes. Chitosan is a cationic polysaccharide. Similar to pectin, it is biocompatible, biodegradable, and nontoxic (Ilium, 1998). As zinc ions produce stronger pectinate matrix than calcium ions, Zn^{2+} was chosen as cross-linking agent. Chitosan can interact with pectin via electrostatic and hydrogen bonds forming polyelectrolyte complex (Chang and Lin, 2000; Marudova et al., 2004; Marudova et al., 2005) which is expected to stabilize the Zn-pectinate matrix. Combination of pectin and chitosan has been employed for colonic drug delivery (Hiorth et al., 2010, 2006; Bigucci et al., 2008, 2009; Maestrelli et al., 2008b).

Resveratrol has shown potential therapeutic activity on colonic diseases, such as colorectal cancer and colitis (Tessitore et al., 2000; Schneider et al., 2001; Martin et al., 2004, 2006). However, along with others (Marier et al., 2002; Walle et al., 2004), our previous research (Das et al., 2008) has shown that resveratrol is rapidly absorbed through the upper gastro-intestinal (GI) tract following oral administration (even from suspension formulation of resveratrol). Resveratrol is also extensively metabolized in the GI tract and liver, which leads to low bioavailability (Marier et al., 2002; Gescher and Steward, 2003; Kaldas et al., 2003; Baur and Sinclair, 2006; Maier-Salamon et al., 2006; Das et al., 2008). Hence, only small amount of resveratrol was expected to reach in the colon following oral administration, which was evident in our previous study (Das et al., 2008). Incorporation of resveratrol into an effective colon-specific carrier was expected to resolve the above-mentioned problem.

Taking into account all the above considerations, the aim of this work was to design and develop an effective colon-specific delivery system of resveratrol. Formulations were prepared by simultaneous cross-linking of pectin chains by Zn^{2+} and chitosan. Effect of cross-linking solution pH, cross-linking time, chitosan concentration, molecular weight of chitosan, pectin to drug ratio (pectin:drug or P:D), formulation technique (single-step versus multi-step) were investigated on drug release from the formulations. The formulation conditions were optimized from the drug release study. Size, shape, weight, weight loss during drying (WL), moisture content (MC), drug loading (L) and encapsulation efficiency (EE) of all batches were evaluated. Relative percent drug content within the optimized formulation after storage at different temperature was determined. Furthermore, *in vivo* pharmacokinetic experiment in rats was performed on zinc-pectinate (Zn-pectinate) particles and optimized zinc–pectin–chitosan (Zn–pectin–chitosan) composite particles. Although some researchers (Munjeri et al., 1997; Chang and Lin, 2000; Kim et al., 2003; Nurjaya and Wong, 2005; Maestrelli et al., 2008b; Mennini et al., 2008; Wong and Nurjaya, 2008) have evaluated the calcium–pectin–chitosan formulations, the Zn–pectin–chitosan composite particles have been completely neglected despite the fact that Zn^{2+} is better cross-linker than Ca^{2+} for pectin (El-Gibaly, 2002; Atyabi et al., 2005; Chambin et al., 2006; Dupuis et al., 2006). Additionally, this study thoroughly investigated the effect of the major formulation parameters on the physicochemical properties, *in vitro* drug release in the simulated GI conditions, and *in vivo* drug release, which have been overlooked in the previous studies.

2. Materials and methods

2.1. Materials

GENU® pectin LM-104 AS-FS (degree of esterification = 28% and degree of amidation = 20%) was a generous gift from CPKelco (Denmark). Medium molecular weight (MMW) chitosan (molecular weight = 190–310 kDa), low molecular weight (LMW) chitosan (molecular weight = 50–190 kDa), sodium hydroxide, sodium phosphate monobasic, zinc acetate dehydrate, and Pectinex® Ultra SP-L (pectinase/pectinolytic enzyme from *Aspergillus aculeatus*, activity > 9500 PG ml^{-1}) were obtained from Sigma (St. Louis, MO, USA). Resveratrol (fine crystalline powder with 99.12% purity) and methanol (HPLC grade) were purchased from Shaanxi Sciphar Biotechnology Co. Ltd. (Xi'an, China) and Tedia Company (Fairfield, OH, USA). Monobasic potassium phosphate and disodium hydrogen phosphate anhydrous were bought from Fluka (Steinheim, Germany). All materials were used as received.

2.2. Formulation procedure

The formulation procedure was modified from our previous publication (Das and Ng, 2010c). Briefly, pectin was dissolved in deionized (DI) water (5%, w/v pectin). Resveratrol was homogeneously dispersed in the pectin solution by a homogenizer. Air bubbles were removed from the dispersion by sonication on a bath sonicator. Chitosan was dissolved in 1% (v/v) acetic acid and mixed with zinc acetate. Then 6 ml pectin–resveratrol mixture was taken into a 10 ml syringe and dropped in the gently agitated 100 ml cross-linking solution (5%, w/v zinc acetate + chitosan) through 23G needle (blunt end) form 5 cm distance at room temperature (RT; 25 °C). Spherical microparticles were immediately formed. Microparticles were separated from the cross-linking solution and repeatedly washed with DI water to remove excess zinc acetate and chitosan. Then the microparticles were dried at RT for 48 h. Different batches were prepared by varying the formulation parameters (Table 1). All batches were prepared in triplicate.

2.3. Shape and size

Fifty microparticles were randomly selected from each batch. Length and breadth of the particles were measured by an optical microscope (LEICA DM IL, Switzerland) as mentioned in our previous publication (Das and Ng, 2010c). Size of the particles was calculated from the following equation (1):

$$\text{size} = \frac{\text{length} + \text{breadth}}{2} \quad (1)$$

Shape of the particles was presented as elongation ratio (ER) which was calculated from Eq. (2).

$$\text{ER} = \frac{\text{length}}{\text{breadth}} \quad (2)$$

Particles with ER < 1.15 were considered spherical (Das and Ng, 2010c).

Table 1
Formulation design.

Variables	Values	Constants					
		Cross-linking solution pH	Chitosan concentration (% w/v)	Cross-linking time (min)	Molecular weight of chitosan	Pectin:drug	Formulation step
Cross-linking solution pH	4.6[a]	–	1	120	MMW	3:1	Single
	1.5	–	1	120	MMW	3:1	Single
Chitosan concentration (% w/v)	0	1.5	–	120	MMW	3:1	Single
	0.1	1.5	–	120	MMW	3:1	Single
	0.5	1.5	–	120	MMW	3:1	Single
	1	1.5	–	120	MMW	3:1	Single
Cross-linking time (min)	5	1.5	1	–	MMW	3:1	Single
	30	1.5	1	–	MMW	3:1	Single
	120	1.5	1	–	MMW	3:1	Single
Molecular weight of chitosan	LMW	1.5	1	120	–	3:1	Single
	MMW	1.5	1	120	–	3:1	Single
Pectin:drug	1:1	1.5	1	120	MMW	–	Single
	3:1	1.5	1	120	MMW	–	Single
Formulation step	Multi[b]	1.5	1	120	MMW	3:1	–
	Single	1.5	1	120	MMW	3:1	–

[a] Unmodified pH of the cross-linking solution.
[b] Multi-step formulation technique: formulation was prepared without chitosan (preformed formulation), thoroughly washed with DI water, put into 1% chitosan solution (pH 1.5), stirred for 2 h, thoroughly washed with DI water, and dried.

