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ORIGINAL ARTICLE

Pectin–metronidazole prodrug bearing microspheres for colon targeting

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Abstract The present study explored the potential of pectin–metronidazole (PT–ME) prodrug bearing microspheres for colon delivery. PT–ME prodrug was synthesized with different degree of substitution. The success of the synthesis was confirmed by spectroscopy. PT–ME microspheres and pectin microspheres bearing plain metronidazole were prepared using emulsion-dehydration technique. Microspheres were evaluated for shape and surface morphology, size distribution, entrapment efficiency and *in vitro* drug release in simulated gastrointestinal fluids (SGF). Microspheres prepared from PT–ME prodrug were not only exhibiting increased the drug entrapment efficiency, but the drug release at the upper part of GIT was also reduced as compared to pectin microspheres having physically entrapped drug. *In vitro* drug release studies were showing no drug release at acidic pH from microspheres prepared by drug polymer prodrug while pectin microspheres having physically entrapped drug showed almost or complete drug release. *In vivo* studies were also performed by assessing the drug concentration in various parts of the GIT at different time intervals which exhibited the potentiality of microspheres prepared from PT–ME prodrug as compared to pectin microspheres having physically entrapped drug. Hence, it can be concluded that microspheres prepared from PT–ME prodrug deliver the drug more efficiently to colon.

1. Introduction

Site-specific drug delivery through site-specific prodrug activation may be accomplished using specific property of the target site, such as altered pH or high activity of certain enzymes. Various strategies are available for targeting drug release selectively to the colon (Chourasia and Jain, 2003). The colon specific drug delivery systems which are based on the use of polysaccharides offer superiority over other systems. Polysaccharides retain their integrity and prevent the release of drug during its passage through the GIT. But when it comes in contact with colonic fluid, it is confronted by the action of microorganisms and consequently entrapped drug is liberated (Rubinstein et al., 1993).

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A large number of enzyme and pH-dependant polysaccharides such as amylose, guar gum, pectin, chitosan, inulin, cyclodextrin, chondroitin sulphate, dextran and locust bean gum, have been investigated for colon specific drug release (Chourasia and Jain, 2004). Prodrug approach involves the formation of a covalent linkage between drug and polysaccharide carriers in such a manner that upon oral administration the moiety maintains its integrity in the hostile environment of stomach and small intestine and is converted into parent drug molecule once it reaches the colon. Among many different delivery systems, microspheres provide a good controlled release of drug entrapped in polymer matrix as polymer networks can provide a diffusion barrier to retard the otherwise rapid drug release (Lorenzo et al., 1998; Wakerly et al., 1996).

Pectins are non-starch, linear polysaccharides of mainly α-(1–4)-linked D-galactouronic acid residues interrupted by 1,2-linked L-rhamnase residues (Vandamme et al., 2002). Pectin was first used by Ashford et al. (1993), for colonic drug delivery. It is resistant to digestion by mammalian digestive enzymes but is broken down in the colon by bacteria. To protect the pectin from dissolution in the upper gastrointestinal tract (GIT), many approaches have been used to create an effective pectin based drug delivery system. Wong et al. (2002) explored the potential of pectin for use in making microspheres for sustained-release of drugs, while Miao et al. (2005) utilized prodrug approach of pectin for colon delivery.

Pectin is water soluble, might not protect loaded drug to release into the upper part of GIT. A successful colon specific oral drug delivery system needs to be developed on the basis of increased resistance of the drug carrier against gastrointestinal enzymes and pH gradients (i.e. from 1 to 3 in the stomach to 6–7 in intestine). However, a better shield effect could be achieved by obtaining multiparticulate system of pectin. In particular, multiparticulate systems based on such specifically biodegradable polymers quickly spread out on their arrival to the colon, and produced rapid drug release due to sharp increase of surface area exposed to bacterial breakdown (Rodriguez et al., 1998). Moreover, a suitable conjugation of drug to pectin could prevent drug release during the transit through stomach and small intestine (Van et al., 1994; Dupuis et al., 2006).

Recently, we have synthesized microspheres of pectin having physically entrapped drug (metronidazole) for colon targeting but they are not able to maintain their integrity in the upper part of GIT and show maximum drug release in the upper part of GIT, thus coating of microspheres is required which maintains their integrity in upper GI tract and releases adequate amount of drug in the lower intestine, i.e. colon (Vaidya et al., 2009).

