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Research Paper

Theme: Advanced Technologies for Oral Controlled Release
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Preparation and Characterization of Microcapsules Based on Biodegradable Polymers: Pectin/Casein Complex for Controlled Drug Release Systems

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Abstract. Controlled release of drugs is an important strategy to diminish the drug dose and adverse side effects. Aqueous mixtures of polysaccharides and proteins are usually unstable above a certain biopolymer concentration and phase separation occurs either because of repulsive (segregative) or attractive (associative) interactions. Herein, pectin/casein microcapsules were prepared by complex coacervation aiming at prolonged drug release. The morphological characteristics, particle size, distribution, and release kinetics of microcapsules were studied using as a model the hydrophilic drug acetaminophen. It was detected that complexation of pectin/casein particles occurs at pH values lower than 6, resulting in the formation of spherical particles after spray drying. Microcapsules had a mean diameter of 3.138 and 4.929 μm without drug, and of 4.680 and 5.182 μm with drug using USP and 8003 pectin, respectively. The *in vitro* release of acetaminophen from microcapsules was slow and the drug release mechanism was controlled by diffusion following first-order kinetics. There was greater release of acetaminophen in simulated gastric fluid than simulated intestinal fluid conditions. Concluding, the polymeric system present herein seemed to be appropriate for a prolonged release of acetaminophen throughout the gastrointestinal tract. Nevertheless, it is likely that it is a promising pectin/casein complex for lipossoluble drugs, which merits further investigation.

KEY WORDS: casein; complex coacervation; microcapsules; pectin; release kinetics.

INTRODUCTION

In recent years, the research on materials obtained from alternative renewable natural sources has markedly increased. Examples of those materials are gelatin (1), dextran (2), collagen (3), chitosan (4), poly(lactide) (5) pectin (6,7), and casein (8,9). As a consequence of having novel materials, there is also constant knowledge improvement on the functional characteristics and applicability of such natural polymers.

Natural polymers such as casein and pectin are proteins and hydrogel-forming polysaccharides, respectively, which receive special attention because they are biodegradable polymers (10,11). An additional advantage of pectin and casein is that they have no toxic effects (11). There is incompatibility of pectin and casein in aqueous solution at pH above 6.5 (12). On the other hand, at low pH values, pectin has been used to stabilize acidified dairy beverages due to pectin adsorption onto casein micelles, preventing the aggregation of casein micelles during the acidification process. Much has been published about the interaction of pectin with casein micelles in acidified milk drinks (11,13–15), but very few studies were performed on pectin/casein mixtures at low pH in calcium-free aqueous solutions (16,17).

Because of the wide applicability of polysaccharide/protein mixtures, the interest in identifying interactions between them has been growing aiming to provide optimum food quality in terms of texture and stability, and also innovative products in cosmetic and pharmaceutical industries (7).

The development of controlled-release systems depends on polymers with appropriate physicochemical characteristic and on effective and economically viable large-scale processes. Among these systems, the microparticulate one has been studied in order to control and/or obtain site-specific release (18), with a strong tendency of applying biodegradable polymers and preparations in aqueous medium (19,20).

Microparticulate systems can be prepared using chemical (21,22), physicochemical (23,24), and mechanical (25,26) processes. Microencapsulation by complex coacervation is accomplished

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by phase separation of one or many hydrocolloids from the initial solution and the subsequent deposition of the newly formed coacervate phase around the active ingredient suspended or emulsified in the same reaction media (27). Microcapsules produced by complex coacervation are water insoluble, possessing excellent controlled-release characteristics and heat-resistant properties (28). The factors influencing the rate of release include drug particle size, loading, drug solubility, and molecular weight and polymer composition (29).

The present study reports an *in vitro* investigation of the potential of interpolymer complex consisting of polysaccharide (pectin) and protein (casein) mixtures to develop microcapsules as a model of multiple-unit system for prolonged drug delivery throughout the gastrointestinal tract. Morphological characteristics of microcapsules and release kinetics studies of acetaminophen microcapsules of polymeric complex were determined. Acetaminophen was selected because of its low molecular weight and high water solubility representing the worst possible scenario to be tested.

MATERIALS AND METHODS

Materials

The following materials were obtained from the indicated sources: pectin USP (68% of esterification) and pectin 8003 (36% of esterification and 14% of amidation) from CPKelco (Limeira, SP, Brazil); casein from Katuffman & Co (Germany); glutaraldehyde (2.6 M) from Fluka Chemicals (USA); citric acid, maleic acid, ethanol, sodium hydroxide, chloride acid, and sodium phosphate from Merck (analytical grade, Germany); pectinex® Ultra SP-L from Novozymes Latin America Limited (Brazil); and acetaminophen from Sigma Chemicals (USA).