2.4. Morphology

Morphology study of surface and cross-section of the microparticles was performed by JEOL scanning electron microscopy (SEM; JSM-5200, Japan). Microparticles were fixed on an aluminum stub and coated with platinum for 30 s (thickness of coating ~2 nm) under vacuum with the JEOL auto fine coater (JFC-1600, Japan). The micrographs were recorded at an excitation voltage of 20 kV.

2.5. Weight, weight loss, and moisture content

Weight, WL and MC were determined by gravimetric method (Das and Ng, 2010c). Briefly, 50 microparticles were randomly selected from each batch. Weights of the microparticles before (W_W) and after (W_D) drying were measured by an analytical balance with readability of 0.00001 g (Mettler Toledo, Switzerland). Then moisture was completely removed by placing the microparticles at 60 °C until no further weight change was noticed. Weight of these completely dry microparticles (W) was measured by the same balance. WL and MC were determined by Eqs. (3) and (4), respectively.

$$\mathrm{WL}(\%) = \frac{W_W - W_D}{W_W} \times 100\% \quad (3)$$

$$\mathrm{MC}(\%) = \frac{W_D - W}{W_D} \times 100\% \quad (4)$$

2.6. Encapsulation efficiency and loading

EE and L of the formulations were measured according to the method used in our previous study with slight modification (Das and Ng, 2010c). Briefly, the formulation (~25 mg) was dispersed in 5 ml phosphate buffer solution (50 mM, pH 7.4) containing 1% (v/v) pectinase. Then methanol (10 ml) was added to the dispersion and thoroughly mixed to dissolve the drug (soluble in methanol). Thereafter, the mixture was centrifuged at 10,000 × g for 10 min. The supernatant was diluted with methanol–water (1:1) to the calibration range (0.1–10 μg ml^{-1}) and drug content in the supernatant (i.e., drug remaining in the formulation) was determined by a UV–Visible Spectrophotometer (UV-1601, Shimadzu) at 320 nm. Drug free formulation was used as control. EE and L were calculated using the following equations (5) and (6):

$$\mathrm{EE}(\%) = \frac{\mathrm{AQ}}{\mathrm{TQ}} \times 100\% \quad (5)$$

$$\mathrm{L}(\%) = \frac{\mathrm{AQ}}{W_F} \times 100\% \quad (6)$$

where AQ is the actual quantity of drug present in the formulation (drug content), TQ is the theoretical quantity of drug (amount of drug used during formulation), and W_F is the weight of the formulation.

2.7. In vitro drug release study

Solubility of resveratrol in SIF was found 0.11 ± 0.01 μg ml^{-1} (Das et al., 2008). Hence, insignificant amount of drug was expected to dissolve in the release media that penetrated into the formulation. Hence, most of the drug released from the formulation was undissolved particles rather than dissolved drug molecules, which remained as undissolved particles in the release media following their release (Das and Ng, 2010c). This phenomenon hindered measurement of drug concentration in the release media. Because of this limited aqueous solubility of the drug, an alternative method was developed for *in vitro* drug release study (Das and Ng, 2010c). Drug remaining in the formulation was measured at different time intervals and drug release was calculated as follows:

$$\text{amount of drug release} = \text{initial amount of drug in the formulation} - \text{drug remaining in the formulation at that time} \quad (7)$$

Detail of the drug release procedure can be found elsewhere (Das and Ng, 2010c, 2010a). Drug release study was performed in the simulated GI conditions (i.e., 0–2 h in SGF, pH 1.2; 2–5 h in SIF, pH 6.8; 5–12 h in SCF, pH 6). SCF was 50 mM phosphate buffer (pH 6) with 300 PG Pectinex® Ultra SP-L. Formulation was weighed (~25 mg) and placed in the screw cap glass test tubes. Separate tubes were used for each time point. Formulations from the same batch were selected for each set of release study. Drug content within the formulation was measured in triplicate for each batch

to ensure the homogeneity of drug distribution in the formulations (data not shown). Release medium (10 ml) was added in the tubes. The tubes were placed on a water bath (37 ± 0.2 °C) with horizontal shaking (100 ± 5 rpm). The designated tubes were withdrawn at the respective time points; intact formulations were isolated from the release medium, and gently washed with DI water to remove the free drug from the surface. Drug content inside the intact formulations was determined following the method described in the previous section (EE and L). Amount of drug release was calculated from Eq. (7) and percent drug release was plotted against time.

2.8. In vivo study

The study design and the animal handling protocol were approved by the Institutional Animal Care and Use Committee of the National University of Singapore (NUS). Adult male Sprague–Dawley rats (250–300 g) were purchased from Laboratory Animal Center of NUS and housed under temperature- (22 ± 1 °C) and humidity- (60–70%) controlled environment at 12 h light–dark cycle in Animal Holding Unit of NUS. Prior to the experiment, a polyethylene tube (i.d. 0.58 mm, o.d. 0.965 mm, Becton Dickinson, MD) was surgically inserted into the right jugular vein of the rat under anesthesia. The rats were randomly divided into three groups ($n = 6$). Group 1 received Zn-pectinate formulation; while Group 2 received optimized Zn–pectin–chitosan formulation (Table 3). The dose of resveratrol in both groups was 25 mg kg^{-1}. Group 3 received drug-free formulation (equivalent weight of drug-loaded formulation; ~100 mg kg^{-1}). The rats were kept in fasting condition from 12 h before the experiment to avoid any interference from food. However, free access to water was allowed. Effective oral gavage of the formulations was almost impossible due to large particle size (~1 mm). As oral gavage method directly administers the formulation to the stomach, an alternative approach was adopted for the oral delivery of these formulations. Briefly, the formulations were surgically placed in the stomach under gas anesthesia (isoflurane) and quickly stitched back to the normal condition. The anesthesia was removed immediately after the surgery. Serial blood samples (200 μl) were withdrawn from each animal at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h through the polyethylene tube, and collected in the heparinized tubes. The polyethylene tube was flushed with an equivalent volume of heparin-saline (5 U ml^{-1} heparin in normal saline) after each draw of blood sample to replace the blood. Plasma samples (supernatant) were collected by centrifuging the blood samples at 5500 × *g* for 10 min and stored at −80 °C.

Sample preparation and assay method was followed from our previous study (Das et al., 2008). Details of the sample preparation and assay method can be found there. Briefly, liquid–liquid extraction (ethyl acetate) was performed to recover the drug from plasma before analysis. Carbamazepine was used as internal standard. The samples were analyzed by high performance liquid chromatography (HPLC; Shimadzu 2010A; Kyoto, Japan) using a reversed phase HPLC column (ODS Hypersil, 5 μm, 250 mm × 4 mm; Agilent, Palo Alto, CA) at 35 °C. Mobile phase consisted acetonitrile and 30 mM phosphate buffer solution pH 7.0 (30:70, v/v) at a flow rate of 1 ml min^{-1} (isocratic). Detection wavelength was 320 nm. Drug recovery from plasma was >95% and limit of quantification (LOQ) of the assay method was 5 ng ml^{-1}.

The peak plasma drug concentration (C_{max}) and the time to reach C_{max} (T_{max}) were obtained from the graph. The area under the plasma concentration versus time curve ($AUC_{0\rightarrow t}$) was calculated by the linear trapezoidal rule from 0 to the last time point.

