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Research paper

Development of enteric-coated calcium pectinate microspheres intended for colonic drug delivery

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Abstract

Enteric-coated calcium pectinate microspheres (MS) aimed for colon drug delivery have been developed, by using theophylline as a model drug. The influence of pectin type (amidated or non-amidated) and MS preparation conditions ($CaCl_2$ concentration and cross-linking time) was investigated upon the drug entrapment efficiency and its release behaviour. Drug stability and drug–polymer interactions were studied by Differential Scanning Calorimetry, thermogravimetry, X-ray diffractometry and FTIR spectroscopy. Enteric coating with Eudragit® S100 enabled maintenance of MS integrity until its expected arrival to colon. The coating was also useful to improve the stability of MS during storage, avoiding morphologic changes observed for uncoated MS stored under ambient conditions. Entrapment efficiency increased by reducing cross-linking time, and (only in the case of non-amidated pectin) by increasing $CaCl_2$ concentration. On the other hand, release tests performed simulating the gastro-intestinal pH variation evidenced an inverse relationship between $CaCl_2$ concentration and drug release rate, whereas no influence of both pectin type and cross-linking time was found. Unexpectedly, addition of pectinolytic enzymes to the colonic medium did not give rise to selective enzymatic degradation of MS. Notwithstanding this unforeseen result, coated MS prepared at 2.5% w/v $CaCl_2$ concentration were able to adequately modulate drug release through a mixed approach of pH and transit time control, avoiding drug release in the gastric ambient, and reaching the colonic targeting where 100% release was achieved within less than 24 h.

Keywords: Colon delivery; Amidated and non-amidated pectin; Calcium pectinate microspheres; Cross-linking conditions; Eudragit S100; Theophylline; Drug release

1. Introduction

In the last years the development of colon-targeted drug delivery systems has received an increasing interest not only for a better treatment of specific local pathologies, but also for the systemic therapy of both conventional and labile molecules, as well as a means of achieving chronotherapy for diseases sensitive to circadian rhythms, such as asthma and arthritis [1,2]. Among the various strategies proposed to target orally administered drugs to the colon [2–4] those based on drug release triggered by colon microflora are generally considered the most effective regarding target selectivity [5,6]. In this field, a particular interest has been directed to the employment of natural biodegradable polymers due to their ability to act as specific substrates for the colonic microflora combined with high safety, non-toxicity and biocompatibility characteristics [6–8]. Among such polymers, pectins, hydrophilic polysaccharides derived from plant cell walls mainly consisting of partially methoxylated poly α-(1–4)-D-galacturonic acids, appear of great and practical interest, due to their low cost, wide availability and variety of types and flexibility in use. However, their solubility and swelling properties in aqueous media prevent them from efficiently avoiding drug release during transit through the upper gastrointestinal

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tract, thus making the combined use of other strategies, such as coating with pH-sensitive polymers, necessary. A recent investigation describing the use of coated pectin-matrices simultaneously exploiting microflora-activated and pH-controlled strategies revealed the validity of this double approach [9].

Most of the colon-specific drug delivery systems developed so far are single-unit systems. On the contrary, multi-particulate systems can offer several advantages compared to single dosage forms, for example more reliable drug release profiles and less local irritation [10]. In particular, multiple-unit systems based on such specifically biodegradable polymers showed to quickly spread out on their arrival to the colon, with a sharp increase of surface area exposed to bacterial breakdown that produces a rapid drug release and thereby improves drug absorption [11]. Drug delivery systems based on calcium pectinate beads have been recently investigated for specific-targeting drugs to the colon [12]. Such systems, obtained by pectin gelatinization in the presence of calcium salts, are less water-soluble than natural pectins, since calcium ions induce non-covalent associations of carbohydrate chains through the formation of the so-called ''egg box complexes'' [13] but they maintain the selective biodegradation by pectinolytic enzymes of colonic bacteria microflora [14,15]. However, a suitable coating of calcium pectinate beads seems to be all the same advisable to prevent drug release during the transit through stomach and small intestine [6,15,16]. Moreover, it has been shown that several formulation factors can affect the drug delivery behaviour from these systems, such as the pectin degree of esterification (DE) and of amidation (DA), the amount of calcium ions in the formulation and the cross-linking time [13,17,18].

Taking into account all these considerations, the aim of the present work was to develop and evaluate the potential of enteric-coated calcium pectinate microspheres as a colon-targeted delivery system. Theophylline was used as model drug since it is well absorbed in the large intestine in humans [19] and both its anti-asthma activity and pharmacokinetic properties make it an interesting candidate for such kind of modified-release preparations. The influence of pectin type, calcium ion concentration and cross-linking time on both drug entrapment efficiency and drug release pattern was investigated. The effect of varying the level of the pH-dependent coating polymer (Eudragit® S100) was also evaluated. In vitro release tests were performed in sequential buffer solutions simulating the physiological pH variation throughout the gastrointestinal tract, in the presence or not of pectinolytic enzymes in the simulated colonic medium, in order to investigate the influence of these enzymes on drug release rate. The morphology of microsphere batches, both freshly prepared and at different storage times, was characterised by Scanning Electron Microscopy. Differential Scanning Calorimetry, thermogravimetry, X-ray diffractometry and FTIR analyses were performed to characterize the microspheres in the solid state and to evidence possible interactions and/or incompatibilities between components.

2. Materials and methods

2.1. Materials

Theophylline (TP) was supplied from Aldrich Chemicals (Italy), Pectin Classic at low methoxylated grade ($36\% > DE > 44\%$, AU701) and Pectin Amid, amidated ($19\% > DA > 23\%$) at low methoxylated grade ($25\% > DE > 31\%$, CF020) were a generous gift from Herbstreith & Fox (Neuenbűrg, Germany). Eudragit® S100 (methacrylic acid copolymer soluble at pH 7) was kindly provided by Röhm Pharma GmbH (Darmstadt, Germany). Calcium chloride (solubility 74% w/v) was supplied by Carlo Erba (Italy). Pectinolytic enzymes extracted from *Aspergillus niger* and having an activity of 26,000 PG/mL at pH 3.5 (Pectinex Ultra SP-L) were kindly granted from Novo Nordisk Ferment (Dittinger, Switzerland). All other materials used were of analytical reagent grade.

2.2. Preparation of microspheres (MS)

Pectins were homogeneously dispersed in 10 mL of distilled water using an high-speed stirrer. Preliminary experiments showed that different final concentrations of AU701 (2% w/v) and CF020 (8% w/v) were necessary to obtain the desired viscosity value (0.16 cP at 25 °C) suitable for the following dropping phase. Two hundred and fifty milligrams of TP was then added under stirring. The dispersions were dropped through a nozzle with an inner diameter of 0.7 mm into a 500-mL room-temperature solution of calcium chloride (at a concentration ranging from 2.5% to 30% w/v) maintained under gentle stirring. Microspheres (MS) were instantaneously produced by ionotropic gelation. The MS formed were allowed to stand in the solution for different cross-linking times (from 5 to 20 min), and then separated from the reaction mixture by filtration, washed with distilled water, and dried at 50 °C in an air-circulated oven for 24 h. MS batches were stored 6 months at room temperature (25 °C) in a desiccator and/or at ambient relative humidity (RH 70–80%) in order to investigate the possible effect on their stability of different storage conditions. The mean diameter of representative MS samples of each batch was determined with a caliper.