Preparation of Microcapsules

Microcapsules were prepared by dispersion in distilled water (solid content 10%, *w*/*v*) of the polymers pectin and casein under constant mechanical shaking. Sodium hydroxide (4.0 M) was used to adjust pH to 8.0±0.1. After complete dispersion, acetaminophen (test drug) was added at the proportion of 1:1 (polymer/drug). Microcapsules were obtained by slow and gradual reduction of pH from 8.0±0.1 to 3.0±0.1 with 1.0 M citric acid or 1.0 M maleic acid. The microcapsules wall was then hardened by the addition of glutaraldehyde (50 μl/g polymer), with constant shaking for an additional 30 min (30). The same methodology was used to prepare microcapsules containing no drug, *i.e.*, empty microcapsules (Table I). The sample was then spray dried with a laboratory scale spray (Lab Plant, model SD-05) using a double-fluid type atomizer nozzle with external mixture and an outlet orifice of 0.7 mm. The process parameters were as follows: inlet air temperature was kept constant at 100°C and the outlet temperature was at 85°C; feed rate was 3 ml/min and airflow was 600 ml/h.

Scanning Electron Microscopic Study

The spray-dried products were coated under argon atmosphere with gold/palladium and examined under a scanning electron microscope (JOEL JSM-T330A). Pictures were taken at ×3,500 magnification. The scanning electron photomicrographs were evaluated.

Particle Size Analysis and Distribution

The samples of microcapsules were analyzed using a LEICA DMRXA optical microscopy with Leica *Qwin Image Analysis System*. Briefly, particles were placed on glass slide and the size measurements of microcapsules were performed using Feret's diameter as parameter, followed by particle size distribution estimation.

Determination of Encapsulation Efficiency and *In Vitro* Dissolution Studies

To determine the encapsulation efficiency, 50 mg of microcapsules (Table I, formulation E, F, G, and H) containing 25 mg of acetaminophen were dispersed in 4 ml of ethanol and then stirred for 5 min, followed by centrifugation at 4,000×*g* for 10 min. The supernatant was filtered through a 0.45 μm nylon syringe filter and the filtrate was analyzed by spectrophotometer at 243 nm to determine drug content. Encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulation efficiency}(\%\text{EE}) = (\text{initial drug added} - \text{free drug}/\text{initial drug added}) \times 100 \quad (1)$$

Dissolution studies of microcapsules (Table I, formulation E, F, G, and H) were performed by measuring the percentage of acetaminophen remaining within the environment at a predetermined sampling time. Drug release patterns were studied during 10 h using USP Apparatus 1 at a rotational speed of 50 rpm and microcapsules weighting the equivalent to 300 mg of acetaminophen. During the first 2 h 375 ml of HCl 0.1 M at pH 1.2±0.05 at 37°C were used to simulate the gastric fluid (SGF) conditions. In the next 4 h, 125 ml of phosphate buffer (Na3PO4 0.2 M) were added to those initial 375 ml of SGF to simulate intestinal fluid (SIF) conditions reaching pH 6.8±0.05. Then after 6 h, 1 ml of pectinolytic enzymes was added in SIF medium as previously reported (24). The results were expressed as mean (±SEM) of three determinations. At the indicated time points, samples were collected and immediately filtered through 0.45-μm Millipore filter paper. Each sample was analyzed spectrophotometrically at 243 nm to determine drug content.

To determine the mode of acetaminophen release by the microcapsule, release data were analyzed using the following mathematical models: zero-order kinetic (Eq. 2); first-order kinetic (Eq. 3); Higuchi equation (square root of time equation; Eq. 4) (31).

$$Q = k_0 t \quad (2)$$

$$\ln(100 - Q) = \ln(Q_0) - k_1 t \quad (3)$$

$$Q = k_H t^{1/2} \quad (4)$$

Table I. Microcapsules Formulations

Formulation	Amount of						
	Pectin GENU® USP (%)	Pectina GENU® 8003 %	Casein (%)	Acetaminophen %	Citric acid 1.0 M	Maleic acid 1.3 M	Glutaraldehyde 50 µl/g of polymer
A	5	–	5	–	×	–	×
B	5	–	5	–	–	×	×
C	–	5	5	–	×	–	×
D	–	5	5	–	–	×	×
E	5	–	5	10	×	–	×
F	5	–	5	10	–	×	×
G	–	5	5	10	×	–	×
H	–	5	5	10	–	×	×

In equations, Q is the percentage of drug released at time t; Q_0 is the percent of drug remaining to be released at time 0, and k_0, k_1, and k_H are the coefficients of the equations.