In continuation of our study to explore more potent drug target microspheres for colon targeting which were prepared from PT–ME prodrug that maintains their integrity in the hostile environment of stomach and small intestine and afterwards releases the drug in the colon. These microspheres are not required to be coated or this technique (first synthesizing the prodrug and then preparing microspheres of the same) is an alternative of coating of microspheres for colon targeting. Hence using this technique, more intact microspheres could be delivered to the colon.

The proposed multiparticulate system, i.e. microspheres which combine a prodrug approach as well as the biodegradability of pectin polymer for colon delivery of metronidazole (ME) for the treatment of amoebiasis. Pectin–drug conjugate protects the drug from its release in acidic environment of GIT and releases drug into the colon due to the cleavage of glycoside linkage as well as degradation of pectin in the colon due to the action of pectinase and glycosidase enzymes released by the microorganism specifically present in the colon.

2. Experimental

2.1. Materials

Pectin was purchased from Otto Kemi (Mumbai, India). Metronidazole was generously supplied by M/s Broshell Remedies (Sagar, M.P., India) as a gift sample. Span-80 was procured from Sigma Chemicals (St. Louis, MO, USA). Isooctane, *N*,*N*-dicyclohexyl carbodiimide (DCC) and dimethylsulphoxide (DMSO) were purchased from Central Drug House (Mumbai, India). N-Hydroxysuccinimide (NHS) was purchased from Spectrochem (Mumbai). All other chemicals used were of analytical reagent grade and were used as received.

2.2. Preparation of pectin–metronidazole (PT–ME) prodrug

Pectin–metronidazole (PT–ME) prodrug was synthesized using the method reported by Miao et al. (2005). Briefly, PT–ME prodrug was synthesized by the reaction of pectin with metronidazole in the presence of *N*,*N*-dicyclohexyl carbodiimide (DCC). PT (1.098 g), ME (0.513 g), DCC (0.618 g) and N-Hydroxysuccinimide (NHS, 0.345 g) were added to anhydrous dimethylsulphoxide (DMSO, 100 mL), the mixture was stirred at room temperature for 72 h, anhydrous ethanol–ether solution (100 mL, 1:1, vol/vol) was added and the product was filtered. The precipitate obtained was dissolved in anhydrous DMSO which was further added into anhydrous ethanol–ether solution (100 mL, 1:1, vol/vol). This process was repeated two times. The precipitate was mounted in 10-cm dialysis bags. Each bag was immersed in 1.0 L of anhydrous ethanol, which was stirred for 48 h at 200 rpm. Then anhydrous ethanol in bags was removed under reduced pressure and the residue was dried at 60 °C for 24 h to yield 1.48 g of PT–ME conjugate.

2.3. Characterization of pectin –metronidazole (PT–ME) prodrug

2.3.1. IR spectrum of PT–ME prodrug

The success of the reaction was confirmed by the IR spectra of PT and PT–ME prodrug which show the characteristic peaks for PT at 3234.73 cm^{-1} (hydroxyl streaching), 943.22 cm^{-1} (hydroxyl out of plane bending), 1747.57 cm^{-1} (carbonyl streaching) while the IR spectrum of PT–ME prodrug shows characteristic peaks at 1238.34 cm^{-1} (ester carbonyl peak), 1747.57 cm^{-1} (carbonyl streaching), 1593.25 cm^{-1} (asymmetric nitro streach) (Figs. 1 and 2).

2.3.2. Determination of degree of substitution

All carboxyl groups in any polymer cannot be esterified; therefore, in the present research attempt has been made to determine the actual performed substitution.