2.9. Effect of storage temperature on relative percent drug content within microparticles

Effect of storage temperature on relative percent drug content within the optimized formulation was performed as stated in our previous publication (Das and Ng, 2010c). Briefly, ~100 mg formulation was stored at 4 °C, RT, and 40 °C in tightly sealed container covered with aluminum foil. Separate containers were used for each time point. Homogeneity of drug distribution in the formulation was checked as mentioned in the drug release study and formulation from same batch was selected for each set of study. Samples were withdrawn at each time interval (1, 3, and 6 months) and drug content inside the formulation was checked by HPLC. Same HPLC assay conditions were used as *in vivo* study. Samples were prepared as described in the EE study. Drug content at 0 day was considered as 100% and percent drug content within the formulation in compare to 0 day's sample were measured in subsequent days to determine relative percent drug content after storage.

2.10. Statistical analysis

All experiments were performed in triplicate and experimental data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using Graph-Pad Prism Version 2.00 software (San Diego, CA). Either one-way ANOVA with the *post hoc* Tukey test or two-tail unpaired *t*-test (where applicable) was performed except T_{max} (non-continuous data). Two-tail Mann–Whitney test were used to compare the T_{max}. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Shape, size, and morphology

All formulations were spherical in shape (Table 2). Size of all formulations was <1 mm and there was no significant difference in particle size among different formulations (except the formulation prepared at P:D = 1:1). However, significant augmentation of size was observed when the formulation was prepared at P:D of 1:1.

SEM image of the microparticles at low magnification (Fig. 1A) indicated spherical particles with size <1 mm. However, slight depression of one side of the surface (black circle in Fig. 1A) was observed. Other researchers (Bourgeois et al., 2008) also noticed such behavior in case of calcium-pectinate particles. This might be due to shrinkage of particle surface during drying. SEM image of the microparticle at both low and high magnifications revealed rough and rugged surface (Fig. 1A and B). Drug crystals (black arrows) were observed as embedded in the matrix (Fig. 1B). A thin surface layer (black arrow in Fig. 1C) was observed in case of cross-section of the microparticle. This layer might be due to more polyelectrolyte complex formation between chitosan and pectin at the microparticle surface than inner matrix.

3.2. Weight, weight loss, and moisture content

Weight of 50 microparticles was 21.19–24.27 mg, WL was 89.83–94.34%, and MC was 8.31–13.25% (Table 2). Weight of the microparticles decreased with increasing chitosan concentration, cross-linking time, molecular weight, P:D; whereas heavier microparticles were produced at higher pH and multi-step technique. There was insignificant difference in WL among the formulations (except the formulation prepared at P:D = 1:1). However, formulation prepared at P:D = 1:1 showed significantly lower WL (89.83 ± 1.57) value than formulation prepared at P:D = 3:1. Formulation parameters exhibited significant impact on MC. MC decreased with increasing chitosan concentration, and cross-linking time; while MC decreased with decreasing cross-linking pH and P:D. MC was almost unaffected by molecular weight of chitosan and formulation technique although slightly higher MC

Table 2
Shape, size, weight of 50 particles, WL, MC, EE, and L of the formulations (data presented as mean ± SD; $n = 3$).

Variables	Values	Shape (ER)	Size (μm)	Weight (mg)	WL (%)	MC (%)	EE (%)	L (%)
Cross-linking solution pH	4.6	1.09 ± 0.05	958.82 ± 34.31	22.49 ± 0.59	93.05 ± 0.95	10.59 ± 1.27	97.58 ± 1.27	20.85 ± 1.04
	1.5	1.07 ± 0.02	944.36 ± 28.79	21.19 ± 0.69	93.85 ± 1.26	9.20 ± 0.56	98.06 ± 1.39	22.85 ± 1.02
Chitosan concentration (% w/v)	0	1.04 ± 0.01	920.48 ± 14.47	23.57 ± 0.61[a]	92.88 ± 0.15	12.25 ± 0.79	96.95 ± 0.19	18.67 ± 1.29[a]
	0.1	1.06 ± 0.04	938.24 ± 25.72	22.85 ± 1.04	94.14 ± 0.57	12.09 ± 1.39	97.40 ± 1.22	19.87 ± 0.82
	0.5	1.09 ± 0.07	959.53 ± 38.93	22.20 ± 0.90	94.34 ± 1.20	10.71 ± 0.98	97.63 ± 1.28	21.14 ± 1.56
	1	1.07 ± 0.02	944.36 ± 28.79	21.19 ± 0.69	93.85 ± 1.26	9.20 ± 0.56[b]	98.06 ± 1.39	22.85 ± 1.02
Cross-linking time (min)	5	1.07 ± 0.04	948.90 ± 23.58	24.27 ± 1.11[c]	94.17 ± 1.24	13.25 ± 1.47[c]	98.85 ± 1.06	17.82 ± 0.85[d]
	30	1.04 ± 0.01	967.06 ± 15.96	22.44 ± 0.75	93.45 ± 1.92	10.93 ± 0.71	98.38 ± 0.87	21.36 ± 0.45
	120	1.07 ± 0.02	944.36 ± 28.79	21.19 ± 0.69	93.85 ± 1.26	9.20 ± 0.56	98.06 ± 1.39	22.85 ± 1.02
Molecular weight	LMW	1.06 ± 0.03	979.57 ± 20.17	21.68 ± 0.84	93.59 ± 1.28	9.45 ± 0.71	98.32 ± 1.25	22.23 ± 1.39
	MMW	1.07 ± 0.02	944.36 ± 28.79	21.19 ± 0.69	93.85 ± 1.26	9.20 ± 0.56	98.06 ± 1.39	22.85 ± 1.02
Pectin:drug	1:1	1.08 ± 0.06	1107.56 ± 41.85[e]	24.10 ± 0.97[e]	89.83 ± 1.57[e]	8.31 ± 1.10	98.62 ± 1.32	48.31 ± 1.04[e]
	3:1	1.07 ± 0.02	944.36 ± 28.79	21.19 ± 0.69	93.85 ± 1.26	9.20 ± 0.56	98.06 ± 1.39	22.85 ± 1.02
Formulation step	Multi	1.08 ± 0.05	982.14 ± 29.53	22.11 ± 0.39	93.42 ± 2.15	9.64 ± 1.30	97.47 ± 1.49	21.09 ± 1.97
	Single	1.07 ± 0.02	944.36 ± 28.79	21.19 ± 0.69	93.85 ± 1.26	9.20 ± 0.56	98.06 ± 1.39	22.85 ± 1.02

[a] $p < 0.05$ between 0 and 1% chitosan concentration.
[b] $p < 0.05$ between 1% chitosan concentration with 0 and 0.1% chitosan concentration.
[c] $p < 0.05$ between 5 and 120 min.
[d] $p < 0.05$ between 5 min with 30 and 120 min.
[e] $p < 0.05$ between P:D of 1:1 and 3:1.

was observed in formulations prepared with LMW chitosan and at multi-step procedure.

3.3. Encapsulation efficiency and loading

EE and L of the formulations are listed in Table 2. EE was very high in all formulations (>97%). EE slightly increased with increasing chitosan concentration, and decreased with increasing cross-linking pH, cross-linking time, molecular weight of chitosan, P:D, and at multi-step formulation procedure. However, there was no significant difference among them. On the other hand, L of the formulations was significantly influenced by the formulation parameters. L increased with increasing chitosan concentration and cross-linking time. Whereas, L was lower at higher cross-linking pH and P:D. Slightly lower L was noticed when LMW chitosan was used or multi-step formulation technique was adopted.