Statistical Analysis

Data were statistically analyzed by one-way ANOVA, followed by Bonferroni's multiple comparisons t test to evaluate the microcapsule size and the constants of kinetic in different mathematical models. Differences were considered significant when $P<0.05$ was obtained. Statistical analyzes were performed using GraphPad Prism® 4.0 software.

RESULTS AND DISCUSSION

Preparation of Microcapsules

In this study, it was investigated the effect of pectin/casein complex polymer preparation by complex coacervation in the obtainment of spherical particles. The process to obtain such particles is performed under mild conditions (*e.g.*, absence of organic solvents), thus, it is of great pharmaceutical interest. The first step was to identify the experimental conditions at which pectin/casein complex would be formed. The complex coacervation of proteins with polysaccharides in aqueous environment occurs by electrostatic interaction between the polysaccharides and the aminogroups of proteins at pH values below the isoelectric point (pI) of polysaccharides (pI_{po}~3.0) and above the pI of the proteins (pI_{pr}~4.55; $pI_{po}<pH<pI_{pr}$; 14,32–34).

As shown in Figs. 1 and 2, pectin/casein complexation occurred at pH below 6 as well as those polymers alone did not form complex coacervation. Complexation occurred since casein becomes significantly positively charged at pH below 6 and attracts the negatively charged pectin molecules, resulting in the formation of pectin/casein particles. These results corroborated previous reports (7).

Scanning Electron Microscopy

The photomicrographs of the microcapsules are shown in Figs. 1 and 2. Pectin GENU® USP/casein microcapsules presented spherical forms with depressions (Fig. 1a, b) while pectin GENU® USP/casein microcapsules containing acetaminophen presented irregular surfaces, showing smaller particles attached or adsorbed on the external surface, and increased size without surface depressions (Fig. 1c, d).

The pectin GENU® 8003/casein microcapsules presented heterogeneous particle size, spherical shape and surface depressions (Fig. 2a, b). The surface depressions could be caused by the drying process (6). The Fig. 2c and d show the acetaminophen loaded microcapsules. The addition of citric acid (Fig. 2c) resulted in microcapsules with spherical shape, without agglomerate formation, uniform particle size, and absence of depressions. The drug load could be an explanation for the absence of depressions. On the other hand, the addition of maleic acid resulted in microcapsules with irregular surface and mainly coalescent particles (Fig. 2d). The spherical form and absence of depressions was similar to citric acid addition.

Particle Size Analysis and Distribution

In the histograms of particle size distribution frequency (%) of formulations not loaded with acetaminophen, it was observed that formulations prepared with the conjugate pectin GENU® USP/casein with citric acid or maleic acid, the mean particle diameter were 3.138±1.489 µm and 3.423±1.675 µm (Table II), respectively, and approximately 82% were between 2.0 and 4.0 µm (Fig. 3a, b). Concerning the pectin GENU® 8003/casein conjugates with citric acid or maleic acid, the mean particle size was 4.929±2.072 and 4.602±2.321 µm (Table II), respectively, and approximately 60% were between 4.0 and 5.0 µm (Fig. 4a, b). Therefore, the particles prepared with pectin GENU® USP/casein were smaller than those prepared with pectin GENU® 8003/casein.

The acetaminophen loading to formulations resulted in an increase of homogeneity and particle size for pectin GENU® USP/casein conjugate and citric acid with 40% of particles with approximately 5.0 µm (Fig. 3c). Changing citric acid by maleic acid resulted in 59.4% of particles within 4.0–5.0 µm (Fig. 3d).

The acetaminophen loaded pectin GENU® 8003/casein with citric acid microcapsules presented 65.29% of particles between 5.0 and 7.0 µm (Fig. 4c) with mean value of 5.182±2.076 µm (Table II). This same formulation prepared with maleic acid presented a peak of 40.17% at approximately 5.0 µm (Fig. 4d).

