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Preparation and characterization of biodegradable or enteric-coated microspheres containing the protease inhibitor camostat

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Abstract

We have produced biodegradable or enteric-coated microspheres containing camostat mesylate, a protease inhibitor, using a water–oil–water emulsion solvent evaporation method. The characteristics of the microspheres were determined. When polylactic acid, a biodegradable polymer, was used as a wall material, the optimized microsphere obtained showed a loading efficiency of almost 95% and had a mean diameter of 30 μm. This microsphere showed a sustained-release profile, with nearly 25% of drug being released at seven days in a dissolution test. When hypromellose acetate succinate (AS-HG type, with a high content of succinyl group) was used as an enteric wall material, optimized microspheres showed a loading efficiency of almost 80%. In this case, pH 3.0 citrate buffer was used as an internal aqueous phase, and citrate buffer containing 0.5% polyvinylalcohol was used as the external aqueous phase. These microspheres showed a rapid release profile in pH 6.8 buffer, whereas the release was extremely slow in pH 1.2 buffer. Hypromellose acetate succinate microspheres were also prepared containing 10% (w/w) *N*-benzoyl-dl-arg-4-nitroanilide as a model substrate for trypsin, with or without 5% (w/w) camostat. These microspheres were incubated in pH 6 or 7 buffer containing trypsin at 37°C. When camostat was included in the microspheres, the substrate was protected from attack by trypsin, while in the absence of camostat, the released substrate was immediately attacked by trypsin to produce the degradation product *N*-benzoyl-dl-arginine.

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Introduction

Camostat mesylate is a protease inhibitor that has been developed as a treatment for acute pancreatitis (Taniguchi et al 1997) or disseminated intravascular coagulation (Naito et al 1989). As the elimination rate of camostat from the body is high, the development of a sustained-release formulation is desirable. In this study, two different materials have been used to produce a microencapsulated formulation of camostat, the biodegradable polymer polylactic acid (PLA) and the enteric polymer hypromellose acetate succinate. The drug release profiles of the prepared microspheres were evaluated.

PLA microspheres have been used in the formulation of long-acting, injectable, drug delivery systems for the past 15 years (Jalil & Nixon 1989; Edman et al 1993; Larionova et al 1999). A wide range of synthetic drugs, as well as biologicals such as enzymes, hormones and proteins, have been microencapsulated by this polyester polymer (Ogawa et al 1988; Kentepozidou et al 1995; Uchida et al 1998).

Even though commercial camostat products are now available in oral dosage forms, the design of appropriate release rates of camostat and substrate such as peptides seems to be essential for appropriate gastrointestinal delivery. Therefore, enteric-coated microspheres containing camostat would be an attractive proposition. We have used the enteric polymer hypromellose acetate succinate to encapsulate camostat because the polymer dissolves easily at pH over 6. In this case, camostat and *N*-benzoyl-dl-arg-4-nitroanilide, a substrate of protease, were encapsulated in the polymer. Model substrate-loaded microspheres, in the presence or absence of camostat, were soaked and incubated at pH 6 or 7 in buffered solution containing trypsin as a protease, and *N*-benzoyl-dl-arginine. HPLC was used to periodically screen for degradation products of the model substrate for trypsin.

Materials and Methods

Materials

Camostat as mesylate was kindly donated by Ono Co. Ltd. (Osaka, Japan). Polylactic acid (MW 18 000) was purchased from Wako Chemical Co. Ltd. (Osaka, Japan). Hypromellose acetate succinate, AS-HG grade, containing 10–14 % (w/w) succinyl group, was kindly donated by Shin-Etsu Chemical Industry Co., Ltd. (Tokyo, Japan). Polyvinyl alcohol (PVA) (MW 85 000–146 000, 87–89 % hydrolysed) was supplied by Aldrich Chemical Co., Ltd. (Milwaukee, WI). Model substrate, *N*-benzoyl-dl-arginine, was purchased from Sigma Co., Ltd. (St Louis, MO) and trypsin was supplied by Nakalai Tesque Co., Ltd. (Kyoto, Japan). Other reagents were all of special grade.