3.4. In vitro drug release

The pH of cross-linking solution in presence of chitosan showed great influence on drug release properties (Fig. 2). Formulation prepared at lower pH (1.5) demonstrated very low drug release in

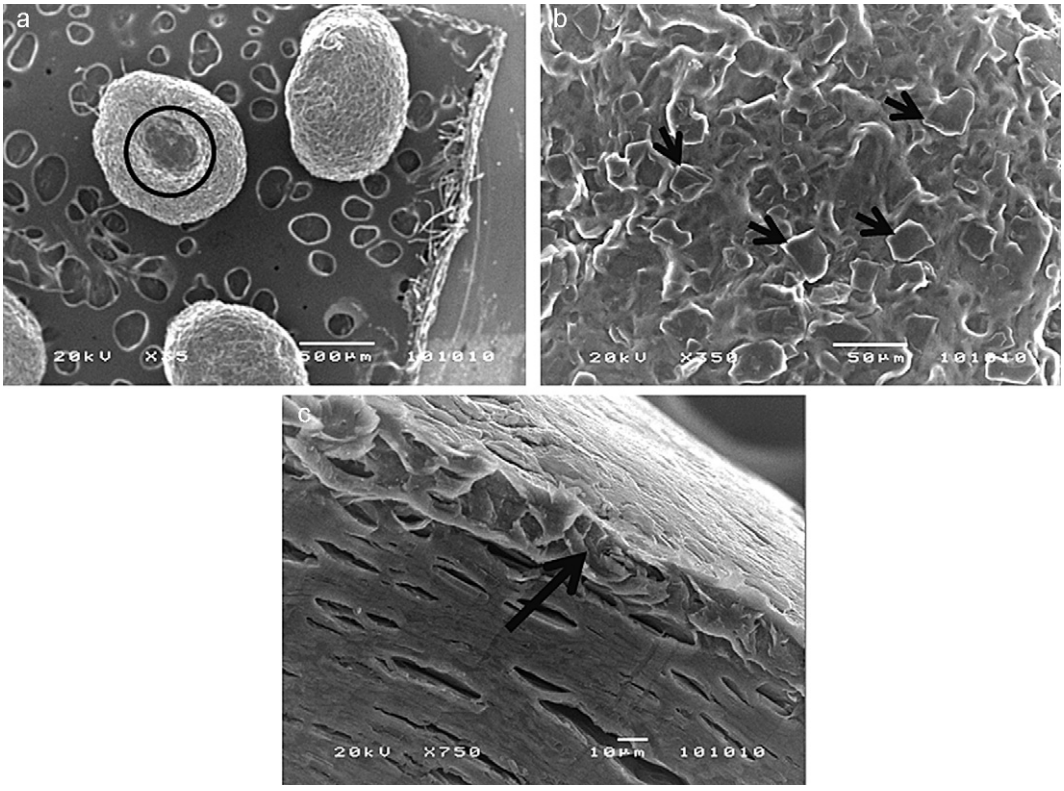

Fig. 1. Scanning electron micrographs of the microparticle surface at 35× (A) and 350× (B), and cross-section at 750× (C).

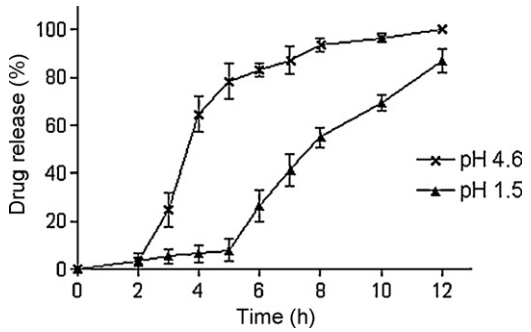

Fig. 2. Effect of cross-linking solution pH on drug release from the formulations. The study was performed in simulated GI conditions (0–2 h: SGF, 2–5 h: SIF, 5–12 h: SCF). Data presented as mean ± SD (n = 3).

the upper GI conditions (<8% drug released after 5 h) followed by rapid but sustained drug release in the colonic condition (>86% drug released after 12 h). In contrary, formulation prepared at higher pH (4.6) demonstrated rapid drug release in the upper GI conditions and most of the drug released in SIF (>78% drug released after 5 h). However, drug release was very low in SGF (<3.5% drug released after 2 h).

Drug release was dependent on concentration of chitosan in the cross-linking solution (Fig. 3). Drug release rate decreased with increasing chitosan concentration. When very low concentration (0.1%) or no chitosan was used, drug release rate was very high in SIF (>80% and >92% drug released after 5 h, respectively) despite low drug release in SGF (<4.5% drug released after 2 h in both cases). However, moderate drug release in SIF (~40% drug released after 5 h) followed by rapid drug release in SCF (>95% drug released after 12 h) was evident when 0.5% chitosan was used. On the other hand, low drug release in SIF (<8% drug released after 5 h) followed by enhanced and prolonged drug release was noticed when formulation was prepared in presence of 1% chitosan (>86% drug released after 12 h).

Effect of cross-linking time on drug release is depicted in Fig. 4. Drug release decreased with increasing cross-linking time. Formulation cross-linked for short duration (5 min) showed rapid drug release in SIF (>84% drug released after 5 h). Moderate cross-linking time (30 min) produced formulation that showed less drug release in SIF (~45% drug released after 5 h) than the formulation cross-linked for short duration. However, this release behavior was not sufficient for colon-specific release. In both cases, drug release in SGF was very low (<5% drug released after 2 h). Nevertheless, formulation cross-linked for long time (120 min) produced sufficiently strong matrix which prevented major drug release in SIF but released drug in SCF in a controlled manner.

This study showed that molecular weight of chitosan did not influence drug release behavior of the formulation (Fig. 5). Drug release in SGF, SIF, and SCF was not significantly different between the formulations prepared with LMW and MMW chitosan.

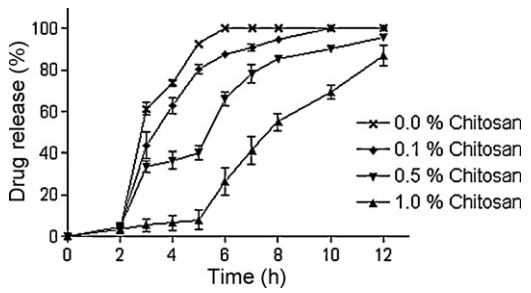

Fig. 3. Effect of chitosan concentration in the cross-linking solution on drug release from the formulations. The study was performed in simulated GI conditions. Data presented as mean ± SD (n = 3).

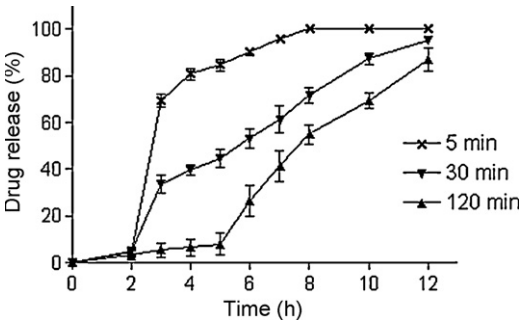

Fig. 4. Effect of cross-linking time on drug release from the formulations. The study was performed in simulated GI conditions. Data presented as mean ± SD (n = 3).