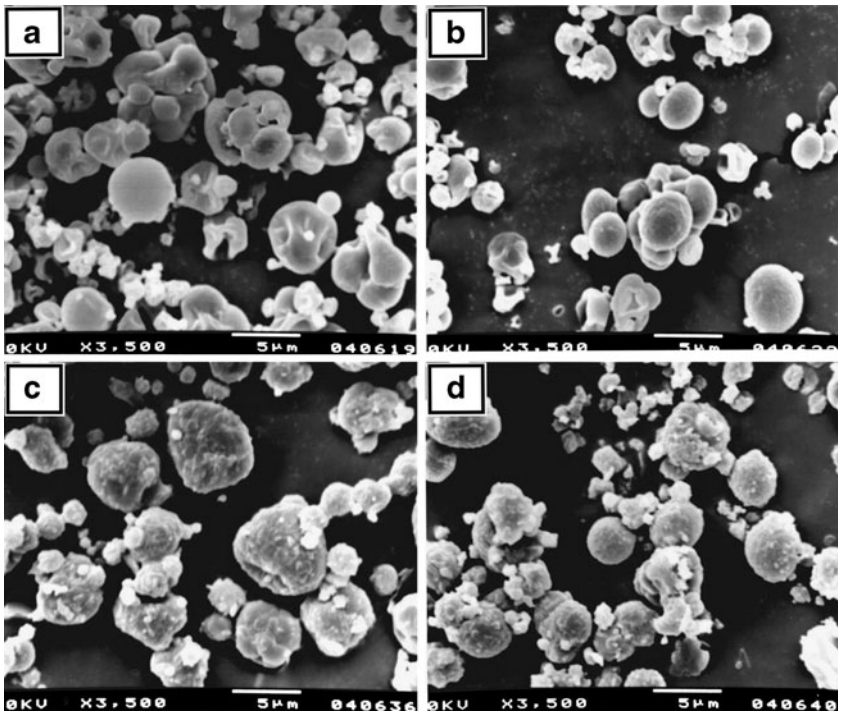

Fig. 1. SEM photomicrographs of spray-dried microcapsules; original magnification ×3,500: **a** formulation A, **b** formulation B, **c** formulation E, **d** formulation F

There was significant statistical difference on particle size comparing formulations of pectin GENU® USP with and without acetaminophen. However, there was no statistical difference between particle size of pectin GENU® 8003 with or without acetaminophen. A possible explanation is that the size of pectin GENU® 8003 microcapsule without drug was already elevated and not being significantly altered after drug addition. It is noteworthy to mention that the starting size of acetaminophen was 3.7 μm of which 79.58% ranged between 3.0 and 5.0 μm as determined by optical microscopy (data not shown). Therefore, the starting size of acetaminophen affected the final size of GENU® USP microcapsule, but not GENU® 8003 microcapsule (Table II).

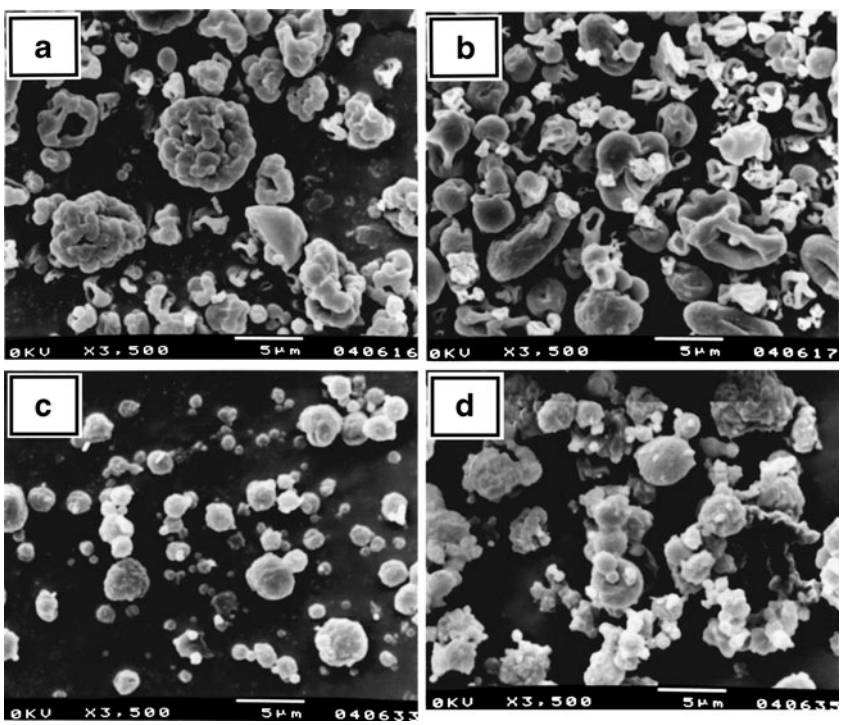

Fig. 2. SEM photomicrographs of spray-dried microcapsules; original magnification ×3,500: **a** formulation C, **b** formulation D, **c** formulation G, **d** formulation H