2.3. Determination of microspheres (MS) water content

Each MS batch was weighed (Mettler M3 microbalance) before and after drying (50 °C for 24 h) and the mean water loss (WL) calculated according to the following equation:

$$WL\% = [(W_O - W_D)/W_O] * 100 \quad (1)$$

where W_O is the initial weight of the batch measured just after filtration and W_D is the weight after drying.

2.4. Entrapment efficiency

Percent of entrapment efficiency (EE%) of TP was indirectly measured by spectrophotometric assay at 266 nm (UV–Vis 1601 Shimadzu) of the residual drug content in the aqueous solution after separating and washing MS, according to the following equation:

$$EE\% = [(Qt - Qr)/Qt] * 100 \quad (2)$$

where Qt is the drug content initially added during the batch preparation and Qr is the sum of the drug content recovered in the aqueous solutions after separating and washing MS.

2.5. Coating of microspheres (MS)

The enteric coating solution was prepared by dissolving Eudragit® S100 in acetone at 12% w/v. This solvent was selected based on preliminary experiments which showed that it allowed complete dissolution of the enteric polymer while maintaining the integrity of MS. Coating was obtained by immersion of MS in the coating solution followed by solvent evaporation in a rotary evaporator. The process was repeated until the desired amount of coating was achieved. MS were coated at different levels (weight increase ranging from 5% to 100% w/w). Samples of coated MS were then dried and weighed and the mean coating weight calculated by difference with respect to the initial MS weight (Mettler M3 microbalance). The mean diameter of representative MS samples of each batch was estimated with a caliper. The mean coating thickness was determined by difference between the mean diameter of MS after and before the coating.

2.6. Morphological characterization of microspheres (MS)

Photographs of each freshly prepared batch were taken with a Canon Power Shot A60 digital camera with 1600×1200 pixel of magnification. The morphology, surface appearance, and inner structure of MS were examined more in depth by Scanning Electron Microscope analysis (SEM Philips XL-30 equipped with an image analysis system) of samples of the different uncoated and coated batches, both freshly prepared and at different time intervals during their storage. Prior to examination, the samples were fixed on a brass stub using double-sided tape and sputter coated with gold–palladium under argon atmosphere (to render them electrically conductive) using a gold sputter module in a high vacuum evaporator. The photographs were then taken using an excitation voltage of 20 kV. The magnifications selected were sufficient to appreciate in detail the morphology of the samples under study.

2.7. Solid-state studies

Fourier-Transform Infra-Red (FTIR) spectroscopy, Differential Scanning Calorimetry (DSC), thermogravimetry (TGA) and X-ray powder diffractometry (XRPD) analyses were carried out in order to investigate the possibility of any interactions between the components and to carefully characterize the MS physical–chemical properties. All solid-state studies were performed on pure components, their simple physical mixtures (in the same w/w ratios as in the MS) and final MS.

FTIR spectra were obtained as Nujol dispersion using a Perkin–Elmer Mod. 1600 apparatus in the 4000 to 400 cm^{-1} region. MS were softly ground before analysis.

DSC analyses were performed with a Mettler TA4000 apparatus equipped with a DSC 25 cell, on 5–10 mg samples (Mettler M3 microbalance) scanned in pierced Al pans at 10 °C min^{-1} between 30 and 300 °C under static air.

Thermogravimetric analyses were carried out on a Perkin-Elmer Pyris 1 TGA apparatus at the heating rate of 20 °C min^{-1} on 10–15 mg samples in open Pt crucibles in the 30–400 °C temperature range, under 20 mL min^{-1} nitrogen purge. In both DSC and TGA analyses, MS were placed in pans as such.

Powder X-ray diffraction patterns were taken using a Bruker D8-advance X-ray diffractometer, with Cu-Kα radiation, voltage 40 kV, current 40 mA, 2–25° 2θ range, scan rate 1° 2θ min^{-1}. MS were softly ground before performing analysis.

2.8. In vitro release studies

In vitro release studies of MS were performed according to the dispersed amount method [20] at 37 ± 0.5 °C and at a stirring rate of 100 rpm. Dissolution medium was varied according to the following sequence, in order to simulate pH conditions in the gastrointestinal tract: 2 h, pH 1.1 0.1 M HCl solution (artificial gastric juice); 2 h, pH 6.8 phosphate buffer (artificial small intestinal fluid); 20 h, pH 7.4 phosphate buffer (artificial intestinal fluid) in the absence or presence of 0.375 mL pectinolytic enzymes, added to evaluate the effect of pectin breakdown brought about *in vivo* by colonic bacteria [9,17]. MS were added in the opportune amount to obtain the final TP concentration of 10 mg l^{-1}, in order to maintain sink conditions (the drug solubility in phosphate buffer at pH 6.8 was determined to be 6.8 g/l). At time intervals, samples were withdrawn with a syringe filter (pore size 0.45 μm) and spectrometrically assayed (UV–Vis 1601 Shimadzu) for drug content at 266 nm. The other components of MS did not interfere with the drug assay. A correction was calculated for the cumulative dilution caused by replacement of the sample with an equal volume of original medium. Each test was repeated four times (coefficient of variation, CV < 1.5%).

3. Results and discussion

3.1. Microsphere characterization

In an initial step, the effect of varying Ca^{2+} concentration in the cross-linking solution on the MS properties

was evaluated, by keeping the cross-linking time constant at 20 min (Table 1). As observed also by other authors [21], the amount of water retained by MS (which can be considered as an index of their water affinity) depended on both the pectin type and counter-ion concentration. In particular, the growing number of intermolecular cross-links formed between carboxyl groups of pectin and counter ions with increasing Ca^{2+} concentration gave rise to less water-permeable MS. This effect was more marked for CF020 than for AU701 pectin, as can be deduced from their water loss values after drying, which decreased, respectively, from 90.7% to 67.3% and from 97.1% to 80.3% by varying the Ca^{2+} concentration from 2.5% to 30% w/v. This may be due to the presence of amide groups on CF020 pectin chains, which reduces the hydrophilicity of pectin and improves its gel forming power [6,21].

The higher hygroscopicity of AU701-based MS was confirmed by TGA data. For example, a 15% w/w mass loss in the 30–120 °C range, associated to water evaporation, was found for MS with non-amidated pectins prepared with a $CaCl_2$ concentration of 20% w/v, with respect to a 2% w/w mass loss recorded, in the same temperature range, for the corresponding amidated ones.

As for the drug entrapment efficiency, it was influenced by Ca^{2+} concentration only in the case of AU701 pectin. This finding could be attributed to the higher number of free carboxyl groups present in non-amidated pectins, able to interact with the counter-ions and thus to positively affect drug entrapment.

Moreover, it was also evidenced that a greater concentration of counter-ions gave rise to MS with more regular shape and morphology. This effect was particularly marked for beads realized with AU701, as can be seen in Fig. 1, where the photographs of MS obtained with the two pectins in the presence of 2.5% (A–A′) and 20% w/v (B–B′) of calcium chloride are shown.