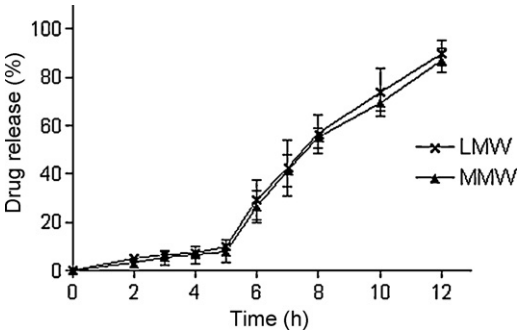

Fig. 5. Effect of molecular weight of chitosan on drug release from the formulations. The study was performed in simulated GI conditions. Data presented as mean ± SD (n = 3).

Formulations with high amount of drug were prepared in this study. However, our study showed that excessive amount of drug incorporation in the formulation (P:D = 1:1) hampered colon-specific drug release (Fig. 6). Formulations prepared at P:D = 1:1 showed faster drug release in SIF (>47% drug released after 5 h) despite little drug release in SGF (<8% drug released after 2 h). Whereas, formulations prepared at P:D = 3:1 showed low drug release in the upper GI conditions (SGF and SIF) and controlled drug release in SCF.

The results from in this study showed that formulation procedure had a critical impact on the drug release pattern from the formulations (Fig. 7). When formulation was prepared via multi-step technique, it could not show colon-specific drug release. The formulation exhibited small drug release in SGF (<3% drug released after 2 h) but quick drug release in SIF (>65% drug released after 5 h). Single-step formulation technique produced stronger matrix

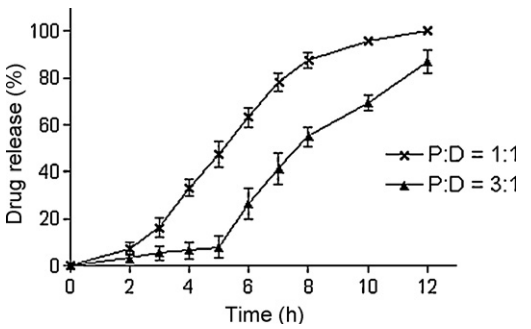

Fig. 6. Effect of polymer to drug ratio on drug release from the formulations. The study was performed in simulated GI conditions. Data presented as mean ± SD (n = 3).

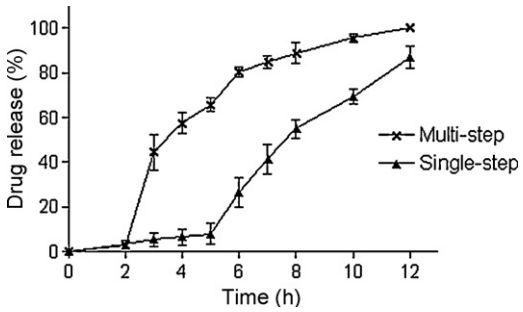

Fig. 7. Effect of formulation technique on drug release from the formulations. The study was performed in simulated GI conditions. Data presented as mean ± SD ($n = 3$).

Table 3
Pharmacokinetic parameters (data presented as mean ± SD; $n = 3$).

	Group 1	Group 2
Formulation	Zn–pectin[a]	Zn–pectin–chitosan[b]
Dose (mg kg^{-1})	25	25
T_{max} (h)	3–4	7–10*
C_{max} (ng ml^{-1})	118.97 ± 28.71	86.15 ± 29.11
AUC (ng h ml^{-1})	451.18 ± 56.39	408.20 ± 30.34

[a] Formulation conditions: cross-linking solution = 5% (w/v) zinc acetate solution, cross-linking solution pH 1.5, cross-linking time = 120 min, P:D = 3:1.
[b] Formulation conditions: cross-linking solution = 5% (w/v) zinc acetate solution + 1% (w/v) MMW chitosan, cross-linking solution pH 1.5, cross-linking time = 120 min, P:D = 3:1, formulation technique = single-step.
* $p < 0.05$ between Groups 1 and 2.

which was able to prevent drug release in the upper GI conditions followed by sustained drug release in SCF.

3.5. *In vivo* pharmacokinetics

In vivo pharmacokinetic studies of the Zn-pectinate and Zn–pectin–chitosan composite particles were performed in rats to compare *in vivo* drug release from the formulations and to confirm the importance of chitosan for colon-specific drug release. The pharmacokinetic parameters are listed in Table 3. The study indicated significantly higher T_{max} value of chitosan-modified formulation than unmodified formulation. However, opposite trend was observed in case of C_{max} and AUC. C_{max} and AUC values were higher in case of unmodified formulation than chitosan-modified formulation. However, C_{max} and AUC values between two formulations were not statistically different ($p > 0.05$). Plasma drug concentrations following administration of the formulations were plotted against time (Fig. 8). The drug in plasma for

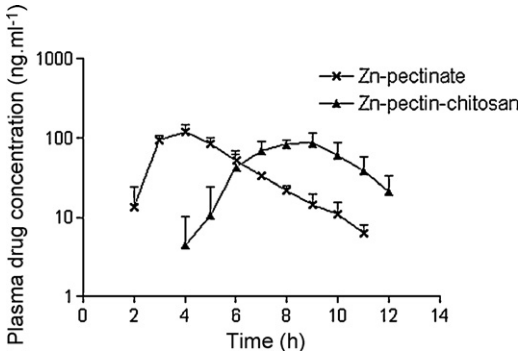

Fig. 8. Plasma concentrations of drug after a single dose of unmodified formulation (Group 1) and chitosan modified formulation (Group 2). The lines represent the predicted values. Symbols represent the mean observed values ± S.D ($n = 6$).

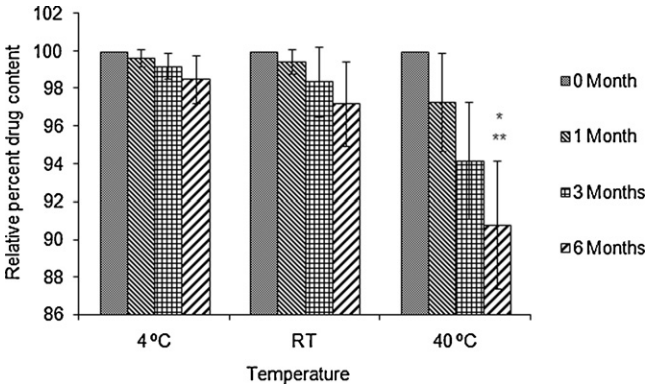

Fig. 9. Effect of storage temperature (4 °C, RT (25 °C), and 40 °C) on relative percent drug content within the microparticles. Data presented as mean ± SD ($n = 3$). *$p < 0.05$ for the difference between 180 days and 0 day, **$p < 0.05$ for the difference between 40 °C with 4 °C and RT.

Zn–pectin particles was undetectable before 2 h and detectable at very low concentration (13.88 ± 10.36 ng ml^{-1}) at 2 h. However, drug concentration rapidly increased after 2 h, reached to max concentration (118.97 ± 28.71 ng ml^{-1}) at 4 h, and then quickly decreased. Drug was no more detectable in plasma at 12 h. In case of chitosan-modified formulation, the drug was not detectable in plasma upto 3 h following their administration. Very small amount of drug was detected in plasma at 4 and 5 h (4.49 ± 5.68 and 10.66 ± 14.03 ng ml^{-1}, respectively). Thereafter, high concentrations of drug were detected in plasma, which gradually increased upto 9 h. Then drug concentration in plasma gradually decreased with time (21.42 ± 11.60 ng ml^{-1} drug was detected in plasma at 12 h). Plasma samples from Group 3 did not show any interference HPLC peak.