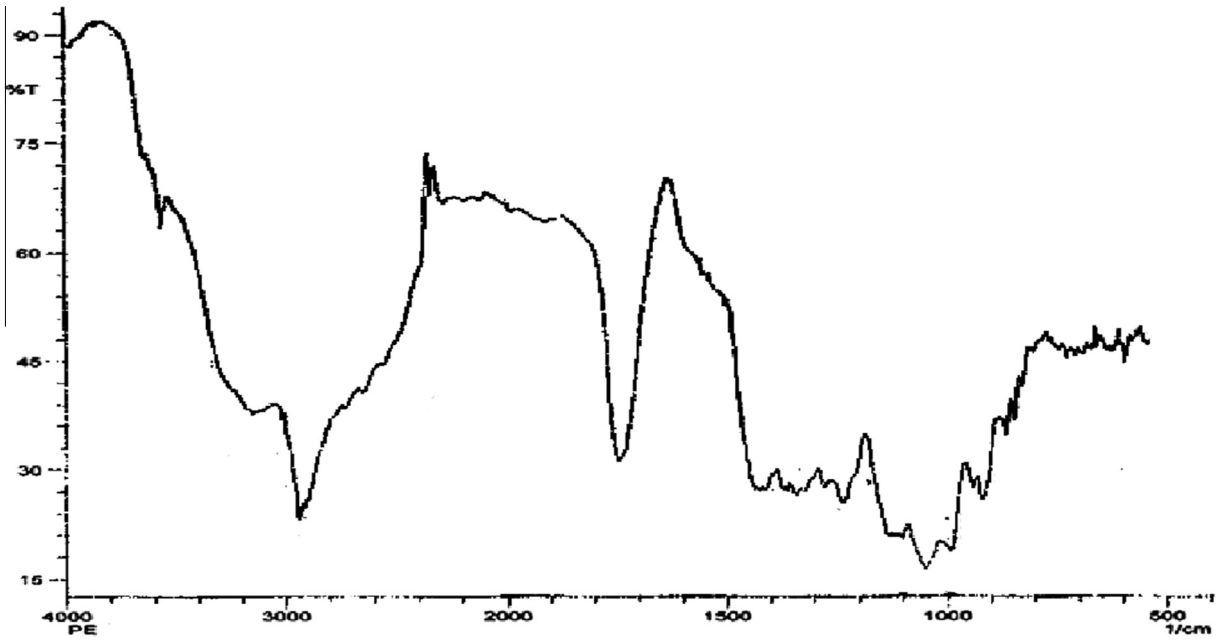

Figure 1 IR spectra of pectin (PT).

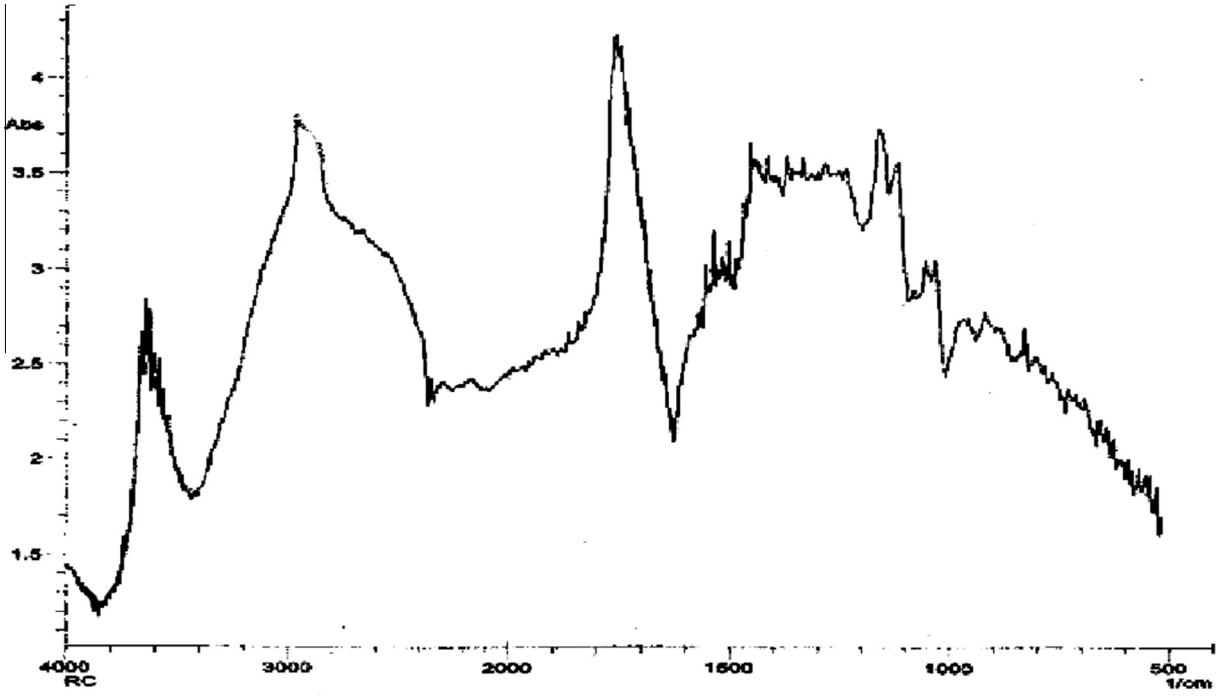

Figure 2 IR spectra of pectin–metronidazole (PT–ME) prodrug.

