International Journal of Pharmaceutics 422 (2012) 151–159

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Coated chitosan pellets containing rutin intended for the treatment of inflammatory bowel disease: In vitro characteristics and in vivo evaluation

Miloslava Rabišková[a,*], Tereza Bautzová[a,b], Jan Gajdziok[a], Kateřina Dvořáčková[a], Alf Lamprecht[b], Yann Pellequer[b], Jiřina Spilková[c]

[a] Department of Pharmaceutics, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Palackého 1-3, 612 42 Brno, Czech Republic
[b] Laboratory of Pharmaceutical Engineering, Faculty of Medicine and Pharmacy, University of Franche-Comté, Besançon, France
[c] Department of Pharmacognosy, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic

ARTICLE INFO

Article history:
Received 28 July 2011
Received in revised form 21 October 2011
Accepted 22 October 2011
Available online 3 November 2011

Keywords:
Colonic delivery
Coated pellets
Rutin
Chitosan
In vitro characteristics
In vivo evaluation

ABSTRACT

Preparation of coated pellets intended for rutin colon delivery, their evaluation in vitro and in vivo in experimental colitis in rats was the purpose of this study. Pellets were obtained using extrusion/spheronization and coated with three types of coatings (caffeic acid/hypromellose/alginic acid; sodium alginate/hypromellose/zinc acetate; sodium alginate/chitosan). Dissolution using buffers of pH values, β-glucosidase and times corresponding to gastrointestinal tract (GIT) was provided. Pellets coated with alginate/chitosan showed low rutin dissolution (12–14%) in upper GIT conditions and fast release (87–89%) under colon conditions; that is a good presumption of intended rutin release. After colitis induction and development, the rats were treated with pellets and rutin solution administered orally, solution also rectally. Colon/body weight ratio, myeloperoxidase activity and histological evaluation were performed. Rutin was able to promote colonic healing at the dose of 10 mg/kg: colon/body weight ratio decreased and myeloperoxidase activity was significantly suppressed. Pellets coated with alginate/chitosan applied orally and rutin solution administered rectally showed the best efficacy. The combination of rutin as natural product, mucoadhesive chitosan degraded in the colon and sodium alginate as the main coating substance in the form of pellets create a promising preparation for therapy of this severe illness.

1. Introduction

Inflammatory bowel disease (IBD) comprises two idiopathic inflammatory diseases of intestinal tract: ulcerative colitis and Crohn's disease. IBD is a refractory, chronic, recurrent and non-specific inflammatory disorder characterized by the development of intestinal inflammation resulting from the transmural infiltration of neutrophils, macrophages, lymphocytes and mast cells, ultimately giving rise to mucosal disruption and ulceration (Baumgart and Sandborn, 2007). IBD is a serious illness, its exact etiology is poorly understood, and individual genetic background, heredity, environmental signals such as stress, and immunologic influences, may all contribute to this disease process (Fiocchi, 2002). The therapy is symptomatic and targets general inflammatory mechanisms (Bresci et al., 2002). Patients often receive intense (i.e., the dosage schedule with multiple tablets several times a day) and long-term therapy that frequently requires lifelong treatment and is followed by several side effects associated with high dose intake (Farup et al., 2001). Drugs currently used in the treatment of IBD include 5-aminosalicylates as a standard therapy, corticosteroids in more severe inflammation, immunosuppressives and biological agents for patients who fail conventional therapy (Sands, 2000). It is well recognized that free radicals, including reactive oxygen metabolites and nitric oxide are produced in excess by the inflamed mucosa in IBD and may be responsible for a great deal of direct injury to the mucosa (Oldenburg et al., 2001). Thus the need of active substances with anti-oxidant activity delivered directly to the inflamed area seems to be an important issue in the treatment of IBD.

As flavonoids such as quercetin, quercitrin or rutin exhibit anti-oxidant and anti-inflammatory effects, they are the subjects of interest as potential drugs for the treatment of several diseases including IBD (Rabiskova et al., 2009). Contrary to quercetin, rutin absorption is limited in upper gastrointestinal (GI) tract due to its highly hydrophilic sugar part. Its use is more advantageous as it reaches the large intestine without significant chemical and biochemical loss. There, rutin is rapidly deglycosylated by colon microbiota to liberate its aglycone quercetin, which is absorbed easily into epithelial cells due to its lipophilicity. Thereafter, it enters the circulation and is subjected to *O*-methylation,

* Corresponding author. Tel.: +42 5 41562860; fax: +42 5 49240589.
E-mail address: rabiskovam@vfu.cz (M. Rabišková).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.10.045

glucuronidation, and/or sulfation in the liver (Murota and Terao, 2003). Water soluble conjugates can be excreted into the urine and the bile (Nemeth and Piskula, 2007). Flavonoid glucuronide and/or sulfate metabolites secreted with bile or in any other way into the small intestine could be hydrolyzed and the liberated aglycones would then pass into enterocytes passively, reabsorbed again, forming thus an enterohepatic cycling (Crespy et al., 1999).

Chitosan, a polysaccharide obtained by *N*-deacetylation from chitin, has been investigated as pharmaceutical excipient and a carrier for new delivery systems thanks to its biocompatibility, biodegradability and safety (Illum, 1998). It has the potential to be degraded by the colon microbiota and can therefore be used for colon-targeted delivery of drugs incorporated in enteric-coated dosage forms (Berthold et al., 1996). Due to its mucoadhesive properties, chitosan based dosage forms, especially multiparticulates such as pellets, micro- and nanoparticles rest in its site of action for a longer time ensuring thus longer therapeutic effect (Lamprecht et al., 2001).

Multiparticulates, at present pellets, play important role in the pharmacotherapy of IBD (Bautzova et al., 2011). They possess many pharmacological (minimal local irritation of GI tract, optimal drug level maintenance, simple dosage regimen) as well as technological (dosage form design flexibility, different drugs combination, easy coating application) advantages over conventional single-unit solid dosage forms. Being small pellets rapidly pass the pylorus regardless of the feeding state of patient and the influence of gastric emptying rate on their upper gastro-intestinal transit time is minimised, thus lowering the intra- and inter-subject variability of drug plasma profiles compared to single-unit formulations (Steckel and Mindermann-Nogly, 2004). For simple treatment schedule, pellets are popular dosage forms possessing good patient compliance.

Pellets based on chitosan designated for colonic delivery are necessary to coat with protective coating due to chitosan solubility in an acidic medium. For this purpose coatings based on components insoluble in acidic conditions are the first choise. Numerous coatings consisting of synthetic, semisynthetic or natural polymers were investigated. Well known and often used are methacrylic acid copolymers soluble at pH above 6.0 or 7.0 (Na and Bae, 2005). Some semisynthetic cellulose derivatives, for instance cellulose acetate phthalate (Rowe et al., 2006), are also used to protect the dosage form from acidic stomach conditions. Natural representatives such as alginic acid and its salts, or some other polysaccharides are also intensively studied to delay or prolong the drug release after oral application (Marcussen and Brandsborg, 2002; Zeng, 2004).