It has been shown that the relationship between Ca^{2+} concentration and bead properties such as EE% and particle size can be affected by several parameters, such as in particular the kind of drug and pectin and the pectin-to-calcium ratio [21–23]. Therefore, we considered it worthy of interest to continue our investigation by evaluating more in depth the role of Ca^{2+} ions on the physicochemical properties, entrapment efficiency and morphological characteristics of the obtained MS. With this aim we selected a series of MS batches (Table 2) that could enable to assess: (a) the effect of Ca^{2+} concentration for a given pectin type (CF020) and a given cross-linking time (20 min) (batches CF_1–CF_4); (b) the effect of the pectin type (AU701 or CF020) for a given Ca^{2+} concentration (20%w/v) and cross-linking time (20 min) (batches AU_1 and CF_4); (c) the effect, for a given Ca^{2+} concentration and a same pectin, of different cross-linking times (5 or 20 min) (batches AU_2–AU_3, and batches CF_4–CF_5, respectively). The selected MS beads are reported in Table 2, with their characteristics of mean weight, diameter and EE%. As expected, the increase in Ca^{2+} concentration (by keeping the cross-linking time constant) gave rise to heavier and larger beads, even though this effect, at least in the case of CF020 pectin, was not related to the drug entrapment efficiency [22]. On the other hand a comparison of the properties of beads obtained with the two different pectins (AU701 or CF020) using a same Ca^{2+} concentration (20% w/v) and cross-linking time (20 min) enabled to exclude a role of the pectin type on the drug entrapment efficiency, since no appreciable differences were observed for their EE% values. On the contrary, a marked increase of EE% was found for both kinds of pectins by reducing the cross-linking time from 20 to 5 min, for a same Ca^{2+} concentration. The inverse relationship found between EE% and the cross-linking time was attributed to the high aqueous solubility of the drug, which is then prone to diffuse outside the beads during the MS production. On the other hand, 5 min of cross-linking time was found to be the minimum time necessary to allow complete MS formation.

The morphology of freshly prepared MS was investigated by SEM analysis (Fig. 2). AU701 MS showed irregular spherical shapes (A) and a rugged surface (C), while the CF020 ones appeared as more homogeneously round particles (B), with a smooth surface (D), as observed also by other authors [16,24]. No crystals of drug were visible on the MS surface, indicating their complete incorporation within the polymeric matrix.

All the prepared MS batches maintained unchanged their organoleptic properties and the drug release behaviour if kept in a desiccator at room temperature (25 °C) until their use. Some physical stability problems were instead observed during storage of MS kept at the same temperature, but under ambient humidity conditions (RH 70–80%). In particular, MS prepared with AU701 completely deliquesced and lost their morphology and consistency a few days after their preparation. This behaviour was attributable to the higher hygroscopicity of calcium pectinate MS prepared with non-amidated pectins. In fact, the greater water loss observed during the drying process (see Table 1) and the higher water content found by TGA analysis seem to be related to the higher water affinity and then greater tendency of the polymer to capture atmospheric humidity. Also CF020 MS presented some alterations in their organoleptic properties during storage under ambient conditions, but in this case the process was more gradual and slower and it was possible to

Table 1
Water loss percent (WL%) and entrapment efficiency (EE%) of microspheres (MS) prepared with increasing Ca^{2+} concentration (cross-linking time 20 min)

$[Ca^{2+}]$ % w/v	WL%		EE%	
	AU701	CF020	AU701	CF020
2.5	97.1	90.7	46.2 ± 2.0	59.8 ± 3.0
5	95.7	87.6	47.7 ± 1.5	55.3 ± 1.6
10	91.6	81.6	48.8 ± 1.3	56.4 ± 1.8
20	82.7	68.5	59.1 ± 1.6	59.2 ± 1.3
30	80.3	67.3	68.8 ± 1.4	60.2 ± 1.1

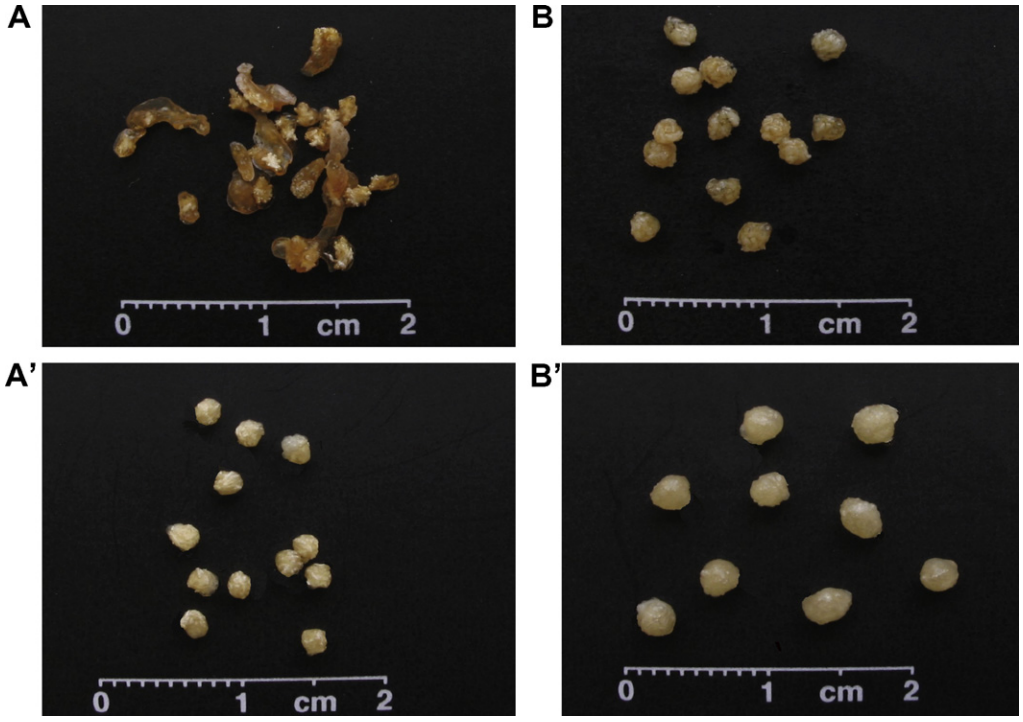

Fig. 1. Photographs of microspheres realized with AU701 pectin (top) or CF020 pectin (bottom) and 2.5% w/v (A–A′) or 20% w/v (B–B′) of calcium chloride.