3.6. *Effect of storage temperature on relative percent drug content*

Drug content (>97%) in the formulation was high after 6 months storage at 4 °C and RT (Fig. 9). *In vitro* drug release profile of the formulation after 6 months storage at 4 °C and RT was not significantly different from the fresh formulation (data not shown). Drug content (<91%) was slightly lower when stored at 40 °C for 6 months. Lower relative percent drug content at 40 °C could be due to degradation of the drug at accelerated temperature.

4. Discussion

Ionotropic interaction between negatively charged carboxylic groups (COO^-) and positively charged divalent zinc cations (Zn^{2+}) led to the instant production of spherical particles (El-Gibaly, 2002). Size of the particles generally depends on diameter of the needle used during formulation and also on the drying method. As both of them were constant for all batches, size was almost similar. However, bigger particles were observed when formulation was prepared at lower P:D (1:1). During drying, particle size primarily decreased due to loss of moisture from the polymer (size of the drug particles remain almost same after drying). Lower amount of polymer was present at P:D = 1:1 than P:D = 3:1. Thus, less amount of polymer was available for drying at low P:D (1:1), which ultimately produced bigger particles.

Reduced weight and MC with increasing chitosan concentration and cross-linking time, and with decreasing cross-linking pH, were mainly due to more compact matrix formation. As mentioned earlier, formulation prepared at P:D = 1:1 contained more amount of drug and less amount of polymer for moisture evaporation. This was responsible for the higher weight and lower WL and MC for the formulation. Slight augmentation of weight and MC was prob-

ably due to loose matrix formation at multi-step procedure (will be discussed later).

EE was very high in all formulations. Such high EE was due to poor aqueous solubility of the drug (Das and Ng, 2010c) and quick formation of the cross-linked matrix (Zn-pectinate or Zn–pectin–chitosan). As the cross-linking solution was aqueous, only small amount of drug leaked in the cross-linking solution and most of the drug remained within the formulation. The drug loading depends on the weight of formulations, which is closely related to MC and EE. L is reciprocal to weight and MC, while proportional to EE. Hence, augmentation of L with increasing chitosan concentration, cross-linking time, molecular weight of chitosan, and with decreasing cross-linking pH and P:D can be explained by corresponding weight, MC, and EE. Similarly, higher L of the formulation prepared using single-step than the formulation prepared using multi-step technique can be explained by their lower weight and MC, and higher EE.

As the drug is poorly soluble in the release media (Das et al., 2008), only small amount of drug (as dissolved drug molecules) was expected to release via diffusion from the matrix. The predominant drug release mechanism is expected to be due to release of drug as undissolved particles following swelling (due to penetration of release media in the matrix) and erosion of the matrix (Das and Ng, 2010c). Enhanced drug release rate in SCF was due to the enzymatic degradation of the matrix by pectinase enzyme.

Pre-exposure to acidic medium caused rapid drug release from the Zn-pectinate formulations in SIF. Other researchers have also reported rapid degradation of Ca-pectinate formulations in the intestinal fluid following their exposure to acidic media (Atyabi et al., 2005). The reasons for such observation have been described elsewhere (Das and Ng, 2010a). The drug release results demonstrated that Zn-pectinate particles were unable to prevent drug release in SIF, while Zn–pectin–chitosan composite particles prevented drug release in the upper GI conditions. The reasons for such observation might be as follows. Pectin is insoluble at low pH and soluble at high pH, while opposite trend is observed for chitosan due to protonation of amine groups under low pH conditions (George and Abraham, 2006). Hence, dissolution of chitosan in low pH was prevented by the pectin network since pectin was insoluble in low pH conditions. On the other hand, dissolution of matrix at higher pH was prevented by chitosan which was insoluble at higher pH. For pectin, electrostatic repulsion was minimized at a low pH due to suppression of ionization, and the matrix exhibited minimum swelling by forming a closer gel network (Munjeri et al., 1997).

In our previous studies (Das et al., 2010b; Das and Ng, 2010c), we have indicated stronger Ca-/Zn-pectinate matrix formation in cross-linking solution pH 1.5 (<pKa of pectin) due to several non-ionic interactions (hydrophobic interaction and hydrogen bonding) and conformational ordering. However, this study revealed that particles prepared in cross-linking solution pH 1.5 in the absence of chitosan did not show colon-specific drug release. In addition to the ionic interaction between Zn^{2+} and COO^{-}, polyelectrolyte complex was formed between chitosan and pectin. Complex formation between pectin and chitosan was mainly due to electrostatic interaction between positively charged amino groups (NH^{3+}) of chitosan and negatively charged COO^{-} groups of pectin (Bigucci et al., 2008). Moreover, intramolecular H-bonding between the COOH groups of pectin or NH_2 groups of chitosan and OH, OCH_3 or $COOCH_3$ groups elsewhere within the complex was also possible (Bigucci et al., 2008). However, these interactions between amidated pectin and chitosan is favoured at low pH (Munjeri et al., 1997; George and Abraham, 2006), which resulted more compact matrix. Due to the formation stronger matrix, less swelling and erosion occurred when formulation was prepared at low pH. This might be the reason for very low drug release in SIF from the formulation prepared at low pH. However, huge drug release in SIF was evident from the formulation prepared at high pH due to loose matrix formation.

Increase of chitosan concentration in the cross-linking solution promoted formation of polyelectrolyte complex between pectin and chitosan, as more chitosan was available in the cross linking solution. Hence, strong matrix was formed at high chitosan concentration. Similarly, increase in cross-linking time produced stronger matrix because of the availability of more time for cross-linking between pectin and Zn^{2+}, and for polyelectrolyte complex formation between pectin and chitosan. Thus, low swelling and erosion of the matrix followed by minimal drug release in the upper GI conditions was anticipated for the formulation prepared at high chitosan concentration or long cross-linking time.

In case of lower P:D, where amount of drug was higher and amount of polymer was lower, higher drug release was expected due to unavailability of sufficient polymer for cross-linking and complex formation. The formulation was more prone to erosion in SIF due to its loose matrix. In case of multi-step technique, only surface of the formulation was cross-linked as preformed particles were placed in the chitosan solution (only outer surface was exposed to chitosan). In case of single-step technique, ionic interaction between zinc and pectin, and complex formation between chitosan and pectin occurred simultaneously during microparticle formation. Therefore, polyelectrolyte complex was formed on the surface as well as at the inner portion of the matrix in this case. As a result, stronger matrix was formed in case of single-step technique than multi-step technique, which prevented drug release in the upper GI conditions due to less swelling and erosion. Despite the presence of chitosan, all formulations were sensitive to pectinase enzyme. Pectinase enzyme (present in SCF) most likely attacked the pectin chains of the Zn–pectin–chitosan complex and degraded the cross-linked structure. In case of the optimized formulation, formulation matrix slowly degraded and consequently released drug in sustained manner.