Different degree of substitution in various PT–ME prodrugs was determined using the method reported by Marcello et al. (2005). Briefly, the known quantity of different substituted PT–ME prodrug was digested in 5% pectinase solution (PBS pH 7.4, 50 mL) with continuous stirring at 1000 rpm for 48 h at 25 °C, the samples were withdrawn at different time intervals, diluted and absorbance was measured at 320.5 nm UV spectrophotometrically (1601, Shimadzu, Japan) (Table 1).

2.4. Preparation of microspheres

Microspheres of these polymeric prodrugs were prepared using the method reported by Esposito et al. (2001), because microspheres provide large surface area for its degradation in colon which may result fast release of drug. Briefly, drug–polymer prodrug solution in distilled water (10 mL) was dispersed in span 80 (1.0% wt/vol) containing isooctane (50 mL). The dis-

Table 1 Degree of substitution.

Formulation code	Aimed substitution (%)	Quantity of pectin	Quantity of metronidazole	Found substitution (%)
PMC-D1	5	1.098	0.0429	4.87 ± 0.55
PMC-D2	10	1.098	0.0858	9.64 ± 0.79
PMC-D3	15	1.098	0.1287	14.38 ± 1.02
PMC-D4	20	1.098	0.1716	15.92 ± 1.34

Values are average of three readings ± standard deviation.

persion was stirred continuously until stable water/oil emulsion was obtained. This solution was rapidly cooled to 10 °C and then acetone (50 mL) was added in order to dehydrate the pectin droplets. This dispersion was continuously stirred at 1000 rpm for 30 min at 30 °C for complete solvent evaporation which resulted in microspheres. These microspheres were freeze dried and kept in airtight containers for further studies. For comparison, microspheres of pectin with physical drug entrapment were also prepared using same methodology.

2.5. Characterization of microspheres

2.5.1. Shape and surface morphology

The shape and surface morphology of microspheres were studied using scanning electron microscopy. The sample was prepared by lightly sprinkling the microsphere powder on a double adhesive tape, which was stuck on an aluminium stub. The stubs were then coated with gold to the thickness of about 300 Å using a sputter coater, then viewed under scanning electron microscope (Leo435 VP, Cambridge, UK) and shown in photomicrographs 1 and 2 (Fig. 3).

2.5.2. Size determination

The size of the microspheres was assessed using Laser diffraction based particle size analyser (1064L, Cilas, Marcoussis, France).

2.5.3. Entrapment efficiency

The drug entrapped in various microspheres was determined using the method reported by Paharia et al. (2007). Briefly, the microspheres were digested in PBS (pH 7.4, 10 mL) containing pectinase solution (4% wt/wt) for 12 h. The digested homogenate was centrifuged at 3000 rpm for 5 min and the supernatant was assayed spectrophotometrically for metronidazole, at 320.5 nm (UV 1601, Shimadzu, Japan).

2.6. In vitro drug release studies

In vitro drug release from microspheres was assessed in USP dissolution test apparatus Type II (paddle type) in various simulated GIT fluids according to the method reported by Souder and Ellenbogen (1985).

The dissolution studies were carried out in 900 mL dissolution medium which was stirred at 100 rpm at 37 ± 0.1 °C.

The scheme for using the simulated gastrointestinal fluids was as follows:

First 2 h: Simulated gastric fluid of pH 1.2.
3rd hour: Mixture of simulated gastric and intestinal fluid of pH 4.5.
4th and 5th hours: Simulated intestinal fluid of pH 6.8.
6th hour: Simulated intestinal fluid of pH 7.5.
7th hour and onwards: Simulated colonic fluid of pH 7.0.