Table 2
Mean weight, diameter and drug entrapment efficiency (EE%) of selected microspheres (MS) prepared at different Ca^{2+} concentration and different cross-linking times

Batch	Pectin type	$[Ca^{2+}]$ (% w/v)	Cross-linking time (min)	Weight (means ± SD) (mg)	Diameter (means ± SD) (mm)	EE (means ± SD) (%)
AU_1	AU701	20	20	4.04 ± 1.03	1.74 ± 0.32	59.1 ± 1.6
AU_2	AU701	30	20	6.59 ± 1.08	2.17 ± 0.41	68.8 ± 1.4
AU_3	AU701	30	5	5.98 ± 1.36	1.90 ± 0.37	80.9 ± 1.1
CF_1	CF020	2.5	20	3.69 ± 0.29	1.75 ± 0.17	59.8 ± 3.0
CF_2	CF020	5	20	5.96 ± 0.53	2.19 ± 0.22	55.3 ± 1.6
CF_3	CF020	10	20	9.2 ± 1.07	2.35 ± 0.32	56.4 ± 1.8
CF_4	CF020	20	20	13.35 ± 2.1	2.61 ± 0.35	59.2 ± 1.3
CF_5	CF020	20	5	9.8 ± 3.37	2.37 ± 0.52	82.0 ± 1.1

monitor the morphologic changes by SEM analysis performed at different time intervals. The SEM images of CF020 MS, freshly prepared and after 1 or 2 weeks of storage at ambient conditions, are shown in Fig. 3. The progressive morphologic modifications and the loss of the original spherical shape of the MS are evident and seem to be generated by the formation of filaments that fray the structure (Fig. 3D). These morphological changes could be attributed to calcium pectinate depolymerization phenomena [25], but this hypothesis needs to be verified by further investigations. No problems of drug chemical stability were detected as demonstrated from 100% drug recovery by UV analysis of dissolved MS. However, even though the observed MS morphologic changes did not affect the drug chemical stability, they prevented their correct use. In fact, as expected, release experiments from "modified" MS showed a very faster drug release rate in comparison with the "unmodified" ones, reaching 100% after only 60 min, as a consequence of the quick flaking and shattering of the polymeric matrix.

Therefore, taking into account these findings, the coating process was necessary not only to obtain a proper lag time before the beginning of drug release but also to improve the MS stability during the storage.

3.2. Solid-state studies

FTIR spectra of pure drug and AU701 pectin, their physical mixture, empty MS and TP charged MS are reported in Fig. 4. TP (a) presents its typical bands at 1717, 1670 and 1567 cm^{-1} [26], while the pectin shows a broad band between 3800 and 3250 cm^{-1}, due to OH stretching, a C=O vibration band of COOH groups at 1732 cm^{-1} and the asymmetric vibration stretching of

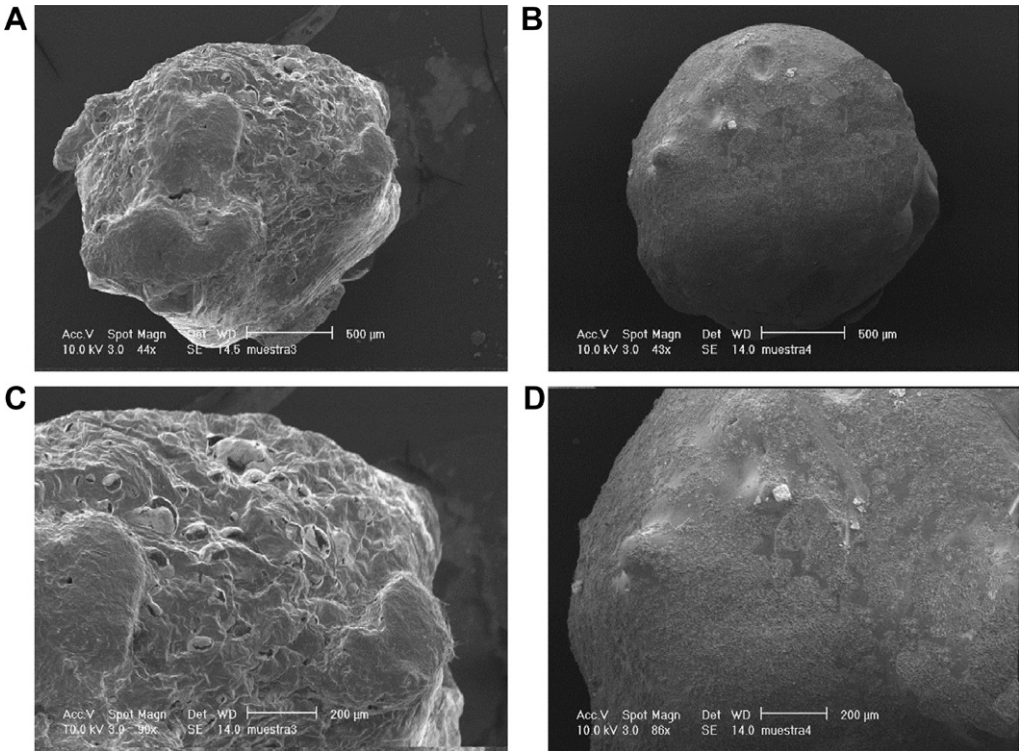

Fig. 2. SEM micrographs of microspheres realized with AU701 pectin (left) or CF020 pectin (right) and 20% w/v of calcium chloride at 40× (A,B) and 90× (C,D) magnification.

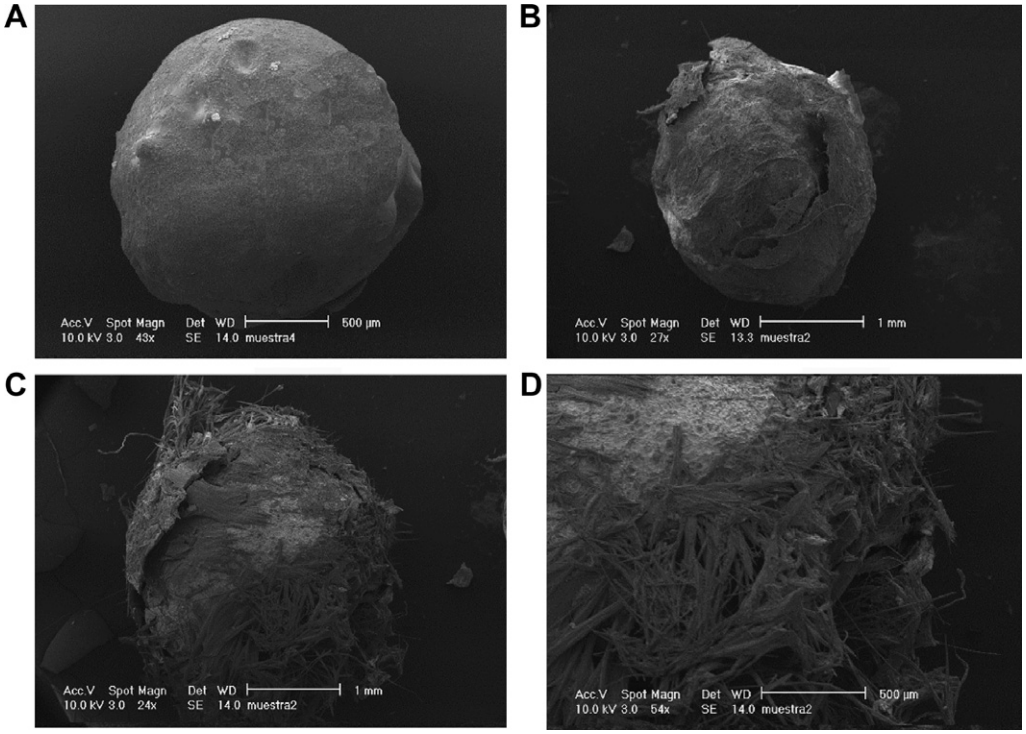

Fig. 3. SEM micrographs of CF020 pectin microspheres realized with 20% w/v of calcium chloride: freshly prepared (A) and after 7 days (B) or 15 days (C,D) of storage under ambient conditions.

