Preparation and Characterization of Enteric Microspheres Containing Bovine Insulin by a w/o/w Emulsion Solvent Evaporation Method

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The objective of this study was to produce enteric microspheres containing bovine insulin as a model drug using a water-in-oil-in-water (w/o/w) emulsion solvent evaporation method, and the preparative conditions were optimized.

When hydroxypropylmethylcellulose acetate succinate (AS-HG type; high content of succinyl group) was employed as an enteric wall material, optimized microspheres showed almost 90% of the loading efficiency of insulin and 30.8 μm of mean volume diameter. The mixture of methylene chloride and acetone (4 : 1) as an oleaginous phase, 400 μl of bovine insulin solution (dissolved in 30% of acetic acid) as an internal aqueous phase, and 1.0% of polyvinylalcohol dissolved in pH 3.0 citrate buffer as an external aqueous phase, were employed in the experiment. In relation to other enteric cellulose derivatives (AS-MG type, AS-LG type; medium and low content of succinyl group, respectively), the microencapsulation using a simultaneous preparation method also resulted in quite high loading efficiencies, whereas the choice of poly(methyl methacrylate) as a wall material caused aggregation or flocculation in the preparative process of every batch.

The AS-HG microspheres showed very fast release profile in pH 6.8 buffer, but no released fraction was observed in pH 1.2 buffer. This phenomenon suggested enteric characteristics of prepared microspheres. Finally AS-HG microspheres containing 4% lauric acid and 9% insulin were prepared, suspended in 0.1% of carboxymethyl cellulose solution, and administered to the rat rectum (corresponding to 50 I.U./kg insulin). The plasma glucose level reached minimum level at 0.5 h after administration then gradually rose to normal.

Key words microsphere; enteric polymer; bovine insulin; hydroxypropylmethylcellulose acetate succinate; lauric acid; plasma glucose level

Microspheres are defined as particle matrix systems where the drug is uniformly dispersed into the polymer. Several encapsulation techniques have been reported in the literature concerning the production of microspheres.[1—3] The solvent evaporation technique has frequently been used for the loading of a drug in polymers.[4,5] The process as originally developed is based on the dissolution or dispersion of a drug into a solution of polymer material in a suitable organic solvent, the subsequent dispersion of the organic phase in an immiscible liquid, and finally drying out of the organic solvent. However, the drawback with this method was the poor loading efficiency for a drug with a high solubility in the external aqueous phase. Recently Ogawa *et al.*[6] and Okada[7] developed a water-in-oil-in-water (w/o/w) emulsion solvent evaporation technique to overcome this problem. They emulsified an aqueous solution of LH-RH (leutenizing hormone - releasing hormone) agonist in polylactic acid organic solution and dispersed the obtained emulsion in an aqueous phase containing 0.25% polyvinyl alcohol (PVA). Even though this technique had already been applied to the biodegradable polymer poly (lactide-co-glycolide (PLGA),[8,9] it had not yet been applied to enteric polymers such as cellulose derivatives or poly(methyl methacrylate).

Our goal in this study was to produce enteric microspheres containing bovine insulin as a model drug using the w/o/w emulsion solvent evaporation method, and *in vitro* preparative conditions were optimized. The pharmacological effect of insulin-loaded enteric microspheres was also evaluated.

Experimental

Materials Hydroxypropylmethylcellulose acetate succinates were kindly donated by Shin-Etsu Chemical Industry Co., Ltd. (Tokyo, Japan). Three grades, *i.e.*, AS-HG, AS-MG, and AS-LG type contained 10—14%, 7—11%, and 5—9% of succinyl group in polymer, respectively. PVA was supplied by Aldrich Chemical Co., Ltd. (Milwaukee, WI, U.S.A.). Bovine insulin was purchased from Sigma Co., Ltd. (St. Louis, MO, U.S.A.) and lauric acid was supplied by Nakalai Tesque Co., Ltd. Other reagents were all of special grade.