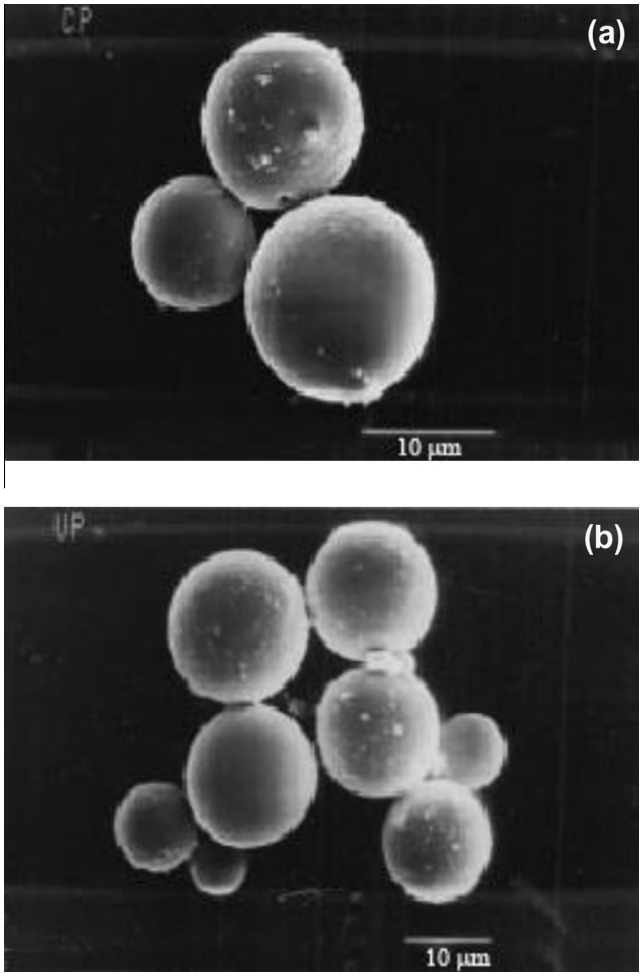

Figure 3 Scanning electron photomicrographs of (a) CP-microspheres prepared from PT-ME prodrug (PMC-D3) and (b) UP- microspheres having physically entrapped drug (PME).

Samples were withdrawn periodically and compensated with an equal volume of fresh dissolution medium. The samples were analysed for drug content by measuring absorbance at 320.5 nm using UV spectrophotometer (UV 1601, Shimadzu, Japan).

2.7. In vitro drug release in presence of rat caecal contents

The rat caecal content release medium was prepared using the method reported by Van den Mooter et al. (1994). Briefly, rats

weighing 150–200 g were maintained on normal diet and 1 mL of 2% dispersion of pectin in water was given orally for 7 days to induce enzyme. After 7 days, the animals were sacrificed and caecal contents were withdrawn from the isolated caecum.

Drug release rate studies for the initial 6 h were performed as described above. From the 7th hour and onwards it was carried out in simulated colonic fluid (pH 7.0) containing 4% rat caecal contents. The PBS (pH 7.0) containing 4% of caecal contents with placebo microspheres served as blank. Samples (2.0 mL) were withdrawn at scheduled time intervals and immediately replaced with equal volume (2.0 mL) of fresh media. The volume of the sample was made up to 10 mL and centrifuged. The supernatant was filtered through Whatman filter paper, and drug content was determined spectrophotometrically at 320.5 nm (UV 1601, Shimadzu, Japan).

2.8. GIT distribution study

Albino rats weighing (100–150 g) were selected for GIT distribution studies. These animals were kept in well spaced ventilated cages and maintained on healthy and fixed diet. The animals were divided equally into four groups of four animals each. The first group served as the control. The second group received plain drug (dose calculated as per the body weight of the animals i.e. 7 mg/kg). Animals of the third group were given microspheres prepared from PT–ME prodrug while fourth group received microspheres prepared with different degree of pectin:drug ratio using physical drug entrapment method, respectively, containing metronidazole equivalent to prescribed dose, i.e. 7 mg/kg. The doses were given orally with the help of cannula. After 2, 4, 6 and 8 h, the animals were sacrificed and stomach, small intestine and colon were isolated. These isolated organs of GIT were homogenized along with a small amount of PBS (pH 7.5) and then centrifuged at 10,000 rpm for 5 min and supernatant was separated. In the separated supernatant, 1 mL of acetonitrile was added and kept for 30 min and filtered. The filtrates were assayed for drug content by measuring the absorbance at 320.5 nm (UV 1601, Shimadzu, Japan) against respective blank solution. The drug content in different parts of GIT was measured at different time intervals. All animal study protocols were duly approved by the Institutional Animal Ethical Committee vide letter no. Animal Eths. Comm/07/274 of Dr. H.S. Gour University, Sagar, M.P., India.