COO^- at 1630 cm^{-1} (b). TP peaks were clearly observed, almost unchanged, in the physical mixture spectrum of the components (c). Spectra of MS, both empty (d) or charged with the drug (e), showed a strong reduction of the pectin band at 1732 cm^{-1}, which appears as a shoulder, and a shift to 1638 $cm^{-1}$of the carboxylate ion vibration

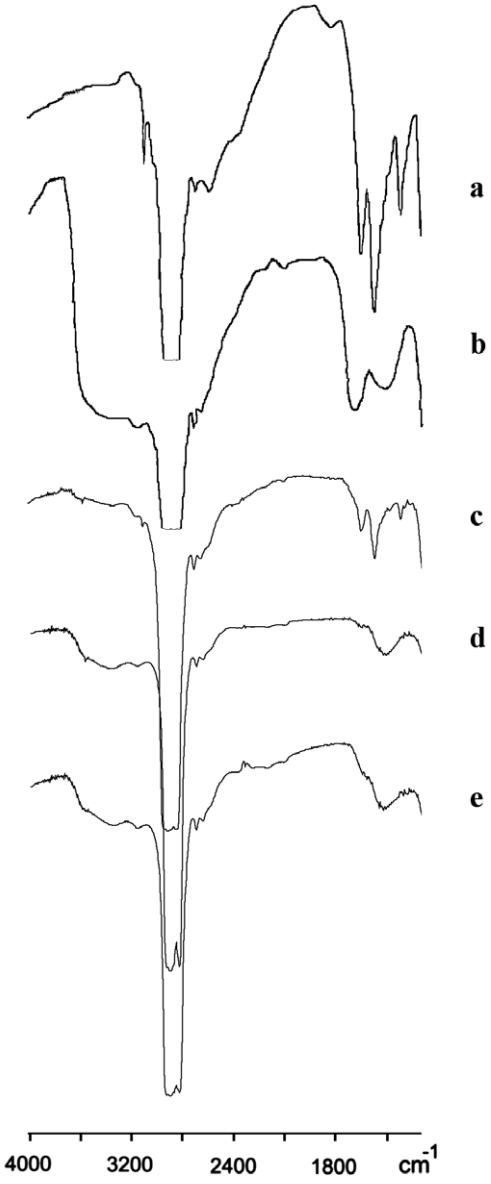

Fig. 4. FTIR spectra of theophylline (a), AU701 pectin (b), their physical mixture (pectin/drug ratio 1:1.25 w/w) (c), and empty (d) and drug-charged (e) microspheres.

stretching band, both indicative of calcium pectinate formation [27]. On the other hand, TP typical peaks were practically not detectable in the spectrum of charged MS (e). This finding, probably due to a loss of drug crystallinity in the formulation, did not allow exclusion of possible interactions between TP and Ca pectinate during MS preparation.

DSC and XRPD analyses were then performed to obtain some more information in this regard. The thermal curves of pure components, physical mixtures, empty and TP-charged MS realized with both AU701 and CF020 pectins are reported in Fig. 5. The thermal curve of TP exhibited a profile typical of a pure, crystalline, anhydrous drug, with a sharp endothermic peak ($T_{peak} = 270.0$ °C, ΔH_{fus} 149.1 J/g) due to its melting process. The DSC curve of

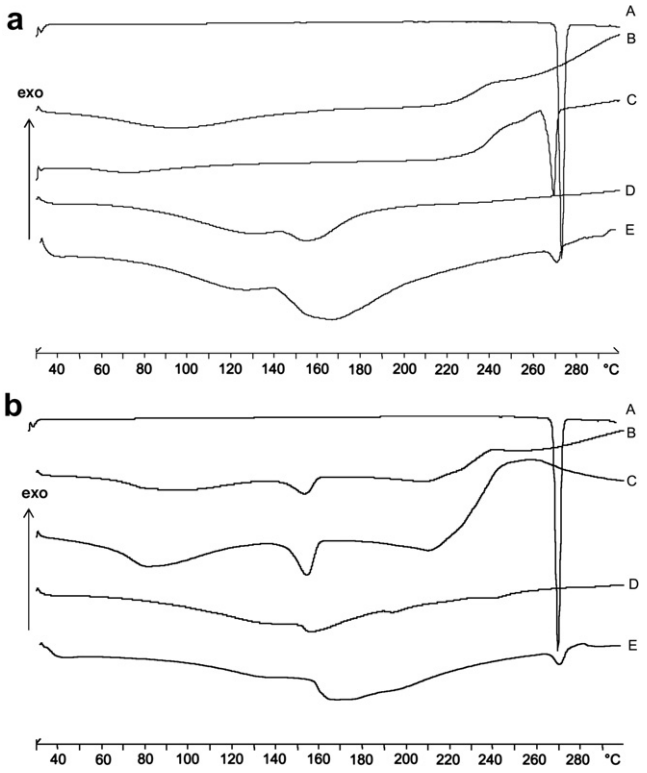

Fig. 5. DSC curves of (a): theophylline (TP) (A), AU701 pectin (B), their pectin:drug 1:1.25 w/w physical mixture (C), and empty (D) and drug-charged (E) microspheres; (b): theophylline (TP) (A), CF020 pectin (B) their pectin:drug 3.2:1 w/w physical mixture (C), and empty (D) and drug-charged (E) microspheres.

unprocessed pectin AU701 showed a broad endothermic band, due to water loss, followed by an almost flat profile, indicative of its amorphous state, and, finally, by the starting of an exothermal effect attributable to decomposition phenomena. The thermal profile of unprocessed pectin CF020 revealed instead, after the dehydration band at around 80–100 °C, a peak at 152.7 °C, attributable to a melting phenomenon, followed by an exothermic event ascribed to a decomposition process [27]. The drug melting peak is still well visible in its physical mixture with AU701, and the little lowering of peak temperature and reduction of associated enthalpy variation ($T_{peak} = 269.2$ °C, $\Delta H_{fus} = 72.4$ J/g) observed are simply due to the mixing of the components. On the contrary, TP melting peak was not more detectable in its physical mixture with CF020, probably due to the strong exothermic effect associated to the pectin decomposition, that overlaps and masks the drug fusion peak. The thermal curves of empty MS prepared with both pectin types (d) were very similar, indicating calcium pectinate formation. In fact, in both cases the phenomenon of enthalpic relaxation, typical of calcium pectinate, appearing as a broad flex [25,27], is well visible, with very similar thermal data for both pectins ($T = 155.0$ °C for AU701 and $T = 150.6$ °C for CF020). Thermal curves of TP-charged MS (e) showed the same broad flex, which appears increased in intensity and shifted at higher temperature (165.0 °C). In these same profiles, the TP melting endo-

therm is still evident, peaked, respectively, at 267.75 °C (ΔH 34.3 J/g) for AU701 and at 270.71 °C (ΔH 31.4 J/g) for CF020, even though strongly reduced in intensity, indicating a marked decrease of crystallinity but not complete drug amorphization in both types of MS. These findings indicate that no solid-state interactions between TP and calcium pectinate or incompatibility problems occurred during MS preparation. In further confirmation, the TGA profiles of both empty or TP-charged MS were almost superimposable for both kinds of examined MS.