Solubilities of camostat in buffers of various pH

Buffers of various pH (pH 1.5, 3, 5, 7, 10, 11.5) were used as solvent. Excess camostat (1 g dL^{-1}) was put into a 30-mL beaker, buffer was added, and the beaker was shaken at 80 rev min^{-1} at 25°C for 10 h. In a pilot study we had confirmed that equilibrium was reached within 10 h. The suspension was filtered using a Millipore filter (0.45-μm pore size) and the filtered solution was diluted with buffer. The camostat concentration of the diluted sample was determined using an HPLC method as described below.

Preparation of PLA or hypromellose acetate succinate microspheres containing camostat

For the PLA microspheres, a water–oil–water (w/o/w) emulsion solvent evaporation method (Heya et al 1991; Uchida et al 1995, 1996) was adopted. Firstly, 20 mg camostat (corresponding to a theoretical loading of 10 %) was suspended in purified water. This medium, as the internal aqueous phase, was emulsified with 5 mL methylene chloride containing 180 mg polylactic acid for 1 min using an ultrasonic disrupter (UD-200, Tomy Seiko Co., Ltd., Tokyo, Japan). This w/o emulsion was poured into 200 mL 0.5 % (w/v) PVA solution (dissolved in buffers of various pH) as the external aqueous phase. Emulsification was continued using a homogenizer (Physcotron, Nichion Irikakikai Co., Ltd., Tokyo) at 3000 rev min^{-1} for 1 min. This dispersion was gently agitated in a 500-mL beaker on a stirring plate with a 3.9-cm stirring bar for 4 h at room temperature. The microspheres were collected by centrifugation at 3000 rev min^{-1} for 15 min (1580 *g*), washed with purified water and freeze-dried (FD-5N, Tokyo Rikakikai Co., Ltd., Tokyo) for at least 4 h. Changing the volume of methylene chloride varied polymer concentration.

The preparation of hypromellose acetate succinate enteric microspheres was essentially the same except that pH 3.0 citrate buffer was used as an internal or external aqueous phase. The preparation of microspheres containing both camostat and *N*-benzoyl-dl-arg-4-nitroanilide as a model substrate of trypsin was also essentially the same, except that the theoretical camostat and substrate loading was 5 and 10 %, respectively.

For all batches, yields were determined as the percentage of weight of the recovered microspheres after drying divided by the initial amount of enteric polymer and the drug employed.

A morphological study was performed using a biological microscope (Aliphaphot-2 YS2-H, Nikon Co., Ltd., Tokyo). A Microtrac Particle Size Analyzer (Model 7995-30, Leeds and Northrup, North Wales, PA) was utilized to determine the microsphere average volume diameters and distribution.

Drug loading

For the PLA microspheres, 200 μL methylene chloride was added to 5 mg microspheres, and 2 mL pH 3.0 citrate buffer was added to the solution, which was then agitated adequately for 5 min. After centrifugation at 3000 rev min^{-1} for 15 min (1580 *g*), the water phase was

Table 1 The effect of preparative conditions on camostat loading efficiency in polylactic acid microspheres theoretically loaded with 10% camostat.

Formulation number	Internal aqueous phase (w) (Camostat 20 mg)		Oily phase (o) methylene chloride volume (mL)	External aqueous phase (w)		Loading efficiency (%)	s.d.
	Solvent	Volume (μL)		PVA concn (%)	Buffer pH		
1	Water	60	1.25	0.5	1.5	8.3	3.2
2	Water	60	1.25	0.5	3	5.9	2.4
3	Water	60	1.25	0.5	5	15.2	3.8
4	Water	60	1.25	0.5	7	23.0	6.8
5	Water	60	1.25	0.5	10	72.0	12.4
6	Water	60	1.25	0.5	11.5	67.8	6.9
7	Water	200	1.25	0.5	10	34.2	4.6
8	Water	400	1.25	0.5	10	28.5	5.3
9	Water	600	1.25	0.5	10	75.1	3.3
10	Water	60	1.0	0.5	10	84.5	4.3
11	Water	60	0.75	0.5	10	95.3	3.9

Each value represents the mean of four to six experiments.