In our experiment, coated chitosan pellets capable to deliver rutin as active ingredient into the colon are presented. Pellets were prepared using extrusion/spheronization and subsequently coated with different coatings to prevent chitosan pellets from the dissolution in acidic surrounding. Pellets' characteristics and their dissolution profiles in vitro were determined and compared. Samples with promising results were checked for their stability and evaluated in vivo in trinitrobenzensulfonic acid (TNBS) induced colitis.

2. Material and methods

2.1. Material

Rutin as an active ingredient, caffeic acid, alginic acid and sodium alginate as coating excipients were purchased from Sigma–Aldrich (Steiheim, Germany). Microcrystalline cellulose (MCC) Avicel® PH 101 as a spheronization enhancer was supplied by FMC (Cork, Ireland), chitosan of deacetylation degree 91% by JBiChem (Hangzhou, China), and hypromellose (HPMC) Methocel® K 100 by Colorcon (Kent, United Kingdom). Pharmacoat 606 (HPMC E) and zinc acetate for pellet coatings were delivered from Zentiva (Praha, Czech Republic) and from Penta (Chrudim, Czech Republic), respectively. Eudragit® FS – a kind gift from Evonik Röhm GmbH (Darmstadt, Germany), glyceryl monostearate, polysorbate 80 (Tween 80) and triethyl citrate sulfate, all from Sigma–Aldrich (Praha, Czech Republic) were used in the comparative pellet coating. The enzyme β-glucosidase (3811 U/mg) used in dissolution testing was purchased from MP Biomedicals (Solon, USA). TNBS and hexadecyltrimethylammonium bromide were obtained from Fluka Biochemika (Steinheim, Germany), o-dianisidine dihydrochloride as a substrate and hydrogen peroxide as a reagent (starting the reaction) from Sigma–Aldrich (Steinheim, Germany). Also all other chemical reagents were purchased from last mentioned companies. Active ingredient and all excipients were of pharmaceutical grade.

2.2. Pellet preparation

Dry powder mixture (100 g) containing of 30% of rutin, 45% of chitosan and 25% of MCC (or 30% of rutin and 70% of MCC – sample 5), was homogenized in a mixer (Tefal Kaleo, Rumilly, France) for 5 min and then wetted by 90 g of 0.25% acetic acid solution to dissolve chitosan (or 97.5 g of water – sample 5). One screw extruder (Pharmex 35T, Wyss & Probst, Ettlingen, Germany) fitted with axialy located die of the thickness 1 mm and perforations 0.8 mm was used to form extrudate. The wetted mass was fed through a hopper on a rotating screw. The extruder operated at a constant speed of 110 rpm. The prepared extrudate was subsequently spheronized in a radial plate spheronizer (Pharmex 35T, Wyss & Probst, Ettlingen, Germany) with a 23 cm diameter serrated plate at rotating speed 640 rpm for 15 min. Formed pellets were dried in ventilated oven (Horo, Ostfildern, Germany) at 40 °C for 3 h. Four pellet batches were prepared to verify the reproducibility of the process.

2.3. Coating of pellets

Pellets (150 g; size of 0.5–1.0 mm) were preheated at 40 °C for 5 min in Wurster type fluid bed unit (M-100, Medipo, Brno, Czech Republic) and then coated with a solution (a, b, c) under following conditions: spray nozzle diameter 0.4 mm; atomization pressure 100 kPa; spray rate 1.8–2.2 g/min and inlet air temperature 40 °C. Final coat represented 18% of total pellets mass (a–c). Coating dispersion (d) was applied on pellets of both types, i.e., with or without chitosan, and used for a comparison. Conditions were almost the same as for previous coatings, only product temperature was lower, i.e., 35 °C, and the coating represented 15% of total pellets mass. Pellets were dried at 45 °C for 10 min. The composition of different pellet samples and their coatings is presented in Table 1.

2.4. Pellet characterization

Pellet size, size distribution and their flow properties were evaluated, and then rutin content, pellet sphericity, intraparticular porosity, hardness and friability were determined in uncoated pellet sample fraction of 0.5–1.0 mm. Particle size and size distribution were measured by sieve analysis using a set of stainless steel sieves with apertures ranging in between of 125–2000 μm (Retsch, AS 200, Haan, Germany). Pellet flow properties, i.e., repose angle and Hausner ratio were evaluated according to the pharmacopoeia recommendations ($n = 3$). Pellet intraparticular porosity was determined from the difference of pycnometric density of pellets and that of initial powder mixture according to Ph. Eur. recommendations (Helium pycnometr, Pycnomatic-ATC, Porotec GmbH, Germany). For pellet sphericity, the image analysis (Leco IA, Leco Instruments, St. Joseph, USA) in 200 particle sample was performed (Scala-Bertola et al., 2009). The hardness of ten randomly selected pellets was tested in a Tablet Hardness &

Table 1
Pellet composition and characteristics.

Sample	1[x]	2[x]	3[x]	4[x]	5[y]
Theoretical content (%)	30	30	30	30	30
Practical content (%)	28.12 ± 0.60	28.37 ± 0.40	28.50 ± 0.38	28.77 ± 0.49	28.55 ± 0.42
Mean diameter (mm)	0.79	0.74	0.77	0.78	0.80
Sphericity	0.83 ± 0.03	0.85 ± 0.04	0.83 ± 0.03	0.83 ± 0.05	0.83 ± 0.04
Porosity (%)	2.82 ± 0.11	3.32 ± 0.09	3.13 ± 0.10	3.06 ± 0.08	2.80 ± 0.01
Hardness (N)	10.54 ± 1.17	11.47 ± 1.16	9.35 ± 1.32	10.71 ± 1.42	18.06 ± 1.69
Friability (%)	0.15 ± 0.02	0.27 ± 0.01	0.21 ± 0.03	0.19 ± 0.02	0.09 ± 0.01
Repose angle	17° 34′ ± 0° 40′	17°28′ ± 0°54′	17°14′ ± 0°35′	17°31′ ± 0°48′	23°18′ ± 0°58′
Hausner ratio	1.07	1.11	1.10	1.08	1.09
Coating[z]	a Caffeic acid 55% Alginic acid 25% HPMC K100M 20%	b – two layers 1. Na alginate 50% 2. HPMC E 25% Zn acetate 25%	c Na alginate 95% Chitosan 5%	d Eudragit® FS 94.1% Polysorbate 80 1.2% Glyceryl monostearate 4.7%	

[x] Composition of uncoated pellets: Rutin 30%, Chitosan 45%, MCC 25%.
[y] Composition of uncoated pellets: Rutin 30%, MCC 70%.
[z] a, b, c – 18%; d – 15%.

Compression Tester (Engineering System, Nottingham, United Kingdom) equipped with a C5 cell for pellet evaluation. For friability testing, a stainless steel drum of the friabilator (Erweka TAR 10, Ensenstam, Germany) was used. Rutin content in the pellets was determined spectrophotometrically at a wavelength of 360 nm (phosphate buffer pH 6.8; Specord® 205, Analytik Jena, Jena, Germany). Friability and content measurements were repeated three times and the results were expressed as an arithmetic mean ± standard deviation (SD).