XRPD studies (Fig. 6) were particularly useful to support and supplement FTIR and DSC results. In particular, the typical crystallinity peaks of TP were clearly detectable in its physical mixtures with both pectin types, without appreciable changes in position and in relative intensities. This confirmed that the disappearance of the drug melting peak observed in the thermal curve of its physical mixture with CF020 was actually attributable to its masking due to the overlapping decomposition phenomena of the polymer and not to possible drug–polymer solid-state interactions and/or drug amorphization. On the other hand, a very strong reduction in intensity of the drug peak was observed in the case of the TP-charged MS, suggestive of a marked loss of crystallinity, in agreement with FTIR and DSC results.

3.3. Coating studies

Eudragit® S100 is a copolymer of methylacrylic acid and methylmethacrylate containing about 30% of methacrylic acid units that tends to dissolve at pH 7. For this reason it is considered a suitable coating material for colonic drug delivery, able to adequately protect the drug core system during its passage through the stomach and small intestine [9]. The coating level was gradually varied from 5% to 100% (w/w) and its influence on the length of the lag time before the beginning of drug release was investigated by performing a series of preliminary release studies under pH-gradient (2 h at pH 1.1 and 2 h at pH 6.8), to simulate the gastrointestinal transit conditions. As shown in Table 3, the lag time increased with increasing the coating level and a 100% w/w of applied film was the minimum amount suitable for achieving colonic targeting, by preventing drug release in the gastric medium (pH 1.2) and assuring a drug release lower than 10% during the first 2 h in the enteric medium (pH 6.8).

The batches selected for the enteric-coating are reported in Table 4 with the relative characteristics of mean weight, diameter and coating thickness. By comparing these data with those of Table 2 it can be observed that a MS weight doubling corresponded to about one-third increase of its diameter, and that, in all cases, the final diameter increase never exceeded 1 mm.

SEM analysis of a section of a coated MS is reported in Fig. 7. Image (A) makes it possible to appreciate both the quality and the thickness of coating. Moreover, a magnification of a section (B) confirmed the presence of residual TP crystals within the polymeric matrix, as indicated by DSC and XRPD studies.

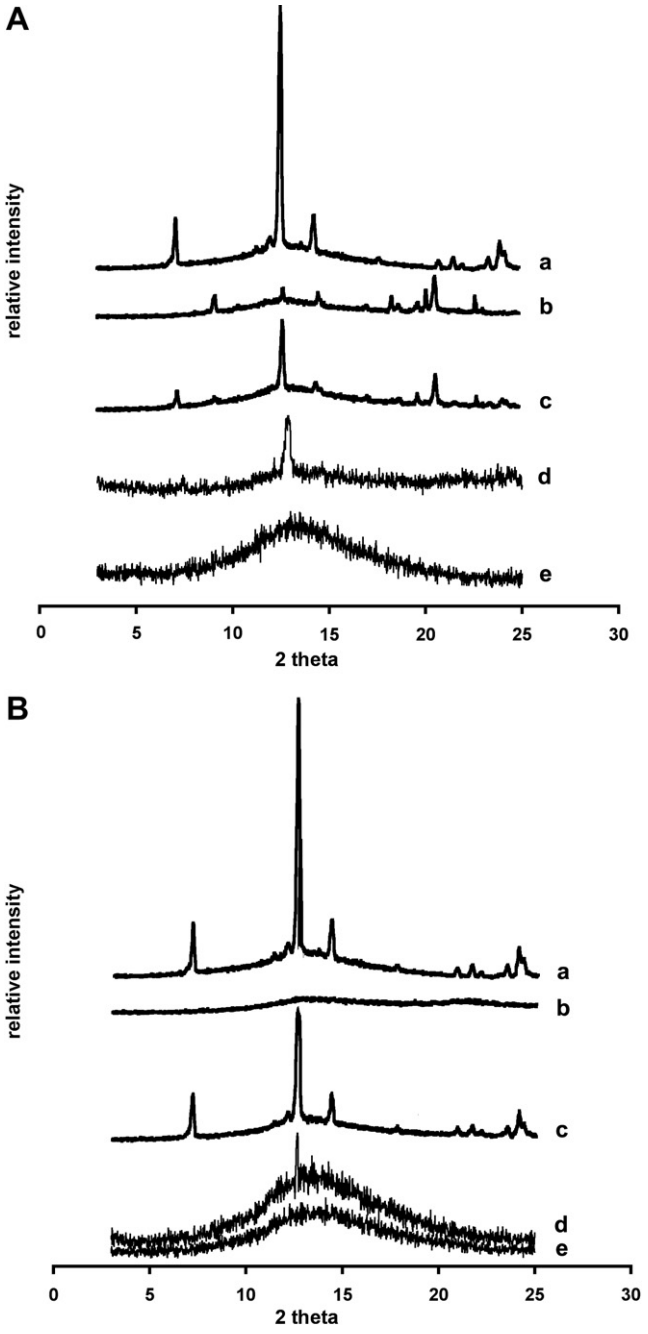

Fig. 6. X-Ray powder diffraction patterns of (A): theophylline (a), CF020 pectin (b), their pectin:drug 3.2:1 w/w physical mixture (c), and drug-charged (d) and empty (e) microspheres; (B): theophylline (a), AU701 pectin (b) their pectin:drug 1:1.25 w/w physical mixture (c), and drug-charged (d) and empty (e) microspheres.

3.4. Release studies

Release tests from the selected enteric-coated MS batches were performed under pH-gradient, by adding or not, in the simulated colonic medium, a commercially

Table 3
Percent of theophylline (TP) released at different times from CF020 microspheres coated with different levels of Eudragit S100®

Coating level (% w/w)	% TP released at different times				
	pH 1.1			pH 6.8	
	30 min	60 min	120 min	180 min	240 min
0	62.3	100			
5	34.6	72.1	100		
7	13.5	34.3	68.3	100	
15	14.3	28.2	55.4	75.1	100
33	2.1	4.3	19.1	22.1	24.5
60	0	0	3.4	8.2	16.1
100	0	0	1.1	4.2	7.5

available mixture of pectinolytic enzymes (Fig. 8). In the tests performed without enzymes (Fig. 8A) an inverse relationship between calcium ions concentration and drug release rate was found, as observed also by other authors [18,22]. This result is also in agreement with the reduction of MS water affinity (and then of water permeability) observed in preliminary studies by increasing Ca^{2+} concentration (see Table 1). Higher calcium concentration leads to a greater degree of cross-linking and aggregation of pectin molecules, inducing higher gel strength and limiting swelling patterns, thus resulting in a subsequent slowing of drug release rate [13,28]. On the contrary, variations of cross-linking time (from 20 to 5 min) did not appreciably affect drug release rate. This result confirmed our previous hypothesis that the higher EE% values found by decreasing the cross-linking time were simply due to the shorter time available for the hydrophilic drug to diffuse out during the MS formation, and not to some possible changes in the MS structure. Also the pectin type (amidated or non-amidated) did not appreciably affect drug release behaviour, the Ca^{2+} concentration being equal.