collected and the methylene chloride phase extracted with 2 mL pH 3.0 buffer. The water phases were combined, and a 50-μL sample was injected onto a chromatograph (Shimadzu LC-10A, Kyoto, Japan) equipped with a UV detector (Shimadzu SPD-10AV), an integrator (Shimadzu C-R4A) and a reversed phase column (Cosmosil 5C18-AR, 4.6 × 150 mm, Nakalai Tesque Co., Ltd, Kyoto, Japan). The following mobile phase systems were used: A, methanol; B, 0.2% sodium heptane sulfonate; C, 0.1% sodium lauryl sulfate; D, glacial acetic acid (A:B:C:D, 200:100:50:1). The flow rate was 1.0 mL min^{-1} and the wavelength was set at 265 nm. Loading was calculated from the weight of the initial microspheres and the amount of camostat incorporated.

For the enteric hypromellose acetate succinate microspheres, the actual camostat loading percentage was determined as follows. A quantity of approximately 5 mg microspheres was precisely weighed and dissolved in 2 mL methanol in a glass vial for further dissolution of the enteric polymer. Two millilitres pH 3.0 citrate buffer was added to the sample solution to precipitate the enteric polymer. The camostat solution containing precipitated polymer was centrifuged at 3000 rev min^{-1} for 15 min. The camostat concentration of the supernatant was determined using the HPLC method described above.

For enteric microspheres containing 10% of substrate, *N*-benzoyl-dl-arg-4-nitroanilide, and 5% camostat, the camostat and substrate were simultaneously extracted by the method described above. The concentration of substrate was determined using a reversed-phase column (Cosmosil 5C18-AR, 4.6 × 150 mm, Nakalai Tesque Co., Ltd., Kyoto) with acetonitrile: phosphoric acid (10 mM) containing 50 mM sodium sulfate (33:67) as the mobile phase. The flow rate was 1.0 mL min^{-1} and the wavelength was set at 265 nm.

In-vitro camostat release from PLA or hypromellose acetate succinate microspheres

The in-vitro release profile of camostat from PLA or hypromellose acetate succinate microspheres was examined as follows. Microspheres corresponding to 1 mg camostat were suspended in 50 mL buffer solution and shaken horizontally at 60 rev min^{-1} at 37°C. For PLA microspheres, 0.02% Tween 80 and sodium azide were added to prevent microspheres from coagulating, and bacterial growth in the dissolution medium, respectively, since the release test was performed for a week. At predetermined intervals, 150-μL suspension was taken as a sample, centrifuged (3000 rev min^{-1} for 5 min), and the concentration of the substrate in the supernatant was analysed by the HPLC method described above.

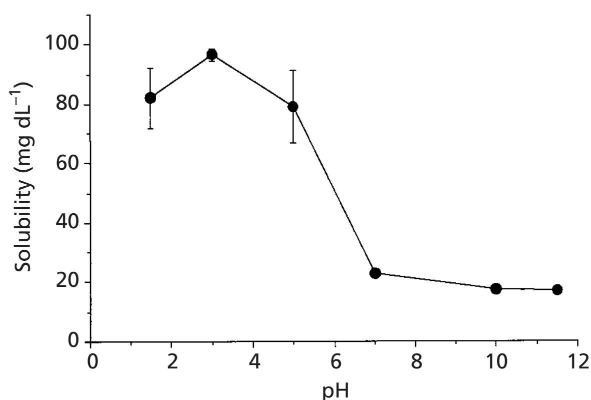

Figure 1 The influence of pH on the solubility of camostat at 37°C. Values are the means of three determinations ± s.d.

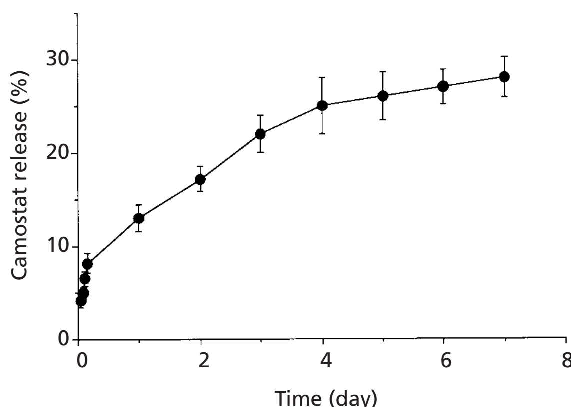

Figure 2 Camostat release from PLA microspheres in pH 6.8 buffer at 37°C. Values are the means of three determinations ± s.d.