2.5. In vitro drug release

First, samples of uncoated and coated pellets (a, b, c) corresponding to the dose of 30 mg of rutin were placed into 1000 mL of phosphate buffer (pH 3.0 or 6.8) for dissolution studies (Ungell and Abrahamsson, 2004). The test was examined using a basket dissolution method at a rotation speed of 100 rpm at 37.0 ± 0.5 °C (Sotax AT 7 Smart on-line, Donau Lab, Basel, Switzerland). Released drug amount was measured spectrofotometrically at 360 nm (Lambda 25, PerkinElmer Instruments, Shelton, USA). All experiments were performed in triplicate and results are expressed as the mean ± SD of the active substance in %, dissolved at given sampling time.

Second, the pellets were also evaluated in dissolution test under simulated GI transit conditions described in Table 2. The buffers needed to be exchanged after 5.5 h of the testing. Samples were withdrawn every 30 min in 0–3 h and then every hour with the exception between 5–6 h.

2.6. Stability testing

Samples of pellets coated with alginate/chitosan were placed into stability boxes (Binder, Tuttlingen, Germany) under 25 °C and 60% of relative humidity (25/60); 30 °C and 65% of relative humidity (30/65); 40 °C and 75% of relative humidity (40/75). Stability tests were provided for 6 months (40/75) and for 12 months (25/60; 30/65), respectively. At the times 0, 3, 6, 9 and 12 months, samples were withdrawn and their dissolution profiles were determined using pH changing dissolution method described in previous chapter, and compared.

2.7. Animal studies

All animal experiments were carried out in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council and National Academy of Sciences, United States). Male Wistar rats (average weight 175–199 g, 5–6 weeks old; $n = 4$/group) were purchased from Société Janvier (Le Genest, St Isle, France). The animals were housed in groups of four and acclimatized to laboratory conditions a week before the experiments, with food and water ad libitum at 23 ± 1 °C, a relative humidity of 60 ± 5% and 12 h light–dark cycle. Animals were randomized into 7 groups according to colitis and drug administration: healthy control group (1); colitis control group – non treated colitis-induced rats (2); rutin solution receiving group per rectum (3); rutin solution receiving group per os (4); rutin pellets receiving group a, b, c: a – coated with caffeic acid/HPMC/alginic acid (5), b – coated with sodium alginate/HPMC/zinc acetate (6), c – coated with sodium alginate/chitosan (7). Twenty-four hours before the experiment the food was withdrawn but free access to tap water was allowed. Colitis was induced according to Morris et al. (1989). Briefly, rats were slightly anesthetized with ether, and then a rubber catheter was inserted into the anus and the tip was advanced to 8 cm to the anus verge. 300 μL of TNBS dissolved in 50% (v/v) ethanol was instilled into the colon through the catheter (dose was 42 mg/kg body weight). The animals were kept in a head-down position for a few minutes to prevent leakage of the intracolonic instillation. Control group received physiological saline instead of the TNBS solution. For 2 days, the rats were housed without treatment to maintain the development of a full inflammatory bowel disease model. Starting day 4, different groups received rutin at the dose 10 mg/kg either as a solution orally or rectally dissolved in phosphate buffer pH 7.4 or as pellet formulation. The rats were treated once daily for 5 continuous days. Colitis controls received physiological saline orally instead. The animals were sacrificed using an

Table 2
Simulating GIT conditions.

Part of GIT	Stomach	Small intesine	Terminal ileum	Colon
pH	3.0	6.8	7.5	4.0/6.0
Dissolution medium	Phosphate buffer	+4.2 g $Na_3PO_4 \cdot 12H_2O$	+6.0 g $Na_3PO_4 \cdot 12H_2O$	Acetate buffer phosphate buffer ± β-glucosidase
Time	2 h	3 h	0.5 h	16.5 h

overdose of anesthetic 24 h after the last drug/particle administration and colons were resected.

2.8. Clinical activity score system, colon/body weight ratio, and myeloperoxidase activity

Colitis activity was quantified with a clinical score assessing weight loss, stool consistency, and rectal bleeding. The mean of these score values was giving the clinical score ranging from 0 (healthy) to 4 (maximal activity of colitis) (Lamprecht et al., 2001).

Resected colon tissue samples were opened longitudinally and rinsed with iced physiological saline to remove luminal content and weighed. The colon/body weight ratio was calculated as a quotient of colon wet weight compared to total body weight of each rat (Lamprecht et al., 2005a).

Myeloperoxidase activity measurement was performed to quantify the severity of the colitis. Activities were analyzed according to Krawisz et al. (1984). One unit of myeloperoxidase activity was defined as the amount that degraded 1 μL of peroxidase per minute at 25 °C.

2.9. Microscopical/histological evaluation

Histologic assessment was carried out by light microscopy of colon tissue samples. Five micrometer thick sections were cut on a rotary microtome (Tissue-Tek®III, Sakura Finetek, Zoeterwoude, Netherlands), and stained with hematoxylin and eosin for histological evaluation of colonic damage and mucus content. All tissue sections were examined by at least two independent investigators with a Leica DMR microscope (Solms Germany) for characterization of histopathological changes and photographed (camera Leica DC500, Solms, Germany).

2.10. Statistical analysis

The results were expressed as mean values ± SD. For analysis of statistical significance, the Anova test was applied. In all cases, $P < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Pellets evaluation

Rutin/chitosan/MCC pellets, i.e., samples 1–4 (or rutin/MCC pellets – sample 5) were prepared by extrusion/spheronization method using acetic acid as the wetting agent and a solvent for partial dissolution of chitosan to obtain matrix pellets (Dvorackova et al., 2009) (or water to prepare sample 5). As moisture content of the extrudate affects pellets shape and size (Perez and Rabiskova, 2002), the optimum amount of acetic acid was evaluated experimentally to be 90 g (or 97.5 g of water in sample 5) for 100 g of the powder mixture, thus forming plastic mass appropriate for extrusion. The size of the pellets produced by extrusion/spheronization was found in narrow interval, i.e., 0.5–1.0 mm (92.15–93.45%), what is typical for this method. Pellet size distribution showed two main fractions: 0.5–0.8 mm and 0.8–1.0 mm giving mean diameter shown in Table 1. These major pellet fractions were used for further evaluation and experiments. Pellets showed shape (Fig. 1A) of suitable sphericity values (Table 1) (Deasy and Law, 1997). Rutin content in pellets was 93.74–95.17% of its theoretical value. Pellet intraparticular porosity was very low indicating good compactness of used substances. Obtained values of pellet friability and their hardness indicated pellet quality sufficient to withstand further processing. Flow properties of pellets showed low repose angle and Hausner ratio close to 1.1 corresponding to excellent flow. For the coated pellets, rutin represented 25% of their total weight. In Fig. 1B–D, representative examples of coated pellets are shown. Fig. 1B illustrates the pellets coated with caffeic acid/HPMC/alginic acid, Fig. 1C with sodium alginate/HPMC/zinc acetate and Fig. 1D with sodium alginate/chitosan. As it can be seen from the cross sections of pellets coated with caffeic acid//HPMC/alginic acid and with sodium alginate/chitosan (Fig. 1E and F), the thickness of the coat was approximately 30 μm. The glinting parts are chitosan particles undissolved in acetic acid. Thus the matrix is composed of rutin, MCC and partialy dissolved chitosan particles.