Unexpectedly, subsequent release studies carried out in the presence of enzymes (Fig. 8B) did not evidence the expected marked improvement in drug release rate, as a consequence of the selective degradation of calcium pectinate. In contrast with some literature data [7,12,13], pectinolytic enzymes seemed to be not very effective in promoting the degradation of such MS. In fact, almost entire MS shells were recovered at the end of the release test (24 h) also in the presence of enzymes, as is shown in the window in Fig. 8B where the SEM photograph of a CF020 MS

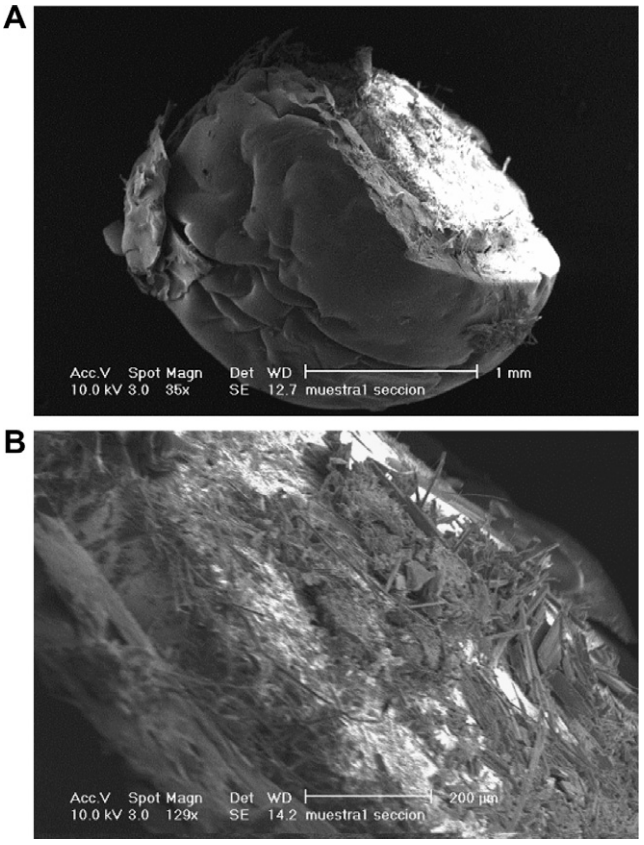

Fig. 7. SEM micrographs of a coated CF020 pectin microsphere (A) and a magnification of its section (B).

after the test is shown. This result cannot be attributed to the pH conditions of the dissolution medium (pH 7.4), which were different than those considered optimal for the activity of pectinolytic enzymes (pH 6). In fact, these same enzymes were effective, under the same pH conditions used in our experiments (pH 7.4), in producing a selective degradation of pectin matrix tablets [9]. Moreover, some other authors too did not find any effect of pectinolytic enzymes on calcium pectinate-coated pellets and beads introduced into enteric capsules [22,29]. Other authors observed that only a small amount of calcium ions actually take part in calcium pectinate matrix formation, while most of them remain in the formulation under free form [21]. Therefore, it could be hypothesized that the free calcium ions present in MS, due to their ability to reduce some

Table 4
Mean weight, diameter and coating thickness of selected batches of microspheres (MS)

Batch	Pectin type	$[Ca^{2+}]$ (% w/v)	Cross-linking time (min)	Weight (mean ± SD) (mg)	Diameter (mean ± SD) (mm)	Coating thickness (mean ± SD) (mm)	$[Ca^{2+}]$ (% w/v)
AU_1	AU701	20	20	9.48 ± 2.60	2.58 ± 0.42	0.83 ± 0.15	20
CF_1	CF020	2.5	20	8.72 ± 2.50	2.50 ± 0.40	0.75 ± 0.22	2.5
CF_2	CF020	5	20	13.01 ± 0.68	3.00 ± 0.45	0.81 ± 0.22	5
CF_4	CF020	20	20	27.51 ± 4.94	3.62 ± 0.35	1.00 ± 0.01	20
CF_5	CF020	20	5	20.77 ± 7.32	3.31 ± 0.50	0.94 ± 0.02	20

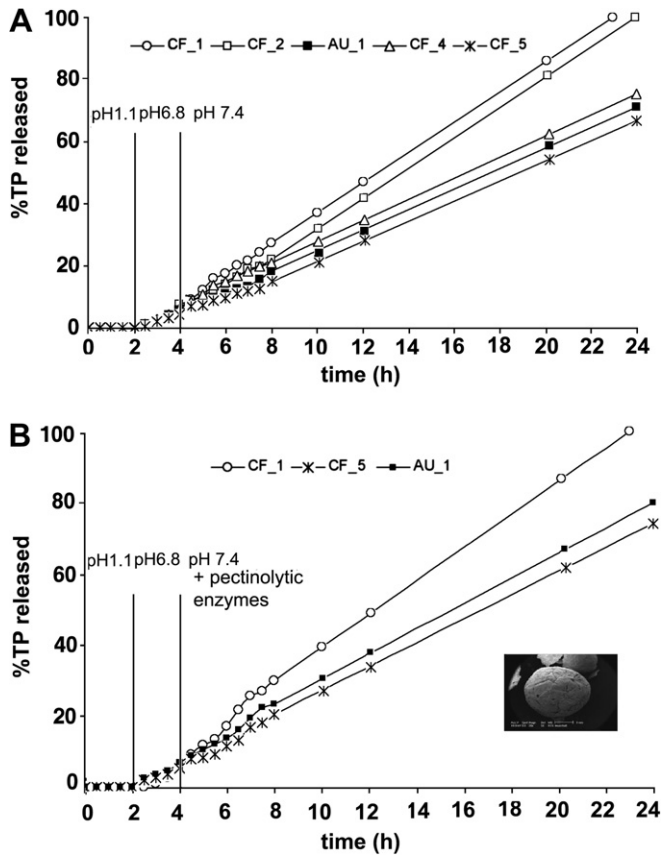

Fig. 8. Theophylline release profiles from coated microsphere (MS) batches (see Table 4) in the absence (A) or in the presence (B) of pectinolytic enzymes. In the window: SEM micrograph of a CF020 MS after the release test in the presence of enzymes.

enzymatic activities, could induce a lack of pectinolytic enzyme activity. In order to verify this hypothesis, enzymes were incubated for 24 h in the presence or not of $CaCl_2$ at 20% w/v. However, it was found that the presence of calcium ions does not block the enzymatic activity, but only gives rise to some slowing down in the degradation rate of the pectin polymeric matrix. Then, the presence of calcium ions plays a role but it cannot be considered the only factor responsible for the loss of enzymatic activity and further and more in-depth investigations will be necessary to fully elucidate the obtained results.

4. Conclusions

Enteric-coated Ca pectinate multiple-unit systems were realized to obtain a specific drug delivery to the colon. Ca pectinate MS prepared with CF020 low-methoxylated amidated pectins were better than those obtained with AU701 low-methoxylated pectins, for both their greater stability during storage (due to less sensitivity to ambient humidity) and more regular and homogeneous morphological properties. Moreover, the shape of CF020 MS was poorly influenced by variations of calcium concentrations, maintaining an almost homogeneous spherical form also at low concentrations of calcium ions. On the contrary, AU701 required at least 20% w/v of Ca^{2+} concentration for obtaining MS of satisfactory and well-reproducible morphological properties. This finding is of particular importance in the choice of the most suitable pectin type, considering that increasing calcium amounts gave rise to a decrease of the drug release rate.