Table II. Mean Diameter of Microcapsules

Formulation	Mean diameter (μm)	SD	*N*
A	3.138	±1.489	628
B	3.423	±1.675	780
C	4.929	±2.072	403
D	4.602	±2.321	516
E	4.680	±1.163	562
F	4.393	±1.467	878
G	5.182	±2.076	435
H	4.944	±1.700	468

Results in the literature showed that size distribution of microcapsules was significantly affected by core-to-wall ratio. The proportion of large microcapsules increased with increase in the core-to-wall ratio. Microcapsules prepared with core-to-wall ratio 1:1.5 and 2:1.5 exhibited similar size distribution ($P>0.05$) and about 95% of the microcapsules were smaller than 450 μm. In contrast, microcapsules prepared with core-to-wall ratio 3:1.5, 4:1.5, and 5:1.5 ($P<0.05$) exhibited about 85% of the microcapsules larger than 700 μm. Therefore, increasing the core-to-wall ratio increases the size of microcapsules and their distribution (35). In agreement, the size distribution of microspheres prepared with ethylcellulose strongly depends on the core-to-wall ration and the stirring speed of the preparation process. In general, increasing the proportion of encapsulating polymer or reducing the velocity of stirring resulted in increase of microcapsules size (36).

In the present study, the formation of bigger particles with greater size variation by altering the polymer and maintaining the amount and stirring speed was observed. This result may be related to the medium viscosity since it was obtained using the conjugate pectin GENU® 8003, which is a polysaccharide of reduced methoxyl groups and contains amidic groups that increase the viscosity.

Determination of Encapsulation Efficiency and *In Vitro* Dissolution Studies

The acetaminophen release by formulations E (pectin GENU® USP/casein/citric acid/drug), F (pectin GENU® USP/casein/maleic acid/drug), G (pectin GENU® 8003/casein/citric acid/drug), and H (pectin GENU® 8003/casein/maleic acid/drug; further details in Table I) in SGF (simulated gastric fluid) condition was 41.98%, 39.57%, 39.93%, and 65.70% in 2 h, respectively (Fig. 5). The initial release of water-soluble drugs is related to their tendency of diffusing and concentrating in the shell of the microcapsules increasing the burst effect (37). In fact, drugs like acetaminophen are used in the initial development of this type of polymer because they represent the worst possible scenario due to their low molecular weight and high solubility in aqueous environment.

The formulation H released a high percentage of acetaminophen in SGF condition, probably due to maleic acid interference in the carbonilic group ligations of glutaraldehyde with the amino groups in the protein chain, and affecting the matrix reticulation. The microcapsules remained for 4 h in SIF (simulated intestinal fluid) condition presenting slow and