In-vitro substrate degradation study of substrate-loaded hypromellose acetate succinate microspheres in the absence or presence of camostat

Microspheres containing approximately 1 mg substrate and approximately 0.5 mg camostat, and control microspheres containing only 1 mg substrate, were used in this study. The two kinds of microspheres were suspended in 50 mL of pH 6 or 7 phosphate-buffered solution containing 2.5 mg trypsin, and shaken horizontally at 60 rev min^{-1} at 37°C. At predetermined intervals, 150-μL suspension was taken as a sample, centrifuged (3000 rev min^{-1} for 5 min) and the concentration of the substrate, *N*-benzoyl-dl-arg-4-nitroanilide, in the supernatant was analysed by HPLC. The determination of the degradation product, *N*-benzoyl-dl-arginine, was performed simultaneously using a mobile phase consisting of: A, 10 mM phosphate buffer containing 50 mM sodium sulfate (pH 2.6); B, acetonitrile. A linear gradient was used: phase B from 5 to 60 % (20 min). The flow rate was 1.0 mL min^{-1} and the wavelength was set at 410 nm.

Results and Discussion

Optimization of camostat-loaded PLA microspheres and their characterization

Table 1 shows the effect of different preparative conditions on the loading efficiency of camostat in PLA microspheres, all of which were theoretically loaded with 10 % camostat. Camostat is a basic compound and so its solubility was expected to be low under alkaline conditions. This expectation was confirmed experimentally (Figure 1). Therefore, the pH of the external aqueous phase was varied in formulations 1–6. In these formulations, the internal aqueous phase volume was fixed at 60 μL, and polymer phase (methylene chloride) volume was fixed at 1.25 mL. As expected, the drug-loading efficiency under alkaline conditions (pH 10, 11) was comparatively high (72.8 and 67.8 %, respectively).

The volumes of the internal phase, oily phase, and external aqueous phase were changed in formulations 7–11. When the internal aqueous phase volume was increased from 60 μL to 200, 400, or 600 μL, the loading efficiency was dramatically decreased, as shown in formulations 7–9. This suggested that the smaller volume of the internal aqueous phase was an important factor for high loading. Entrapment efficiency seemed to depend on the phase volume of oil (methylene chloride) relative to the internal aqueous phase, since the smaller the oil phase volume/internal aqueous phase volume, the smaller the exposure probability of the internal aqueous phase.

Finally, when the volume of the oily phase was decreased to 0.75 mL, the highest loading efficiency (95 %) was achieved (formulation 11), which suggested that a high concentration of methylene chloride might be a significant barrier to drug diffusion from the internal to the external aqueous phase. For the three batches, the yield was almost 80 % and the mean average diameters of the microspheres were 30 ± 4 μm.

The most favourable sustained release profile was obtained with formulation 11. Almost 10 % of the camostat was released within 4 h, and this was followed by a secondary slow-release phase (Figure 2). Approximately 75 % of the drug remained unreleased one week later. The remaining camostat was extracted from the PLA matrix using the same method as described under Drug loading and a good mass balance was reached (data not shown).

Table 2 The effect of preparative conditions on camostat loading efficiency in hypromellose acetate succinate microspheres theoretically loaded with 10 % camostat.

Formulation number	Internal aqueous phase (w) (Camostat 20 mg)		Oily phase (o) methylene chloride volume (mL)	External aqueous phase (w)		Loading efficiency (%)	s.d.
	Solvent	Volume (μL)		PVA concn (%)	Buffer pH		
1	Citrate buffer (pH 3)	60	5.0	0.5	3	14.3	3.1
2	Citrate buffer (pH 3)	200	5.0	0.5	3	3.5	1.2
3	Citrate buffer (pH 3)	400	5.0	0.5	3	2.7	0.8
4	Citrate buffer (pH 3)	60	2.5	0.5	3	69.0	4.1
5	Citrate buffer (pH 3)	60	1.25	0.5	3	81.0	5.2

Each value represents the mean of four to six experiments.