3. Results and discussion

3.1. Preparation of pectin–metronidazole (PT–ME) prodrug

Pectin–metronidazole (PT–ME) prodrug was synthesized successfully using conjugation technique. The IR spectrum indicated that the carbonyl group of PT is covalently bonded to one of the hydroxyl groups of ME through an ester bond (1238.34 cm^{-1} of ester carbonyl peak in IR spectra). Degree of substitution in drug–polymer prodrug was calculated by digestion in pectinase solution (5% wt/wt) (Table 1), which revealed increased degree of substitution up to 15% drug:polymer ratio, which was not changed on further increasing the drug:polymer ratio possibly due to the saturation of pectin matrix.

3.2. Preparation of microspheres

Pectin microspheres were prepared using the emulsion-dehydration technique. Various formulation variables, viz degree of substitution, emulsifier concentration and process variables, viz. stirring speed and stirring time, which could affect the preparation and properties of microspheres, were identified and optimized by varying one variable and keeping other constant (Table 2).

3.3. Particle size of microspheres

The size of the microspheres was assessed using laser diffraction based particle size analyser. The effective particle size of microspheres increased on increasing the degree of substitution. Mean particle size of microspheres prepared from PT–ME prodrug increased from 14.94 ± 0.73 to 15.42 ± 0.83 μm as the degree of substitution increased from 5% to 20%. The increase in the size of microsphere could be explained on the basis of increased drug concentration and percent drug entrapment efficiency. The effective particle size of microspheres prepared from 15% substituted PT–ME prodrug was found to be 15.36 ± 0.92 μm while the particle size of microspheres prepared with 85:15 pectin drug ratio using physical drug entrapment method was 14.02 ± 1.03 μm. The increase in the particle size of microspheres prepared from PT–ME prodrug could be due to higher drug entrapment as compared to microspheres prepared from physical entrapped drug. Shape

Table 2 Average particle size, entrapment efficiency and *in vitro* drug release from microspheres prepared from drug:polymer prodrug and physically entrapped drug.

Formulation code	Degree of substitution	Conc. of emulsifier span-80 (wt/vol)	Stirring speed (rpm)	Stirring time (min)	Particle size (μm)	Entrapment efficiency (%)	*In vitro* drug release after 8 h (%)
Microspheres prepared from PT–ME prodrug							
PMC-D1	5%	1.5	1000	30	14.94 ± 0.73	92.43 ± 1.89	18.62 ± 0.23
PMC-D2	10%	1.5	1000	30	15.16 ± 0.37	93.40 ± 2.13	19.06 ± 0.57
PMC-D3	15%	1.5	1000	30	15.36 ± 0.92	94.52 ± 2.25	20.68 ± 0.91
PMC-D4	20%	1.5	1000	30	15.42 ± 0.83	80.61 ± 2.42	22.73 ± 1.42
Microspheres having physically entrapped drug							
PME[a]	15:85[b]	1.5	1000	30	14.02 ± 1.03	70.28 ± 2.59	93.47 ± 3.32

Values are average of three readings ± standard deviation.
[a] Optimized formulation.
[b] Drug:polymer ratio (unconjugated).

and surface of the microspheres were studied by Scanning Electron Microscopy which showed smooth surface and spherical shape of all the formulations.

3.4. Entrapment efficiency

Entrapment efficiency was calculated by taking the ratio of weight of metronidazole in the final formulation and the initial amount of metronidazole used. Significant quantity of metronidazole is lost during encapsulation. Drug entrapment efficiency was increased from 92.43 ± 1.89% to 94.52 ± 2.42% as the degree of substitution increases from 5% to 15%; but after 15% degree of substitution it decreases (80.61 ± 2.42 for microspheres prepared with 80:20 pectin drug ratio) which may be due to saturation of pectin matrix. The microspheres prepared from PT–ME prodrug show 94.52 ± 2.25% entrapment efficiency for all formulations with 15% degree of substitution, while those microspheres prepared with 85:15 pectin drug ratio using physical drug entrapment method shows 70.28 ± 2.59% entrapment efficiency. Higher entrapment efficiency in microspheres prepared from PT–ME prodrug is due to chemical bonding or conjugation of drug to polymer, which will not allow the drug to diffuse from polymer matrix during encapsulation.

3.5. Drug release

Microspheres prepared from PT–ME prodrug were assumed to remain intact in upper GIT, i.e. the physiological environment of stomach and small intestine, but once they reached in the colon, they were acted upon by the bacterial pectinase enzyme, which resulted in the degradation of the matrices and subsequently released the drug from the microspheres. These multiparticulate systems have biodegradability property in the colon.