The formulation for *in vivo* study was selected from the *in vitro* drug release study. The formulation prepared at cross-linking solution pH of 1.5, MMW chitosan concentration of 1%, cross-linking time of 120 min, P:D of 3:1, and single-step technique showed *in vitro* colon-specific release. Hence, this formulation was selected for the *in vivo* study. Also the formulation prepared at the same conditions and procedure without chitosan was used for *in vivo* study to find out the effect of chitosan in modifying *in vivo* drug release. Our previous study showed that resveratrol is quickly absorbed into blood through the GI tract upon oral administration of both solution and suspension formulations (Das et al., 2008). Other researchers also reported that GI permeability of resveratrol is very high (Kaldas et al., 2003; Walle et al., 2004). Thus, measurement of drug concentration in blood was performed to determine drug release in the GI tract following administration of the formulations. Other researchers also used pharmacokinetic study to predict *in vivo* colon-specific drug release (Munjeri et al., 1998; Musabayane et al., 2000; Krishnaiah et al., 2003; Bourgeois et al., 2008; Fan et al., 2008; Wu et al., 2008; Zhao et al., 2008; Kaur and Kim, 2009). Additionally, pharmacokinetic study is more ethical as less animals are required than the experiment where rats are required to be sacrificed at each time point to measure the released drug in different part of the GI tract (Jain et al., 2007). Although Zn-pectinate formulation showed delayed appearance of drug in plasma, it did not exhibit colon-specific release as most of the drug appeared in blood before 5 h of administration. It is well established that any particle needs about 5–6 h for its arrival to the colon (Liu et al., 2003). Hence, Zn-pectinate formulation cannot be considered as colon-specific formulation. On the other hand, only small amount of drug was detected in plasma before 5 h of administration of Zn–pectin–chitosan formulation followed by steady increase in plasma drug concentration up to 9 h. This sig-

nifies that the modified formulation showed *in vivo* colon-specific drug release. Hence, Zn–pectin–chitosan composite particles can be considered as colon-specific formulation.

5. Conclusion

A multi-particulate colon-specific drug delivery system was developed. The biopolymers (pectin and chitosan) were used to prepare the formulation, which were nontoxic, biocompatible, and biodegradable. The results emphasized on the importance of formulation procedure and optimization of the formulation parameters. Cross-linking solution pH, cross-linking time, and chitosan concentration in the cross-linking solution exhibited major influence on drug release pattern. This study also revealed that certain amount of drug could be incorporated without hampering the colon-specific drug release behavior. EE of the formulations was very high and the drug was stable within the formulation during their storage. Single-step procedure was found to be better than multi-step procedure for the production of colon-specific formulation. In addition, single-step procedure is cost-effective as it cuts down formulation steps, production time, and manpower requirement. Hence, this technique is economical for industrial production and easy to scale up. Most importantly, *in vivo* pharmacokinetic study in rats suggested *in vivo* colon-specific drug release from the optimized zinc–pectin–chitosan composite particle formulation. In future, this formulation may be tested *in vivo* colitis or colorectal cancer disease model.

Acknowledgments

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References

Ahishali, E., Uygur-Bayramicli, O., Dolapcioglu, C., Dabak, R., Mengi, A., Isik, A., Ermis, E., 2009. Chemical colitis due to glutaraldehyde: case series and review of the literature. Digest. Dis. Sci. 54, 2541–2545.

Atyabi, F., Majzoob, S., Iman, M., Salehi, M., Dorkoosh, F., 2005. In vitro evaluation and modification of pectinate gel beads containing trimethyl chitosan, as a multi-particulate system for delivery of water-soluble macromolecules to colon. Carbohydr. Polym. 61, 39–51.

Basit, A.W., 2005. Advances in colonic drug delivery. Drugs 65, 1991–2007.

Baur, J.A., Sinclair, D.A., 2006. Therapeutic potential of resveratrol: the in vivo evidence. Nat. Rev. Drug Discov. 5, 493–506.

Bigucci, F., Luppi, B., Cerchiara, T., Sorrenti, M., Bettinetti, G., Rodriguez, L., Zecchi, V., 2008. Chitosan/pectin polyelectrolyte complexes: selection of suitable preparative conditions for colon-specific delivery of vancomycin. Eur. J. Pharm. Sci. 35, 435–441.

Bigucci, F., Luppi, B., Monaco, L., Cerchiara, T., Zecchi, V., 2009. Pectin-based microspheres for colon-specific delivery of vancomycin. J. Pharm. Pharmacol. 61, 41–46.

Bourgeois, S., Tsapis, N., Honnas, H., Andremont, A., Shakweh, M., Besnard, M., Fattal, E., 2008. Colonic delivery of beta-lactamases does not affect amoxicillin pharmacokinetics in rats. J. Pharm. Sci. 97, 1853–1863.

Chambin, O., Dupuis, G., Champion, D., Voilley, A., Pourcelot, Y., 2006. Colon-specific drug delivery: influence of solution reticulation properties upon pectin beads performance. Int. J. Pharm. 321, 86–93.

Chang, K.L.B., Lin, J., 2000. Swelling behavior and the release of protein from chitosan–pectin composite particles. Carbohydr. Polym. 43, 163–169.

Chourasia, M.K., Jain, S.K., 2003. Pharmaceutical approaches to colon targeted drug delivery systems. J. Pharm. Pharm. Sci. 6, 33–66.

Das, S., Lin, H.S., Ho, P.C., Ng, K.Y., 2008. The impact of aqueous solubility and dose on the pharmacokinetic profiles of resveratrol. Pharm. Res. 25, 2593–2600.

Das, S., Ng, K.Y., 2010a. Colon-specific delivery of resveratrol: optimization of multi-particulate calcium-pectinate carrier. Int. J. Pharm. 385, 20–28.

Das, S., Ng, K.Y., 2010b. Impact of glutaraldehyde on *in vivo* colon-specific release of resveratrol from biodegradable pectin-based formulation. J. Pharm. Sci. 99, 4903–4916.

Das, S., Ng, K.Y., 2010c. Resveratrol-loaded calcium-pectinate beads: effects of formulation parameters on drug release and bead characteristics. J. Pharm. Sci. 99, 840–860.

Das, S., Ng, K.Y., Ho, P.C., 2010a. Design of a pectin-based microparticle formulation using zinc ions as the cross-linking agent and glutaraldehyde as the hardening agent for colonic-specific delivery of resveratrol: in vitro and in vivo evaluations. J. Drug Target., doi:10.3109/1061186X.2010.504272.

Das, S., Ng, K.Y., Ho, P.C., 2010b. Formulation and optimization of zinc-pectinate beads for the controlled delivery of resveratrol. AAPS PharmSciTech 11, 729–742.

Dupuis, G., Chambin, O., Genelot, C., Champion, D., Pourcelot, Y., 2006. Colonic drug delivery: influence of cross-linking agent on pectin beads properties and role of the shell capsule type. Drug Dev. Ind. Pharm. 32, 847–855.

El-Gibaly, I., 2002. Oral delayed-release system based on Zn-pectinate gel (ZPG) microparticles as an alternative carrier to calcium pectinate beads for colonic drug delivery. Int. J. Pharm. 232, 199–211.

Fan, L.F., He, W., Bai, M., Du, Q., Xiang, B., Chang, Y.Z., Cao, D.Y., 2008. Biphasic drug release: permeability and swelling of pectin/ethylcellulose films, and in vitro and in vivo correlation of film-coated pellets in dogs. Chem. Pharm. Bull. 56, 1118–1125.