Release tests performed under pH gradient and in the presence of pectinolytic enzymes in the simulated colonic medium revealed that the Ca-pectinate MS did not undergo the foreseen selective colonic-microflora triggered drug release mechanism. The observed slowing down effect of free calcium ions in the enzymatic degradation rate of the pectin matrix can only partially concur to explain the almost complete lack of activity shown by pectinolytic enzymes. However, in spite of this unexpected result, CF020 MS, realized with low calcium chloride concentrations (2.5% w/v) and coated with an appropriate thickness of a pH-dependent polymeric film (100% w/w), demonstrated to be suitable to adequately modulate drug release through a mixed approach of pH and transit-time control, completely avoiding drug release during the first 2 h in gastric ambient, limiting to less than 10% the release in the following 2 h, and reaching 100% release in the colonic simulated medium within less than 24 h.

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References

[1] M. Ashford, J.T. Fell, Targeting drugs to the colon: delivery systems for oral administration, J. Drug Target. 2 (1994) 241–257.
[2] L. Yang, J.S. Chu, J.A. Fix, Colon specific drug delivery: next approaches and in vitro/in vivo evaluation, Int. J. Pharm. 235 (2002) 1–15.
[3] M. Ashford, J.T. Fell, D. Attwood, P. Woodhead, An in vitro investigation into the suitability of pH-dependent polymers for colonic targeting, Int. J. Pharm. 91 (1993) 241–245.
[4] M.K. Chourasia, S.K. Jam, Pharmaceutical approaches to colon targeted drug delivery systems, J. Pharm. Pharmaceut. Sci. 6 (2003) 33–66.
[5] A. Rubinstein, Microbially controlled drug delivery to the colon, Biopharm. Drug Dispos. 1 (1990) 465–475.
[6] V.R. Sinha, R. Kumria, Polysaccharides in colon-specific drug delivery, Int. J. Pharm. 224 (2001) 19–38.
[7] T.F. Vandamme, A. Lenourry, C. Charrueau, J.C. Chaumeil, The use of polysaccharides to target drugs to the colon, Carbohydr. Polym. 48 (2002) 219–231.
[8] D.R. Friend, New oral delivery systems for treatment of inflammatory bowel disease, Adv. Drug Del. Rev. 57 (2005) 247–265.
[9] P. Mura, F. Maestrelli, M. Cirri, M.L. Gonzalez Rodriguez, A.M. Rabasco Alvarez, Development of enteric-coated pectin-based matrix tablets for colonic delivery of theophylline, J. Drug Target. 11 (2003) 365–371.
[10] J. Krämer, H. Blume, Biopharmaceutical aspects of multiparticulates, in: Y. Ghebre-Sellasie (Ed.), Multiparticulate Oral Drug Delivery, Marcel Dekker, New York, 1994, pp. 307–332.

[11] M. Rodriguez, J.L. Vila-Jato, D. Torres, Design of a new multiparticulate system for potential site-specific and controlled drug delivery to the colonic region, J. Control. Release 55 (1998) 67–77.
[12] L. Liu, M.L. Fishman, J. Kost, K.B. Hicks, Pectin-based systems for colon-specific drug delivery via oral route, Biomaterials 24 (2003) 3333–3343.
[13] P. Sriamornsak, Investigation of pectin as a carrier for oral delivery of proteins using calcium pectinate gel beads, Int. J. Pharm. 169 (1998) 213–220.
[14] A. Rubinstein, R. Radaï, M. Ezra, S. Pathak, J.S. Rokem, In vitro evaluation of calcium pectinate: a potential colon-specific drug delivery carrier, Pharm. Res. 10 (1993) 258–263.
[15] A. Rubinstein, R. Radaï, In vitro and in vivo analysis of colon specificity of calcium pectinate formulations, Eur. J. Pharm. Biopharm. 41 (1995) 291–295.
[16] O. Dupuis, C. Chambin, D. Génelot, Y. Champion, Y. Pourcelot, Colonic drug delivery: influence of cross-linking agent on pectin beads properties and role of the shell capsule type, Drug Dev. Ind. Pharm. 32 (2006) 847–855.
[17] Z. Wakerly, J. Fell, D. Attwood, D. Parkins, Studies on amidated pectins as potential carriers in colonic drug delivery, J. Pharm. Pharmacol. 49 (1997) 622–625.
[18] P. Sriamornsak, J. Nunthanid, Calcium pectinate gel beads for controlled release drug delivery. I: Preparation and in vitro release studies, Int. J. Pharm. 160 (1998) 207–212.
[19] A.H. Staib, D. Loew, S. Harder, E.H. Graul, R. Pfab, Measurement of theophylline absorption from different regions of the gastrointestinal tract using a remote controlled drug delivery device, Eur. J. Clin. Pharmacol. 30 (1986) 691–697.
[20] H. Nogami, T. Nagai, I. Yotsuyanagi, Dissolution phenomena of organic medicinals involving simultaneous phase changes, Chem. Pharm. Bull. 17 (1969) 499–509.
[21] S. Bourgeois, M. Gernet, D. Pradeau, A. Andremont, E. Fattal, Evaluation of critical formulation parameters influencing the bioactivity of β-lactamases entrapped in pectin beads, Int. J. Pharm. 324 (2006) 2–9.
[22] O. Chambin, G. Dupuis, D. Champion, A. Voilley, Y. Pourcelot, Colon-specific drug delivery: influence of solution reticulation properties upon pectin beads performance, Int. J. Pharm. 321 (2006) 86–93.
[23] S. Bourgeois, M. Gernet, D. Pradeau, A. Andremont, E. Fattal, Design and characterization of pectin beads for colonic delivery, in: Proceedings of the Fourth World Meeting Adritelf-APGI-APV, Florence, 2002, p. 805.
[24] P. Sriamornsak, N. Thirawong, Use of back-scattered electron imaging as a tool for examining matrix structure of calcium pectinate, Int. J. Pharm. 267 (2003) 151–156.
[25] V. Pillay, R. Fassihi, In vitro release modulation from crosslinked pellets for site-specific drug delivery to the gastrointestinal tract. II. Physicochemical characterization of calcium-alginate, calcium-pectinate and calcium-alginate–pectinate pellets, J. Control. Release 59 (1999) 243–256.
[26] Clarke's Analysis of Drug and Poison, third ed., Pharmaceutical Press, London, UK, 2004.
[27] S. Nurjaya, T.W. Wong, Effect of microwave on drug release properties of matrices of pectin, Carbohydr. Polym. 62 (2005) 245–257.
[28] S. Sungthongjeen, P. Sriamornsak, T. Pitaksuteepong, A. Somsiri, S. Puttipipatkhachorn, Effect of degree of esterification of pectin and calcium amount on drug release from pectin-based matrix tablets, AAPS Pharm. Sci. Technol. 5 (2004) 1–8.
[29] R. Semdè, K. Amighi, M.J. Devleeschouwer, A.J. Moës, Effect of pectinolytic enzymes on the theophylline release from pellets coated with water insoluble polymers containing pectin HM or calcium pectinate, Int. J. Pharm. 197 (2000) 169–179.