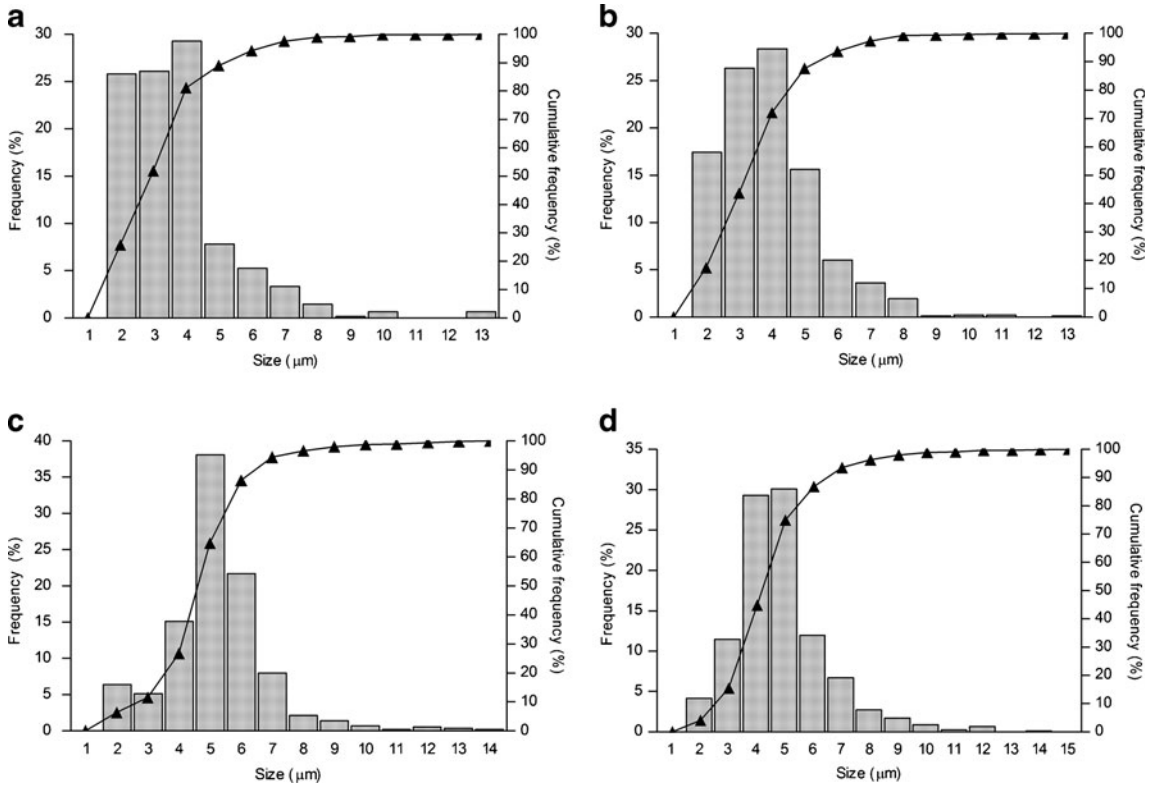

Fig. 3. Size distribution of microcapsules. **a** Formulation A, **b** formulation B, **c** formulation E, **d** formulation F; size frequency distribution (*bars*) and size cumulative frequency distribution (*line*). The particle size class interval is 1.0 μm

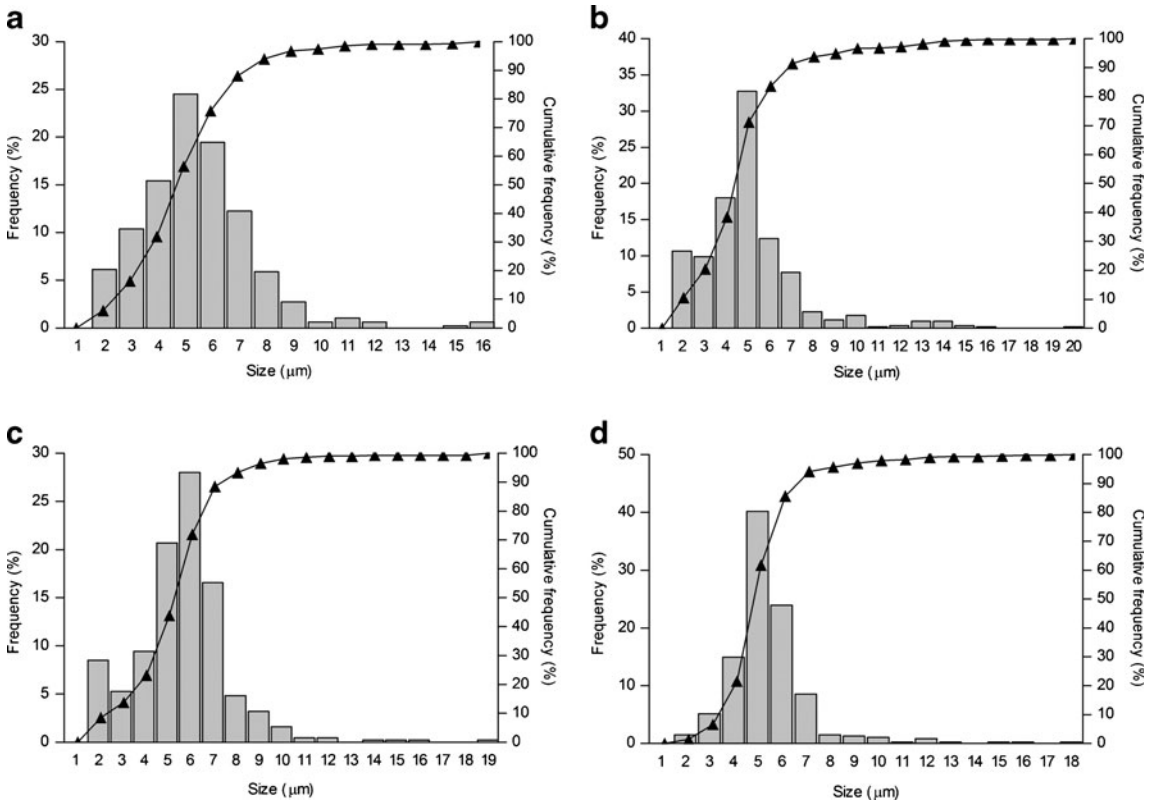

Fig. 4. Size distribution of microcapsules. **a** Formulation C, **b** formulation D, **c** formulation G, **d** formulation H: size frequency distribution (*bars*) and size cumulative frequency distribution (*line*). The particle size class interval is 1.0 μm