Optimization of camostat-loaded hypromellose acetate succinate microspheres and their characterization

In a pilot study, we found that the critical factor for the successful preparation of enteric microspheres was the choice of an acidic external aqueous phase, such as pH 3.0 citrate buffer containing 0.5 % PVA solution. When 0.5 % PVA (pH 5.5) was used as an external aqueous phase for microencapsulation, the prepared enteric microspheres were very fragile and were ruptured by centrifugation in the recovery process (data not shown). Therefore, in this study we used pH 3.0 citrate buffer containing 0.5 % PVA.

Table 2 summarizes the effect of preparative conditions on camostat loading efficiency in hypromellose acetate succinate microspheres theoretically loaded with 10 % camostat. Formulations 1–3 showed the effect of internal aqueous phase volume on loading efficiency. As the internal aqueous phase volume increased from 60 μL to 200 or 400 μL, the loading efficiency dramatically decreased to 3.5 % or 2.7 %, respectively.

Secondly, the effect of oily phase volume (polymer concentration of methylene chloride) on loading efficiency was examined. In this case, the internal aqueous phase volume was fixed at 60 μL. In formulation 5, when 1.25 mL methylene chloride was used as an oleaginous phase, over 80 % loading was observed. As suggested for PLA microspheres, the high concentration of methylene chloride might present a significant barrier to drug diffusion from the internal to the external aqueous phase.

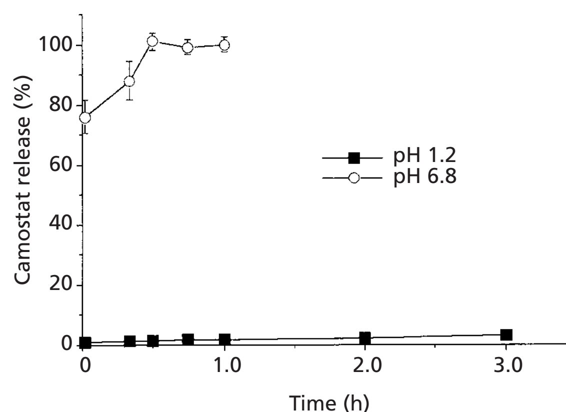

Figure 3 Camostat release from hypromellose acetate succinate microspheres in pH 1.2 or 6.8 buffer at 37°C. Values are the means of three determinations ± s.d.

Figure 3 shows the camostat release profiles from hypromellose acetate succinate microspheres at pH 1.2