The *in vitro* drug release studies showed that no measurable drug release was observed for microspheres prepared by PT–ME prodrug (with 15% degree of substitution) in simulated stomach fluid (pH 1.2 for 2 h). Only 1–5% drug release was observed in a mixture of simulated gastric and intestinal fluid (pH 4.5, 6.8 and 7.5 for 6 h) and it showed approximately 10–20% of drug release in simulated colonic fluid (pH 7.0) in subsequent 2 h (Fig. 4). For comparison microspheres prepared with 85:15 pectin:drug ratio using physical drug entrapment method were also assayed. Drug release from microspheres having physically entrapped drug was high in the upper part of GIT (i.e. SGF and SGIF of pH 4.5, 6.8 and 7.5), i.e. 93.47 ± 3.32% within 8 h (Fig. 4), while no measurable drug release was observed for microspheres prepared by PT–ME prodrug in simulated stomach fluid (pH 1.2 for 2 h). The greater drug release from microspheres having physically entrapped drug could be due to physical entrapment of drug in matrix, which leached out from polymer matrix on its swelling in gastrointestinal fluids; while microspheres prepared from PT–ME prodrug and the drug was chemically bonded to the polymer which prevented its leaching in the upper part of GIT.

In vitro drug release from microspheres prepared by PT–ME prodrug showed 18.62 ± 0.23% to 22.73 ± 1.42% within 8 h as the degree of substitution was increased from 5% to 20%, possibly due to the increasing entrapment efficiency.

Further, the biodegradability of pectin by colonic microflora was assayed by performing the *in vitro* drug release studies in PBS (pH 7.0) containing 4% rat caecal content. The drug release was found to be improved in the presence of rat caecal content in comparison to those that were carried out without using rat caecal content. In colonic fluid 40–50% drug release was observed within 6–8 h from microspheres prepared by PT–ME prodrug (Fig. 5). The higher amount of drug release in the presence of rat caecal contents in dissolution media clearly enlightens the degradative effect of polymer matrix by rat caecal content. Polymer in the presence of dissolution media containing rat caecal content swells and forms gel layer, which allows the entry of dissolution media inside the formulation leading to cleavage of drug–polymer bond and allow the drug to diffuse from polymer matrix, showing higher drug release.

The *in vivo* studies indicate that maximum concentration of metronidazole (57.9%) was observed in stomach 2 h after oral administration of plain metronidazole and in subsequent hours, very less amount of drug reached the small intestine and no drug was found in the colon. Only 26.4% of total drug load of conventional dosage form reached the colon after 8 h (Fig. 6). While the microspheres having physically entrapped drug showed maximum amount of drug (41.6%) in the small intestine after 6 h of orally administered microspheres due to leaching of drug from pectin microspheres on its swelling in gastrointestinal fluids and 37.5% into the colon after 8 h of its administration which is higher than the conventional dos-

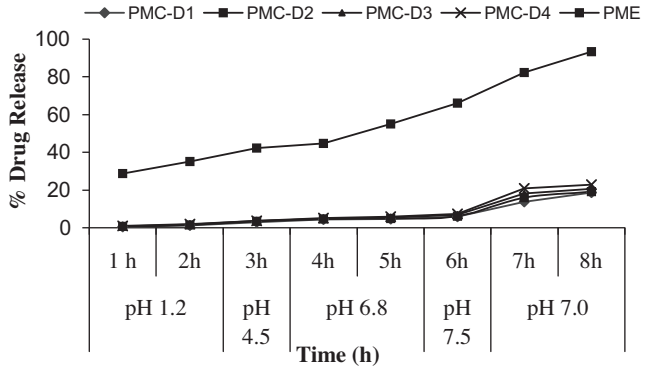

Figure 4 *In vitro* percent drug release from microspheres prepared from PT–ME prodrug and microspheres having physically entrapped drug.

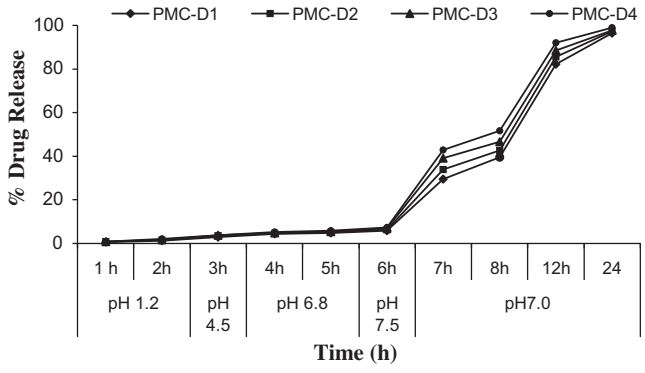

Figure 5 *In vitro* percent drug release from microspheres prepared from PT–ME prodrug in rat caecal content.