3.2. Dissolution tests

After pellets characterization, in vitro dissolution profiles of uncoated and coated (a–c) pellets were determined in phosphate buffers of pH 3.0 or 6.8, respectively. In man, in fasted state pH reaches the values 1.0–3.0, but in fed state pH from 2.0 to 5.0 has been observed (Chuong et al., 2008). Thus, pH 3.0 could be considered as a good compromise of pH values in fed state to simulate gastric conditions. Generally, pH value of 6.8 is used to simulate conditions in the small intestine, therefore pH 6.8 was the second value used in preliminary dissolution tests. Rutin dissolution profiles are presented in Fig. 2. All pellet samples showed prolonged rutin release in media of both pH values. As evident from Fig. 2A the drug release from uncoated pellets was significantly faster (77.72 ± 3.80%) than that from coated samples due to chitosan dissolution in acidic media (Rowe et al., 2006). Pellets coated with caffeic acid/HPMC/alginic acid showed faster rutin release (38.45 ± 3.87%) than two other samples (16.55 ± 3.77% and 22.19 ± 3.69%, respectively). This coating consists from two acids and HPMC helping to form a gel layer. Hypromellose K is known for its fast hydration rate and thus, when distributed homogeneously within the layer, could cause higher coating permeability. Second coating b consisted of two layers: sodium alginate, and hypromellose E and zinc acetate. The gelation and crosslinking of the polymers are achieved by the exchange of sodium and zinc ions and stacking of glucuronic groups to form egg-box structure (Gombotz and Wee, 1998) able to control drug release within 24 h (Zeng, 2004). Hypromellose E has slower hydration rate and when used in a different layer, it seems to have less influence on the coating permeability than HPMC K in the previous coating. Third coating c was based on sodium alginate with 5% of chitosan and ensured slow rutin dissolution profile similar to that of coating b. Alginate is a natural, biocompatible, biodegradable and cost acceptable polysaccharide, insoluble in aqueous solutions with pH less than 3 (Rowe et al., 2006). Containing carboxyl end groups, it is classified as an anionic mucoadhesive polymer, resistant to digestive enzymes and fermented by colonic microbiota (Patel et al., 2007). Alginate and chitosan are known to interact and form polyelectrolyte complex decreasing the drug release in low as well as high pH values (Mi et al., 2002). In pH 6.8 (Fig. 2B), rutin release was very slow in all tested samples: 19.8–27.5% after 12 h of dissolution test. These results were expected as chitosan is not soluble at pH values higher than 6.5 (Rowe et al., 2006). In vivo, chitosan degradation takes place in the colon due to the activity of enzymes produced by colonic microbiota. Following the degradation, dosage forms based on chitosan can release the drug included (Jose et al., 2009). In dissolution studies, commercially available β-glucosidase with similar degradation function on chitosan as that of colonic enzymes is used instead (Zhang and Neau, 2001).

Following the above described testing, rutin release from pellets with coat c using buffers with changing pH values and the final buffers of pH 4.0 and 6.0, respectively, was provided (Fallingborg et al., 1993; Nugent et al., 2001). Dissolution medium of pH 6.0 contained β-glucosidase in the concentration of 0.1%. Fig. 3 compares dissolution profiles of rutin/chitosan/MCC pellets with the coating c and d, and rutin/MCC pellets with the same coating d to

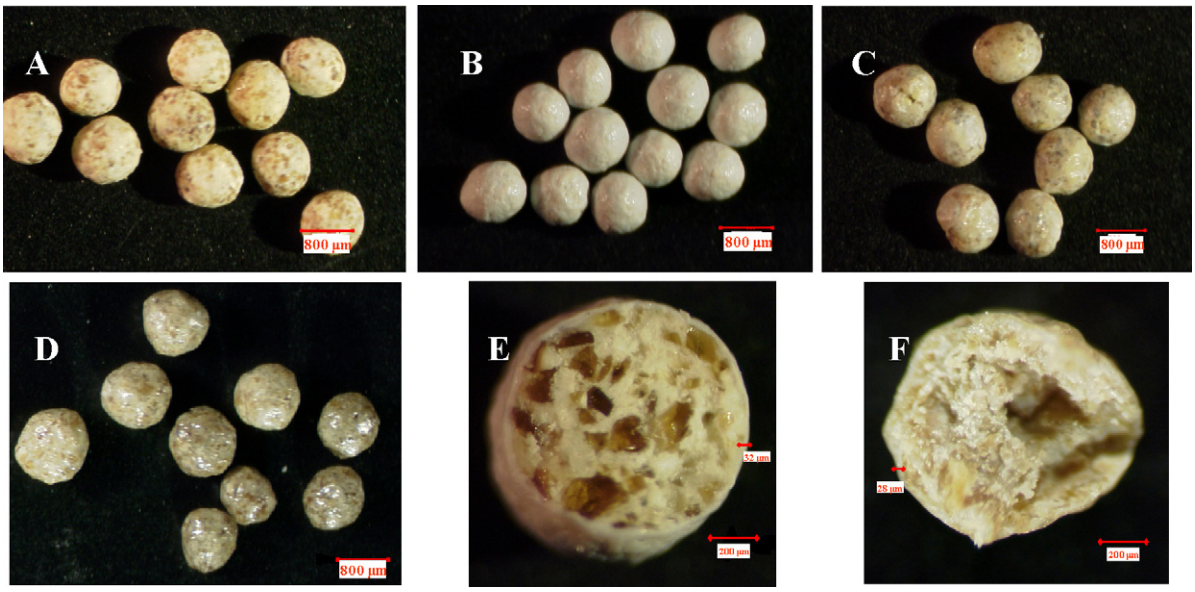

Fig. 1. Optical microscopic images showing the general morphology of rutin pellets uncoated (A) and coated with different coatings: caffeic acid/HPMC/alginic acid (B), sodium alginate/HPMC/zinc acetate (C), sodium alginate/chitosan (D), a cross-section of caffeic acid/HPMC/alginic acid coated pellet (E) and a cross-section of sodium alginate/chitosan coated pellet (F). The scale bars represent 800 μm (A–D), 200 μm (E–F); 32 μm (E) and 28 μm (F) for the coating thickness, respectively.

see the possible influence of chitosan inside the pellets. No difference was found in promising rutin dissolution profiles from pellets with polysaccharide coating no matter of pH value of the last buffer used: during first 5 h of dissolution test only small amount (<14%) of rutin was released and 90 min after the pH change simulating terminal ileum more than 80% of rutin was determined in the medium. On the other hand, great difference was determined in drug release from pellets coated with polyacrylates depending on the final buffer value. When acetic buffer (pH 4.0) was applied, the course of rutin dissolution profile was more similar to those measured for pellets coated with sodium alginate/chitosan. However in phosphate buffer of pH 6.0, very slow release ending at 27% of rutin was determined. Similar values were found also when β-glucosidase was added into the last medium. It is evident that short period in buffer of pH 7.5 was not sufficient to dissolve coating d and facilitate rutin release. Similar results were recently reported also by Poelvoorde et al. (2008). It seems however that dissolution buffer activity was able to cause some fractures in this coating explaining fast rutin release in acetic buffer of pH 4.0. This buffer passing through the coating could dissolve chitosan inside the coated pellets helping thus the drug release. This finding can be supported by the results obtained from rutin/MCC pellets with coat d, i.e., the dissolution profiles ending at 27% of rutin when final pH of 4.0 was applied and 17% in pH 6.0, respectively. These results were expected as inside of these pellets was no excipient promoting drug release through polyacrylic coating and rutin solubility is low, only slightly different in pH 4.0 (219.64 mg/L) and pH 6.0 (90.95 mg/L) at 37 °C. Considering in vitro results, the administration of dosage forms with polyacrylic coating d and intended for IBD treatment need not lead to the drug release in the appropriate site of GI tract in some IBD patients exhibiting lower colonic pH values.