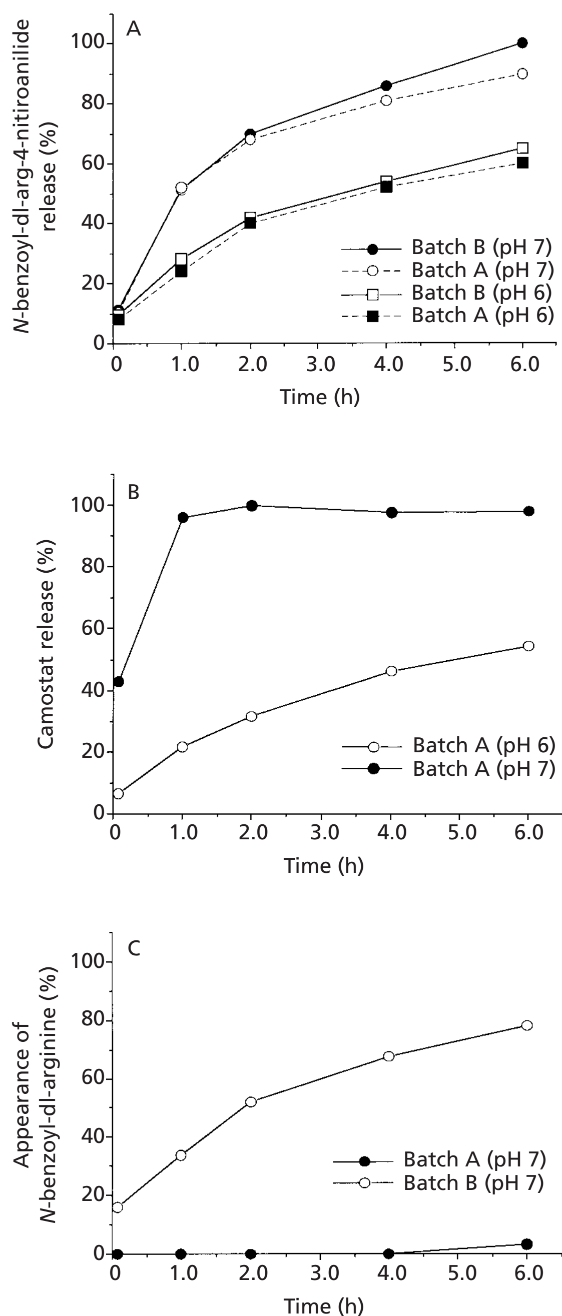

Figure 4 A. Release profile of *N*-benzoyl-dl-arg-4-nitroanilide from hypromellose acetate succinate microspheres with and without camostat at pH 6 or 7 at 37°C. Batch A contained 4.5% camostat and 9.5% *N*-benzoyl-dl-arg-4-nitroanilide; batch B contained 9.2% *N*-benzoyl-dl-arg-4-nitroanilide. B. Release profile of camostat from batch A at pH 6 or 7. C. Appearance of the degradation product *N*-benzoyl-dl-arginine from batch A or B at pH 7 in the presence of trypsin. Each value represents the mean of two or three experiments.

and 6.8. In pH 6.8 buffer, comparatively fast release was observed, suggesting the enteric characteristics of the microspheres; all drug was released within 45 min. In the pH 1.2 buffer, only a low level of release was observed. This information confirmed that enteric-coated microspheres had been prepared successfully.

Preparation and characterization of hypromellose acetate succinate microspheres loaded with *N*-benzoyl-dl-arg-4-nitroanilide and camostat

Two batches of hypromellose acetate succinate microspheres were prepared, one containing 4.5% camostat and 9.5% *N*-benzoyl-dl-arg-4-nitroanilide as a substrate (batch A), and a second containing 9.2% substrate only (batch B). For the two batches, the yield was almost 80% and the mean average diameters of the microspheres were 34 ± 5 and 36 ± 7 μm, respectively.

Figure 4A shows the cumulative substrate released from the two batches of microspheres in pH 6 or 7 buffer medium without trypsin. As expected, at pH 7 a comparatively fast release was observed for both batches, and almost all substrate was released within 6 h. Incorporation of camostat did not affect the release rate of the substrate significantly. The release rate at pH 6 was slightly slower.

Figure 4B shows the release profile of camostat from batch A at pH 6 or 7 in a medium without trypsin. Even in this case, comparatively fast release of camostat was observed at pH 7, while the release rate at pH 6 was more moderate. The difference in the release rates of camostat and *N*-benzoyl-dl-arg-4-nitroanilide seemed to be due to differences in their solubilities. This experiment also showed that the substrate was released from hypromellose acetate succinate microspheres together with camostat. Once the substrate was released from the microspheres into the medium in the presence of trypsin, it would be degraded immediately and the degradation product would be formed. Therefore, as shown in Figure 4A and B, we confirmed the substrate was released with camostat from hypromellose acetate succinate microspheres, even though the buffer medium did not contain trypsin.

Finally, Figure 4C shows the appearance of degradation product when batch A or B microspheres were incubated in pH 7 buffer containing trypsin as a protease. For hypromellose acetate succinate microspheres containing substrate only (batch B), the substrate degradation product *N*-benzoyl-dl-arginine was produced almost immediately at pH 7 in a trypsin-containing medium, in contrast with the hypromellose acetate succinate microspheres containing camostat and substrate (batch A), in which the released camostat almost completely prevented the degradation of the substrate.

In the formulation design, the release rates of substrate and camostat seemed to be important criteria. Not only the substrate release rate but also the release rate of camostat seems to be important in drug delivery.

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