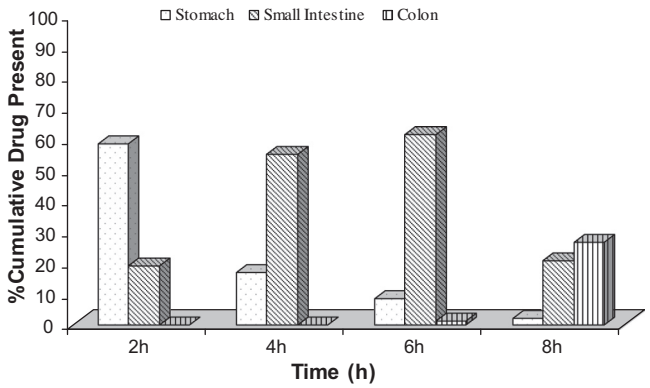

Figure 6 Biodistribution of drug in various organs after oral administration of plain drug.

age (Fig. 7). The microspheres prepared from PT–ME prodrug were observed relatively intact in the upper part of GIT (Fig. 8). Approximately 1–5% of the total drug load was released during its transit through upper GIT (1–5 h) and showed the maximum amount of drug in the colon (67.9%) after 8 h. The maximum drug which is observed in the colon from microspheres prepared from PT–ME prodrug could be due to chemical binding of drug to polymer matrix, which was degraded by enzymes released from colonic microflora and subsequently released the drug. The drug content in different parts of GIT at different time intervals was calculated and shown graphically in Figs. 6–8.

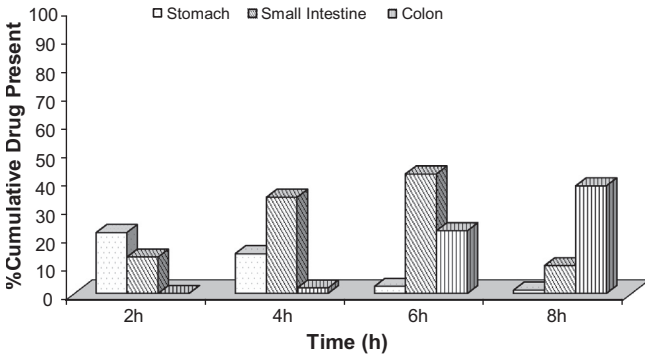

Figure 7 Biodistribution of drug in various organs after oral administration of microspheres having physically entrapped drug.

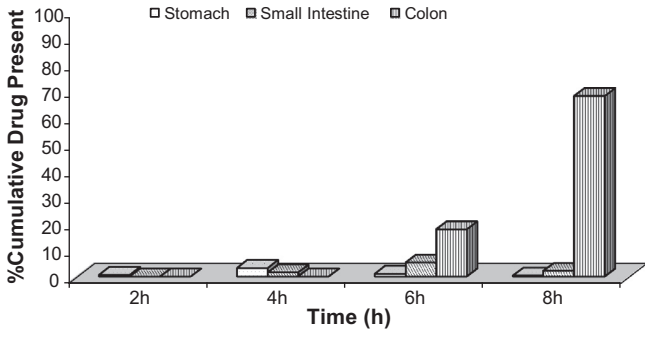

Figure 8 Biodistribution of drug in various organs after oral administration of microspheres prepared from PT–ME prodrug (PMC-D3) in rat caecal content.

4. Conclusion

In conclusion, amoebiasis can be treated effectively by delivering appropriate amount of drug directly to the colon by microspheres prepared by PT–ME prodrug. The microspheres of pectin were prepared by emulsification method, which showed optimum performance in terms of size and entrapment efficiency. Microspheres which were prepared from PT–ME prodrug maintained its integrity in the hostile environment of stomach and small intestine and afterwards released the drug into the colon. A sufficient quantity of drug was delivered into colon from microspheres prepared from PT–ME prodrug as compared to the microspheres having physically entrapped drug. Thus, it can be concluded that conjugation of drug to polymer is an effective carrier system for the delivery of the drug to the colon.

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Authors report no declaration of interest.

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