3.3. Stability testing

Pellets coated with alginate/chitosan were placed to stability boxes under 25 °C and 60% of relative humidity (25/60); 30 °C and 65% of relative humidity (30/65); 40 °C and 75% of relative humidity (40/75). Stability tests were provided for 6 months (40/75) and for 12 months (25/60; 30/65), respectively. At the times 3, 6, 9 and 12 months, samples were withdrawn and their dissolution profiles were determined using pH changing dissolution method and compared with rutin dissolution profiles at the time 0 (Rabiskova et al., 2011). Differences between obtained results are presented in Table 3. No significant differences in rutin release have been

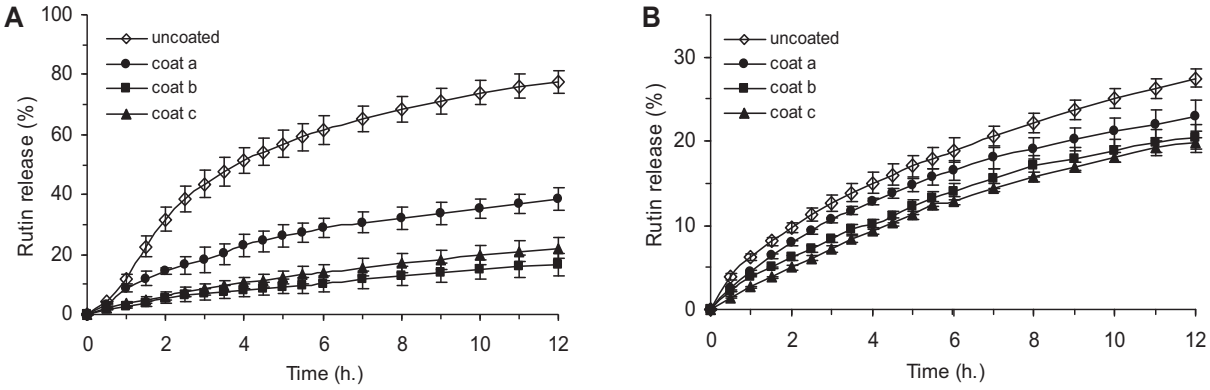

Fig. 2. Dissolution profiles of rutin pellets with different coatings in phosphate buffer systems pH 3.0 (A) and 6.8 (B); coat a: caffeic acid/HPMC/alginic acid; coat b: sodium alginate/HPMC/zinc acetate; coat c: sodium alginate/chitosan.

Table 3
Differences in rutin release from pellets coated with sodium alginate/chitosan within stability testing.

Temperature (°C)/RH/*t*	Differences in rutin release (%)								
Time (h)	1	3	5	5.5	6	7	8	14	22
25/60/3M	−0.82	−1.13	−1.41	3.27	5.43	4.27	3.38	2.52	1.97
25/60/6M	−3.30	−3.84	−4.28	6.87	11.88	10.53	9.21	7.17	7.01
25/60/9M	0.40	−1.63	−1.98	9.71	12.89	9.92	7.89	5.42	4.39
25/60/12M	0.76	0.12	−0.32	10.43	12.98	11.12	9.26	6.77	6.50
30/65/3M	0.39	−0.32	−0.10	0.26	4.66	4.57	4.42	4.15	4.74
30/65/6M	0.19	−1.26	−2.04	5.01	9.35	9.64	8.61	8.98	10.06
30/65/9M	0.36	−3.38	−5.55	5.12	7.75	6.05	4.29	3.75	4.39
30/65/12M	1.14	−1.15	−2.40	6.22	9.67	8.97	7.44	7.10	7.93
40/75/3M	0.46	−1.25	−1.62	2.92	4.20	3.91	3.46	2.75	2.66
40/75/6M	1.08	−1.63	1.76	5.77	8.28	6.87	6.01	4.57	4.15

found; the maximum difference was less than 13%. Nevertheless these small differences indicating excellent stability of the pellets, one can admit the tendency in the dissolution profiles change in all stability regimens, i.e., slowing down of the rutin release within first 5 h and mild acceleration in the release from the 5.5 h of the testing. This tendency is in harmony with the demanded dissolution profile, i.e., no or low amount of the drug released under conditions simulating upper GI tract and fast release of the drug under the conditions mimicking the colon.

3.4. Animal studies

Several investigations were realized in order to evaluate the therapeutic value of rutin, on a preexisting colitis model in rats. Cruz et al. (1998) demonstrated the anti-inflammatory effect of rutin in acute and chronic colitis. Rats were treated with aqueous solution of rutin at different doses administered orally with optimal effect at the dose of 10 or 25 mg/kg of rutin. Kwon et al. (2005) reported that rutin ameliorates experimental colitis, presumably by suppressing the induction of pro-inflammatory cytokines. In our study, we investigated the effect of rutin solution or rutin/chitosan/MCC pellets coated with polysaccharides for diminution the inflammation in TNBS-induced colitis. The animals were divided into 7 groups and starved for 24 h before induction of colitis. On day 3, the animals received an intrarectal application of TNBS except the healthy control group. Before this time point, animals showed no clinical problems. After inducing the experimental colitis, the clinical score increased rapidly and consistently for the next 2 days for all groups as represented by clinical activity score (Fig. 4), and the rats developed prostration, piloerection and hypomotility. The diarrhea in the majority of TNBS-administrated animals occurred during the first 3–4 days. The inflammatory response showed similar characteristics to those reported elsewhere: experimental animals had anorexia and loss of weight as well as diarrhea (Cruz et al., 1998; Magro et al., 2006).

Starting from day 5, rats received rutin solution either rectally or orally or rutin pellets orally once a day for five following days, with the exception of the colitis control group. Healthy control group received saline instead. The clinical activity score was used to evaluate the severity of the colonic inflammation and the colitis control group proved to be an excellent model of inflammation as evidenced by the highly increased clinical activity. During the whole treatment period, rutin lowered the clinical activity so that all drug-receiving groups showed decreasing index values after a lag time of 24 h. The difference between drug-treated groups and colitis controls became significant on day 7. The treatment with rutin resulted in a diminution of diarrhea compared to the TNBS control group, where we detected diarrhea still at the day of sacrifice. The most pronounced decrease in clinical activity score was observed in group treated with pellets coat c. Other treatment resulted in nearly identical clinical activity score.