Friend, D.R., 2005. New oral delivery systems for treatment of inflammatory bowel disease. Adv. Drug Deliv. Rev. 57, 247–265.

George, M., Abraham, T.E., 2006. Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan – a review. J. Control. Release 114, 1–14.

Gescher, A.J., Steward, W.P., 2003. Relationship between mechanisms, bioavailibility, and preclinical chemopreventive efficacy of resveratrol: a conundrum. Cancer Epidemiol. Biomarkers Prev. 12, 953–957.

Hiorth, M., Skoien, T., Sande, S.A., 2010. Immersion coating of pellet cores consisting of chitosan and calcium intended for colon drug delivery. Eur. J. Pharm. Biopharm. 75, 245–253.

Hiorth, M., Versland, T., Heikkila, J., Tho, I., Sande, S.A., 2006. Immersion coating of pellets with calcium pectinate and chitosan. Int. J. Pharm. 308, 25–32.

Ilium, L., 1998. Chitosan and its use as a pharmaceutical excipient. Pharm. Res. 15, 1326–1331.

Jain, A., Gupta, Y., Jain, S.K., 2007. Potential of calcium pectinate beads for target specific drug release to colon. J. Drug Target. 15, 285–294.

Kaldas, M.I., Walle, U.K., Walle, T., 2003. Resveratrol transport and metabolism by human intestinal Caco-2 cells. J. Pharm. Pharmacol. 55, 307–312.

Kaur, K., Kim, K., 2009. Studies of chitosan/organic acid/Eudragit RS/RL-coated system for colonic delivery. Int. J. Pharm. 366, 140–148.

Khandare, J., Mohr, A., Calderon, M., Welker, P., Licha, K., Haag, R., 2010. Structure–biocompatibility relationship of dendritic polyglycerol derivatives. Biomaterials 31, 4268–4277.

Kim, T.H., Park, Y.H., Kim, K.J., Cho, C.S., 2003. Release of albumin from chitosan-coated pectin beads in vitro. Int. J. Pharm. 250, 371–383.

Krishnaiah, Y.S., Satyanarayana, V., Dinesh Kumar, B., Karthikeyan, R.S., Bhaskar, P., 2003. In vivo pharmacokinetics in human volunteers: oral administered guar gum-based colon-targeted 5-fluorouracil tablets. Eur. J. Pharm. Sci. 19, 355–362.

Liu, L., Fishman, M.L., Kost, J., Hicks, K.B., 2003. Pectin-based systems for colon-specific drug delivery via oral route. Biomaterials 24, 3333–3343.

Maestrelli, F., Cirri, M., Corti, G., Mennini, N., Mura, P., 2008a. Development of enteric-coated calcium pectinate microspheres intended for colonic drug delivery. Eur. J. Pharm. Biopharm. 69, 508–518.

Maestrelli, F., Zerrouk, N., Cirri, M., Mennini, N., Mura, P., 2008b. Microspheres for colonic delivery of ketoprofen–hydroxypropyl-beta-cyclodextrin complex. Eur. J. Pharm. Sci. 34, 1–11.

Maier-Salamon, A., Hagenauer, B., Wirth, M., Gabor, F., Szekeres, T., Jager, W., 2006. Increased transport of resveratrol across monolayers of the human intestinal Caco-2 cells is mediated by inhibition and saturation of metabolites. Pharm. Res. 23, 2107–2115.

Marier, J.F., Vachon, P., Gritsas, A., Zhang, J., Moreau, J.P., Ducharme, M.P., 2002. Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. J. Pharmacol. Exp. Ther. 302, 369–373.

Martin, A.R., Villegas, I., La Casa, C., de la Lastra, C.A., 2004. Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates apoptosis during early colonic inflammation in rats. Biochem. Pharmacol. 67, 1399–1410.

Martin, A.R., Villegas, I., Sanchez-Hidalgo, M., de la Lastra, C.A., 2006. The effects of resveratrol, a phytoalexin derived from red wines, on chronic inflammation induced in an experimentally induced colitis model. Br. J. Pharmacol. 147, 873–885.

Marudova, M., Lang, S., Brownsey, G.J., Ring, S.G., 2005. Pectin–chitosan multilayer formation. Carbohydr. Res. 340, 2144–2149.

Marudova, M., MacDougall, A.J., Ring, S.G., 2004. Pectin–chitosan interactions and gel formation. Carbohydr. Res. 339, 1933–1939.

Mennini, N., Furlanetto, S., Maestrelli, F., Pinzauti, S., Mura, P., 2008. Response surface methodology in the optimization of chitosan-Ca pectinate bead formulations. Eur. J. Pharm. Sci. 35, 318–325.

Munjeri, O., Collett, J.H., Fell, J.T., 1997. Hydrogel beads based on amidated pectins for colon-specific drug delivery: the role of chitosan in modifying drug release. J. Control. Release 46, 273–278.

Munjeri, O., Hodza, P., Osim, E.E., Musabayane, C.T., 1998. An investigation into the suitability of amidated pectin hydrogel beads as a delivery matrix for chloroquine. J. Pharm. Sci. 87, 905–908.

Musabayane, C.T., Munjeri, O., Bwititi, P., Osim, E.E., 2000. Orally administered, insulin-loaded amidated pectin hydrogel beads sustain plasma concentrations of insulin in streptozotocin-diabetic rats. J. Endocrinol. 164, 1–6.

Nurjaya, S., Wong, T.W., 2005. Effects of microwave on drug release properties of matrices of pectin. Carbohydr. Polym. 62, 245–257.
Rodriguez, M., Vila-Jato, J.L., Torres, D., 1998. Design of a new multiparticulate system for potential site-specific and controlled drug delivery to the colonic region. J. Control. Release 55, 67–77.
Schneider, Y., Duranton, B., Gosse, F., Schleiffer, R., Seiler, N., Raul, F., 2001. Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis. Nutr. Cancer 39, 102–107.
Sriamornsak, P., 1999. Effect of calcium concentration, hardening agent and drying condition on release characteristics of oral proteins from calcium pectinate gel beads. Eur. J. Pharm. Sci. 8, 221–227.
Tessitore, L., Davit, A., Sarotto, I., Caderni, G., 2000. Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21(CIP) expression. Carcinogenesis 21, 1619–1622.
Walle, T., Hsieh, F., DeLegge, M.H., Oatis Jr., J.E., Walle, U.K., 2004. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab. Dispos. 32, 1377–1382.
Wong, T.W., Nurjaya, S., 2008. Drug release property of chitosan-pectinate beads and its changes under the influence of microwave. Eur. J. Pharm. Biopharm. 69, 176–188.
Wu, B., Deng, D., Lu, Y., Wu, W., 2008. Biphasic release of indomethacin from HPMC/pectin/calcium matrix tablet. II. Influencing variables, stability and pharmacokinetics in dogs. Eur. J. Pharm. Biopharm. 69, 294–302.
Yang, L., Chu, J.S., Fix, J.A., 2002. Colon-specific drug delivery: new approaches and in vitro/in vivo evaluation. Int. J. Pharm. 235, 1–15.
Zhao, X.L., Li, K.X., Zhao, X.F., Pang, D.H., Chen, D.W., 2008. Study on colon-specific 5-Fu pH-enzyme di-dependent chitosan microspheres. Chem. Pharm. Bull. 56, 963–968.