gradual release of drug, and the release percentage for formulations E, F, G and H were 62.79%, 78.89%, 71.95%, and 95.01%, respectively. Colonic bacteria that produce pectinases degrade pectin (38,39). Therefore, it was evaluated whether acetaminophen release was improved by adding Pectinex® Ultra SP-L (pectinase) in SIF to induce hydrolysis of the

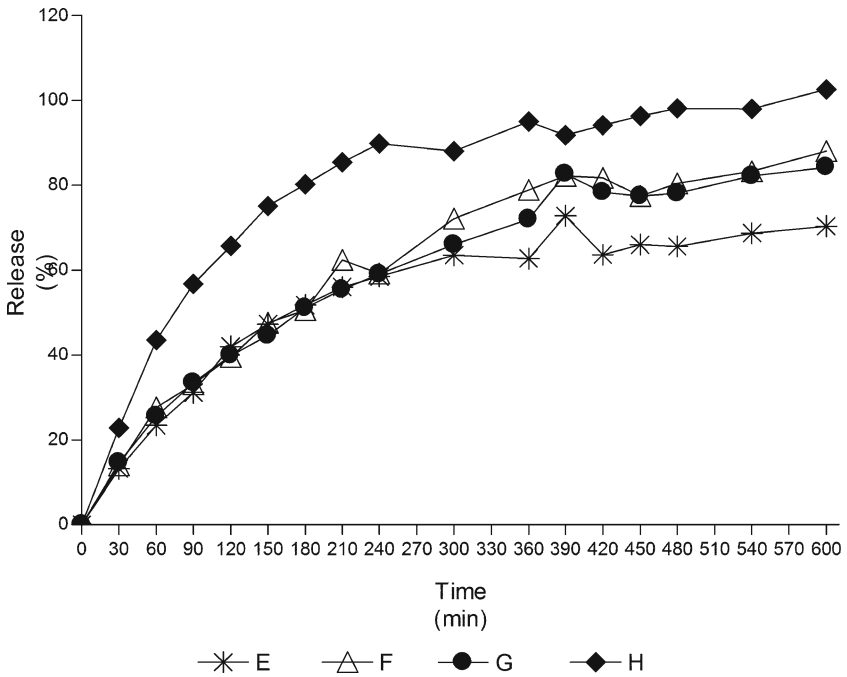

Fig. 5. *In vitro* release profile of microcapsules in simulated gastric fluid (SGF; 120 min) condition, and simulated intestinal fluids (SIF) condition before (120–360 min) and after (360–600 min) addition of pectinolytic enzymes. Formulation E, formulation F, formulation G, formulation H. In all cases, mean values (n=3) are presented

Table III. Encapsulation Efficiency of Acetaminophen in Microcapsules of Pectin/Casein (n=3)

Formulation	Drug encapsulation efficiency mean±SD (%)
E	57±0.9
F	46±1.4
G	32.9±3.2
H	38±2.4

microcapsules wall. However, there was no significant improvement of drug release. It is possible that the reticulation process altered the binding sites of pectinase, thus, reducing pectinase effects.

As shown in Table III, formulation E (pectin GENU® USP/casein/citric acid/drug) presented higher drug encapsulation efficiency (57%) and lower drug release within 10 h (72.76%) than the other formulations. In agreement, others (5) have also shown that an increase of drug encapsulation efficiency from 54.94% to 70.80% results in a decrease in release from 16.95% to 4.5%, respectively, in the SIF using poly(lactide) microcapsules prepared by combining membrane emulsification technique and double-emulsion solvent evaporation method. Thus, increasing the encapsulation efficiency diminishes the drug release in SIF. It is known that the efficiency of encapsulation in formulations depends on the rate and extend of diffusion of the drug into the external aqueous phase during the in-water solvent evaporation following microencapsulation (37). In this sense, the encapsulation efficiency was possibly under-estimated due to the low molecular weight and high solubility in aqueous environment of acetaminophen. These characteristics increase the tendency the drug to diffuse out the microcapsules as discussed above.