On day 10 (24 h after the last drug administration), the animals were sacrificed, and colon/body weight ratio and myeloperoxidase activity were determined to quantify the inflammation. Inflammatory changes of the intestinal tract after intracolonic administration of TNBS are associated with transmural inflammation of the colonic segments and presence of adhesions of the rat colon to adjacent

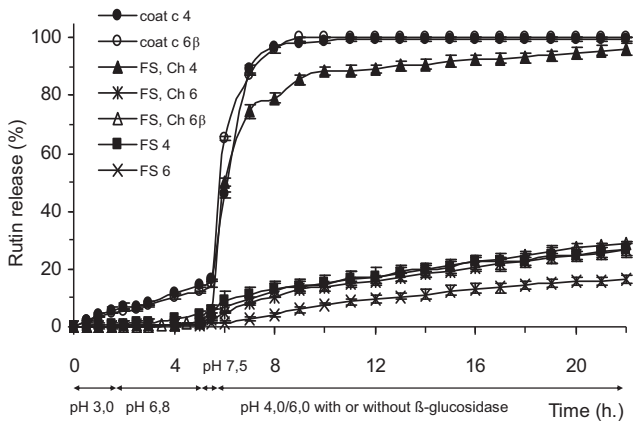

Fig. 3. Rutin dissolution profiles from rutin/MCC/chitosan or rutin/MCC pellets with different coatings obtained using dissolution method with continual pH change of buffers: c – sodium alginate/chitosan coat; d – Eudragit® FS coat; Ch – rutin/MCC/chitosan pellets; M – rutin/MCC pellets; 4 – final pH 4.0; 6 – final pH 6.0; 6β – final pH 6 with β-glucosidase. Data are shown as mean ± SD.

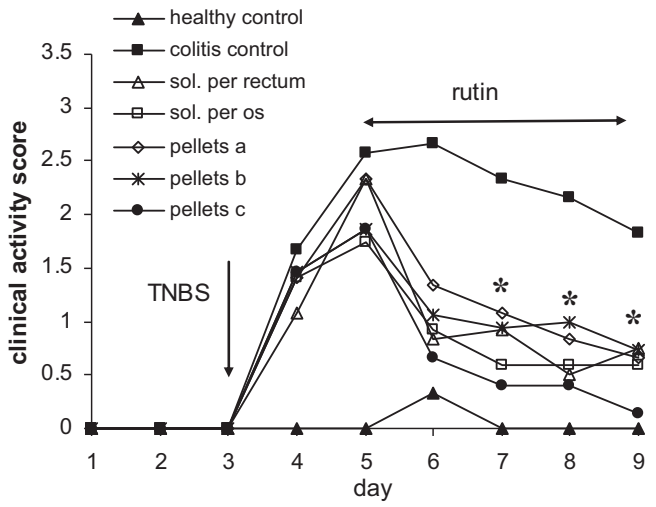

Fig. 4. Clinical activity score in TNBS rat model during the whole experimental period after rectal and oral administration always determined for $n = 4$ animals. Error bars are not shown for clearness. *$P < 0.05$ compared with colitis control rats given saline for all treated group, except pellets at administration on day 7.

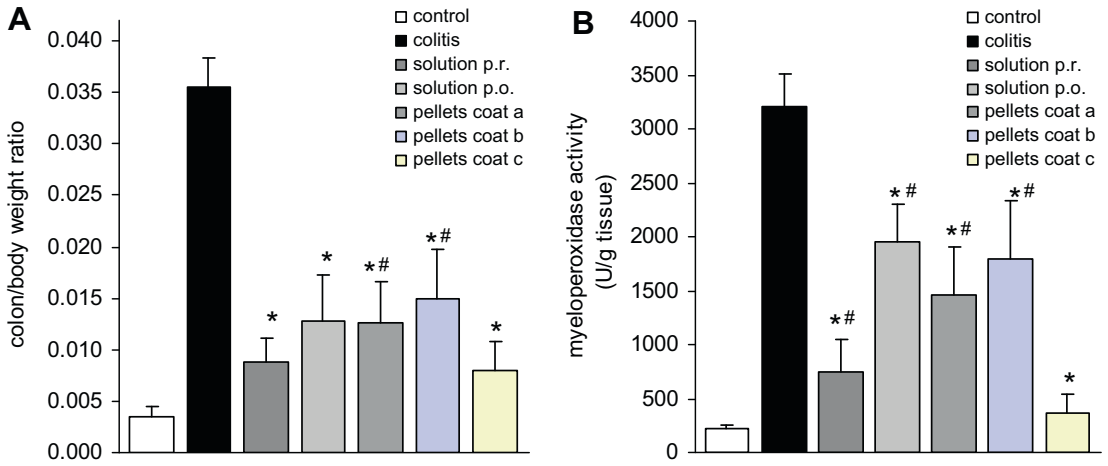

Fig. 5. Determination of colon/body weight ratio (A) and myeloperoxidase activity (B) on day 10 in TNBS colitits model after rectal and oral rutin administration at a dose of 10 mg/kg. Data are shown as mean ± S.D. for $n = 4$ animals. $^{*}P < 0.05$ compared with colitis control rats given saline, $^{\#}P < 0.05$ compared with rats given pellets coat c.

organs (Galvez et al., 2000). Macroscopic inspection in colitis control group showed evidence of bowel wall thickening and stiffness, bleeding or intense hyperemia, focal ulceration of the mucosa and diffuse hemorrhagic necrosis of the epithelium. Amelioration in macroscopic changes without the sign of bleeding, ulceration and necrosis, and reduced presence of adhesions to adjacent organs was observed in all treated groups. Only bowel wall thickening and moderate hyperemia were detected in flavonoids receiving groups. In Fig. 5A colon/body weight ratio is depicted. Significant increase of values up to 0.036 was observed in colitis control group and reported also elsewhere (Lamprecht et al., 2005b). Similarly as in the case of clinical activity score, the drug-treated groups showed decreased values in the colon/body weight ratio. The best results were obtained in groups receiving pellets coated with sodium alginate/chitosan (0.008) or rutin solution rectally (0.009). In healthy control group, colon/body weight ratio was also calculated and reached the value of 0.004. A marked increase in myeloperoxidase activity, an indicator of the colonic infiltration with polymorphonuclear leukocytes, characterizes the severity of colitis as well (Cuzzocrea et al., 2005). Rutin administration, either rectally or orally, significantly reduced myeloperoxidase activity when compared to the corresponding colitis control group (Fig. 5B): best results were achieved in group treated with pellets coat c and then the group treated with rutin solution administered rectally. This reduction of neutrophil infiltration seemed to be a consequence of the accelerated healing of colonic ulcers, facilitating the elimination of neutrophil accumulation from the inflamed colon (Cruz et al., 1998). In groups treated with rutin solution administrated orally and pellets coated either with caffeic acid/HPMC/alginic acid or sodium alginate/HPMC/zinc acetate the decrease in myeloperoxidase activity was less significant. It seems that pellets with coat c pass through the stomach and intestines without the structural changes. Reaching the colon, both coating substances are decomposed by microbiota, uncoated particles adhere to the bowel wall due to bioadhesive properties of chitosan, and released rutin can be effective in the site of inflammation, similarly as after its rectal administration. As mentioned above, the coating based on caffeic acid/alginic acid, starts to release the small amount of rutin already in pH 3.0, i.e., in the stomach. This can lead to the decomposition of pellets within intestinal transit and thus the same efficacy as after the oral administration of rutin solution was observed. Surprisingly, the pellets coated with sodium alginate/HPMC/zinc acetate possessing better dissolution profiles than pellets coated with caffeic acid/alginic acid in vitro did not prove an appropriate amelioration of the inflammation in vivo. Taking into consideration the article published by Dhalleine et al. recently (2011), the interaction between Zn^{2+} ions present in pellet coating and phosphate ions from GI tract could occur forming the insoluble layer on the pellet surface. This zinc phosphate layer can be responsible for minimal drug liberation in the colon and subsequently less important therapeutic effect of rutin.

Histological pictures are shown in Fig. 6. Colonic inflammation of TNBS-induced colitis is characterized by strong damages of intestinal tissue, e.g., crypt destruction, mucosal ulceration, erosions and infiltration of immune related cells into the mucosal tissue. In some sections of ulcerated areas necrotic tissue could be observed. Goblet cells were totally absent at the surface epithelium (A) of colitis group compared to the healthy one (D). The

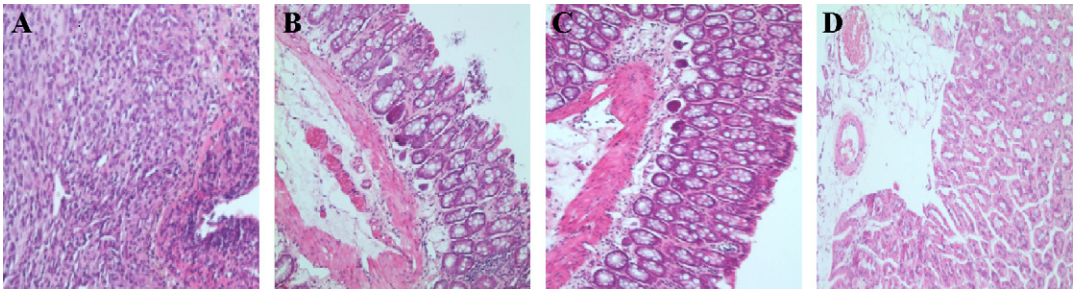

Fig. 6. Microscopic images of a colon section through a tissue sample after hematoxylin and eosin stain of the colitis group (A) showing massive ulceration with dissappearance of 3 typical layers and site of necrosis, (B) the group treated with pellets coated with sodium alginate/HPMC/zinc acetate presenting decreased inflammation, still visible erosion of mucosa, (C) group treated with pellets coated with sodium alginate/chitosan showing a rest of focus of inflammation, but without any erosion of mucosa and (D) healthy group without any histological modification. Original magnifications 100×.

inflammation extended through three layers of the colon tissue, i.e., mucosa, muscularis mucosae and submucosa. Histological figures confirmed the results presented in previous figures (Figs. 4 and 5), i.e., that the treatment with rutin reduced the morphological alterations associated with TNBS administration showing ulcers in the process of healing (B, C). After the treatment with pellets coated with sodium alginate/HPMC/zinc acetate we observed persisting erosion of mucosa and some focus of inflammation (B). Thanks to favorable effect of rutin to damaged tissue, after the treatment we could anew distinguish three layers of colonic epithelium (mucosa, muscularis mucosae, submucosa), which disappeared during the inflammation. After the application of the pellets coated with sodium alginate/chitosan, histological cut of tissue (C) demonstrates the reconstitution of the mucosa surface with the rest of few focus of inflammation. Histological examination of healthy group represented typical features of the colon and normal structure (D).

4. Conclusions

Rutin pellets based on chitosan and coated with several natural substances were successfully prepared and showed very good characteristics. Pellets coated with sodium alginate/chitosan presented promising rutin dissolution profiles and excellent stability. The results of in vivo study demonstrate that the administration of rutin at the dose of 10 mg/kg daily during 5 days in all groups ameliorated clinical activity score observed in TNBS control rats. In this way, rutin treatment also decreased the colon/body weight ratio. In addition, rutin administrated rectally as solution or orally as pellets coated with sodium alginate/chitosan, significantly reduced the inflammatory response. The beneficial effect exerted by rutin resulted in diminution of myeloperoxidase activity, a marker of neutrophil infiltration into the colon, which has been previously described to be upregulated in experimental colonic inflammation (Cuzzocrea et al., 2005). This reduction of neutrophil infiltration seemed to be a consequence of the accelerated healing of colonic ulcers, facilitating the elimination of neutrophil accumulation from the inflamed colon.

Thus, rutin pellets coated with sodium alginate/chitosan could become the valuable preparation for mild-to-moderate IBD treatment. The combination of rutin as natural product, chitosan as a mucoadhesive excipient degraded in the colon, sodium alginate as the main natural coating substance and pellets as multiparticulate dosage form having numerous advantages in IBD treatment, could form a promising preparation free of side effects for life long therapy of this severe illness.

Acknowledgements

The authors would like to thank to Dr. Jarmila Klusakova, Ph.D. from Department of Histopathology, University Hospital Brno, Czech Republic for histological preparations. Financial support for Research Project Ministry of Health, Czech Republic NS10222-2/2009, Research Project MSM 0021620822 and from Institut Universitaire de France are announced.