Furthermore, the pectin GENU® 8003 presents higher viscosity than pectin GENU® USP, probably because of the amidation. Therefore, the increased viscosity of pectin GENU® 8003 decreases the shear stress during agitation, which might have negative influence in the encapsulation efficiency compared to pectin GENU® USP (Table III). Different of what was observed in the dissolution assay (Fig. 5), there was no clear influence of citric acid and maleic acid in the encapsulation efficiency (Table III).

The kinetic evaluation of formulations started at 30 min (Fig. 5) until the release rate reached steady state. The drug release curve profile was obtained with the dissolution test of the microcapsules. There was a partial linearization of acetaminophen release with a cumulative amount of acetaminophen proportional to the root square of time, which was highlighted by the equation of Higuchi, indicating that the drug release was controlled by diffusion. There was a higher coefficient of correlation in the formulations E, F, and G in Higuchi equation than in zero- and first-order kinetics (Table IV).

Regarding formulation H, the release kinetics was considered to be first order displaying almost total release in 360 min. Thus, suggesting that the release kinetics was not influenced by the swelling of the microcapsules.

Microcapsules that are prepared by classic process hardly present zero-order kinetics. In general, the release curve is of first-order kinetics and the linearization is obtained by the logarithmic of retained drug over time that represents the sum of release of all particles. For a zero-order release from microcapsules, a monodispersion system in which the permeability and particle sizes are related would be necessary. Furthermore, the homogeneity of encapsulation of drug is important (40).

The constant of kinetic models (K) of formulations E, F, and G followed the Higuchi model without significant statistical differences among them (P>0.05). On the other hand, for first-order equations, there were statistical differences (P<0.05) for K values among formulation except by the comparison between E and G. The release values indicated that the change in the polymer (pectin GENU® USP/casein or pectin GENU® 8003/casein) did not modify the release mechanism, but rather changed the release rate of acetaminophen with exception of formulation H, which was prepared with pectin GENU® 8003/casein and maleic acid.

The formulations prepared with maleic acid (F and H) presented the greatest release rate in the Higuchi and first-order equations (Table IV). The best particle size distribution was observed with formulation H. However, its release profile indicated that it is not adequate to reach the colon region.

CONCLUSIONS

The pectin/casein mixture in aqueous dispersion form multiparticle organized systems determined by pH values lower than the pI of the original compounds. The microcapsules present predominantly spherical forms and are resistant to the

Table IV. Correlation Coefficient (R^2) and Constant (k_0, k_1, k_H) of Different Kinetic Models for Acetaminophen Microcapsules Formulations

Formulation	Zero order	First order	Higuchi equation
	$Q=k_0\,t^a$	$\ln(100-Q)=\ln(Q_0)-k_1\,t^a$	$Q=k_H\,t^{1/2a}$
E (pectin GENU® USP/casein/citric acid/drug)	k_0=0.4365	k_1=−0.001244	k_H=11.80
	r^2=0.8850	r^2=0.9455	r^2=0.9623
F (pectin GENU® USP/casein/maleic acid/drug)	k_0=0.5404	k_1=−0.001838	k_H=14.21
	r^2=0.9608	r^2=0.9825	r^2=0.9885
G (pectin GENU® 8003/casein/citric acid/drug)	k_0=0.5078	k_1=−0.001437	k_H=12.91
	r^2=0.9551	r^2=0.9972	r^2=0.9973
H (pectin GENU® 8003/casein/maleic acid/drug)	k_0=0.912	k_1=−0.004073	k_H=20.01
	r^2=0.9312	r^2=0.9973	r^2=0.9836

[a] Q the percent of drug released is at time t; Q_0 the percent of drug remaining to release at time 0; k_0, k_1, k_H are the coefficients of the equations

drying and spray-drying process. The microencapsulated systems with acetaminophen showed uniform distribution concerning particle size compared to microcapsules without acetaminophen. The release of acetaminophen from the microcapsules was slow, gradual, driven by a diffusion mechanism, and modulated by the organic acids used in the preparations. This controlled release is even more important considering that acetaminophen is a small particle of easy diffusion. Furthermore, the release kinetics was not influenced by the swelling of the microcapsules. Therefore, the polymeric system present herein seemed to be appropriate for a sustained release of acetaminophen throughout the gastrointestinal tract despite the low molecular weight and high water solubility of the drug. Nevertheless, it is likely that it is a promising pectin/casein complex for lipossoluble drugs, which merits further investigation.

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