References

Baumgart, D.C., Sandborn, W.J., 2007. Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet 369, 1641–1657.
Bautzova, T., Rabiskova, M., Lamprecht, A., 2011. Multiparticulate systems containing 5-ASA for the treatment of inflammatory bowel disease. Drug Dev. Ind. Pharm. 37, in print.
Berthold, A., Cremer, K., Kreuter, J., 1996. Preparation and characterization of chitosan microspheres as drug carrier for prednisolone sodium phosphate as model for anti-inflammatory drugs. J. Controlled Release 39, 17–25.
Bresci, G., Parisi, G., Bertoni, M., Capria, A., 2002. Long-term maintenance treatment in ulcerative colitis: a 10-year follow-up. Dig. Liver Dis. 34, 419–423.
Chuong, M.C., Christensen, J.M., Ayres, J.W., 2008. New dissolution method for mesalamine tablets and capsules. Dissolution Technol. 15, 7–14.
Crespy, V., Morand, C., Manach, C., Besson, C., Demigne, C., Remesy, C., 1999. Part of quercetin absorbed in the small intestine is conjugated and further secreted in the intestinal lumen. Am. J. Physiol. Gastrointest. Liver Physiol. 277, 120–126.
Cruz, T., Galvez, J., Ocete, M.A., Crespo, M.E., Sanchez de Medina, L.H.F., Zarzuelo, A., 1998. Oral administration of rutoside can ameliorate inflammatory bowel disease in rats. Life Sci. 62, 687–695.
Cuzzocrea, S., Rossi, A., Mazzon, E., Di Paola, R., Genovese, T., Muia, C., Caputi, A.P., Sautebin, L., 2005. 5-Lipoxygenase modulates colitis through the regulation of adhesion molecule expression and neutrophil migration. Lab. Invest. 85, 808–822.
Deasy, P.B., Law, M.F.L., 1997. Use of extrusion-spheronization to develop an improved oral dosage form of indomethacin. Int. J. Pharm. 148, 201–209.
Dhalleine, C., Assifaoui, A., Moulari, B., Pellequer, Y., Cayot, P., Lamprecht, A., Chambin, O., 2011. Zinc-pectinate beads as an in vivo self-assembling system for pulsatile drug delivery. Int. J. Pharm. 414, 28–34.
Dvorackova, K., Skrabakova, G., Rabiskova, M., 2009. Influence of formulation technology on theophylline release from chitosan-based pellets. Ces. Slov. Farm. 58, 216–224.
Fallingborg, J., Christensen, L.A., Jacobsen, B.A., Rasmussen, S.N., 1993. Very low intraluminal colonic pH in patients with active ulcerative colitis. Dig. Dis. Sci. 38, 1989–1993.
Farup, P.G., Hinterleitner, T.A., Lukas, M., Hebuterne, X., Rachmilewitz, D., Campieri, M., Meier, R., Keller, R., Rathbone, B., Oddsson, E., 2001. Mesalazine 4 g daily given as prolonged-release granules twice daily and four times daily is at least as effective as prolonged-release tablets four times daily in patients with ulcerative colitis. Inflamm. Bowel Dis. 7, 237–242.
Fiocchi, C., 2002. Inflammatory bowel disease: current concepts of pathogenesis and implications for therapy. Minerva Gastroenterol. Dietol. 48, 215–226.
Galvez, J., Garrido, M., Merlos, M., Torres, M.I., Zarzuelo, A., 2000. Intestinal anti-inflammatory activity of UR-12746, a novel 5-ASA conjugate, on acute and chronic experimental colitis in the rat. Br. J. Pharmacol. 130, 1949–1959.
Gombotz, W.R., Wee, S.F., 1998. Protein release from alginate matrices. Adv. Drug Deliv. Rev. 31, 267–285.
Illum, L., 1998. Chitosan and its use as a pharmaceutical excipient. Pharm. Res. 15, 1326–1331.
Jose, S., Dhanya, K., Cinu, T.A., Litty, J., Chacko, A.J., 2009. Colon targeted drug delivery: different approaches. J. Young Pharmacists 1, 13–19.
Krawisz, J.E., Sharon, P., Stenson, W.F., 1984. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Gastroenterology 87, 1344–1350.
Kwon, K.H., Murakami, A., Tanaka, T., Ohigashi, H., 2005. Dietary rutin, but not its aglycone quercetin, ameliorates dextran sulfate sodium-induced experimental colitis in mice: attenuation of pro-inflammatory gene expression. Biochem. Pharmacol. 69, 395–406.
Lamprecht, A., Ubrich, N., Yamamoto, H., Schafer, U., Takeuchi, H., Maincent, P., Kawashima, Y., Lehr, C.M., 2001. Biodegradable nanoparticles for targeted drug delivery in treatment of inflammatory bowel disease. J. Pharmacol. Exp. Ther. 299, 775–781.
Lamprecht, A., Yamamoto, H., Ubrich, N., Takeuchi, H., Maincent, P., Kawashima, Y., 2005a. FK506 microparticles mitigate experimental colitis with minor renal calcineurin suppression. Pharm. Res. 22, 193–199.
Lamprecht, A., Yamamoto, H., Takeuchi, H., Kawashima, Y., 2005b. A pH-sensitive microsphere system for the colon delivery of tacrolimus containing nanoparticles. J. Controlled Release 104, 337–346.
Magro, F., Fraga, S., Azevedo, I., Soarez-Da-Silva, P., 2006. Intestinal 5-hydroxytryptamine and mast cell infiltration in at experimental colitis. Dig. Dis. Sci. 51, 495–501.
Marcussen, J., Brandsborg, E., 2002. Enteric coating, comprising alginic acid, for an oral preparation. US Patent 6455052.
Mi, F.L., Sung, H.W., Shyu, S.S., 2002. Drug release from chitosan–alginate complex beads reinforced by a naturally occurring crosslinking agent. Carbohydr. Polym. 48, 61–72.
Morris, G.P., Beck, P.L., Herridge, M.S., Depew, W.T., Szewczuk, M.R., Wallace, J.L., 1989. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 96, 795–803.
Murota, K., Terao, J., 2003. Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism. Arch. Biochem. Biophys. 417, 12–17.
Na, K., Bae, Y.H., 2005. pH-sensitive polymers for drug delivery. In: Kwon, G.S. (Ed.), Polymeric Drug Delivery Systems. Taylor and Francis, Boca Raton, pp. 129–180.
Nemeth, K., Piskula, M.K., 2007. Food content, processing, absorption and metabolism of onion flavonoids. Food Sci. Nutr. 47, 397–409.
Nugent, S.G., Kumar, D., Rampton, D.S., Yazaki, E., Evans, D.F., 2001. Intestinal luminal pH in inflammatory bowel disease: possible determinants and implications for therapy with aminosalicylates and other drugs. Gut 48, 571–577.
Oldenburg, B., van Kats-Renaud, H., Koningsberger, J.C., van Berge Henegouwen, G.P., van Asbeck, B.S., 2001. Chemiluminescence in inflammatory bowel disease patients: a parameter of inflammatory activity. Clin. Chim. Acta 310, 151–156.
Patel, G., Patel, R., Patel, J., Bharadia, P., Patel, M., 2007. Sodium alginate: physiological activity, usage and potential applications. Drug Deliv. Technol. 7, 28–37.
Perez, J.P., Rabiskova, M., 2002. Influence of the drying technique on theophylline pellets prepared by extrusion-spheronization. Int. J. Pharm. 242, 349–351.
Poelvoorde, N., Huyghebaert, N., Vervaet, C., Remon, J.P., 2008. Optimisation of an enteric coated, layered multi-particulate formulation for ileal delivery of viable recombinant *Lactococcus lactis*. Eur. J. Pharm. Biopharm. 69, 969–976.

Rabiskova, M., Bautzova, T., Dvorackova, K., Spilkova, J., 2009. Beneficial effects of rutin, quercitrin and quercetin on inflammatory bowel disease. Ces. Slov. Farm. 58, 47–54.
Rabiskova, M., Dvorackova, K., Kofronova, L., 2011. Stability testing of alginate–chitosan films. Ces. Slov. Farm. 60, in print.
Rowe, R.S., Sheskey, P.J., Owen, S.C., 2006. Handbook of Pharmaceutical Excipients, fifth ed. Pharmaceutical Press, Washington.
Sands, B.E., 2000. Therapy of inflammatory bowel disease. Gastroenterology 18, 68–82.
Scala-Bertola, J., Gajdziok, J., Rabiskova, M., Bonneaux, F., Lecompte, T., Sapin, A., Maincent, P., 2009. Pellets for oral administration of low-molecular-weight heparin. Drug Dev. Ind. Pharm. 35, 1503–1510.
Steckel, H., Mindermann-Nogly, F., 2004. Production of chitosan pellets by extrusion/spheronization. Eur. J. Pharm. Biopharm. 57, 107–114.
Ungell, A.L., Abrahamsson, B., 2004. Biopharmaceutical support in candidate drug selection. In: Gibson, M. (Ed.), Pharmaceutical Formulation and Preformulation: A Practical Guide from Candidate Drug Selection to Commercial Dosage Form. Informa Healthcare, New York, pp. 129–171.
Zeng, W.M., 2004. Oral controlled release formulation for highly water-soluble drugs: drug–sodium alginate–xanthan gum–zinc acetate matrix. Drug Dev. Ind. Pharm. 30, 491–495.
Zhang, H., Neau, S.H., 2001. In vitro degradation of chitosan by a commercial enzyme preparation: effect of molecular weight and degree of deacetylation. Biomaterials 22, 1653–1658.