Preparation of Enteric Microspheres Containing Bovine Insulin A w/o/w emulsion solvent evaporation method was adopted; the procedure was essentially the same as that used previously. First of all, 20 mg of bovine insulin (corresponding to 10% of theoretical loading) was dissolved in 30% aqueous acetic acid or suspended in purified water. This solution as the internal aqueous phase was emulsified with 5 ml of methylene chloride or a mixture of methylene chloride and acetone containing 180 mg of enteric polymer for 1 min using an ultrasonic disruptor (UD-200, Tomy Seiko Co., Ltd., Tokyo, Japan) . This w/o emulsion was poured into 200 ml of 0.1—1.0% (w/v) PVA solution (dissolved in pH 3.0 citrate buffer) as the external aqueous phase. Emulsification was continued using a homogenizer (Physcotron, Nichion Irikakikai Co., Ltd., Tokyo) at 2000 rpm 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 rpm for 10 min, and those obtained were washed with pH 3.0 citrate buffer and freeze-dried (FD-5N, Tokyo Rikakikai Co., Ltd., Tokyo) for at least 4 h. In the case of microencapsulation of insulin with lauric acid, the acid was dissolved in the organic phase, then incorporated into microspheres to enhance insulin permeability through the gastrointestinal membrane.

The microsphere yield was determined as the percentage of weight of the recovered microspheres after drying divided by the initial amount of enteric polymer and the drug employed. Morphology study was performed using a biological microscope (Alphaphot-2 YS2-H, Nikon Co., Ltd., Tokyo).

Insulin Loading The actual insulin loading percentage in enteric microspheres was determined in the following way. About 5 mg of microspheres was precisely weighed and dissolved in 2 ml of methanol for further dissolution of enteric polymer in a glass vial. Then, 2 ml of pH 3.0 citrate buffer was added to the sample solution to precipitate the enteric polymer. The insulin solution containing suspended polymer was centrifuged at 3000 rpm for 10 min. The concentration of the bovine insulin of supernatant was determined using the HPLC method: Twenty microliters 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

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reversed phase column (Cosmosil 5C18-AR, 4.6×150 mm, Nakalai Tesque Co., Ltd., Tokyo). The following mobile phase systems were used: A, 0.1% trifluoroacetic acid (TFA) in H_2O; B, 0.1% TFA in acetonitrile. A linear gradient was used: phase B from 30% to 50% (10 min). The flow rate was 1.5 ml/min and the wavelength was set at 220 nm. Loading was calculated from the weight of the initial microspheres and the amount of drug incorporated.

For insulin-loaded enteric microspheres containing lauric acid as an enhancer, the insulin loading was determined simultaneously. Lauric acid incorporated into these microspheres was extracted by the same method as insulin, and lauric acid concentration was determined using HPLC method. HPLC was performed using reversed phase column (Cosmosil 5C18-AR, 4.6×150 mm, Nakalai Tesque Co., Ltd.) with acetonitrile: 5 mM phosphoric acid (70 : 30) as the mobile phase. The flow rate was 1.5 ml/min, the wavelength was set at 205 nm and the column was operated at 40 °C.

***In Vitro* Insulin Release from Microspheres** The *in vitro* release profile of insulin from enteric microspheres was examined as follows. Microspheres corresponding to 1 mg of insulin were suspended in 10 ml of buffer solution containing 0.02% of Tween 80 and sodium azide, and shaken horizontally at 50 rpm at 37 °C. Tween 80 was added to prevent microspheres from coagulating during the release test. Sodium azide was also added to avoid bacterial growth in the dissolution medium. At predetermined intervals, 150 μl of the suspension was taken as a sample, centrifuged (6400 rpm, 5 min) and the concentration of the supernatant was analyzed by the HPLC method described above.

***In Vivo* Experiment** Normal male Wistar rats (8 weeks of age, weighing 170—180 g) were fasted for 20 h. After insulin and lauric acid-loaded enteric microspheres (corresponding to 50 I.U./kg insulin) were suspended in 0.2 ml of pH 3.0 citrate buffer dissolved in 0.1% CMC-Na and administered to the normal rat rectum, the glucose levels were measured periodically. Assay of glucose levels in plasma was performed using a Glucose-test kit (Wako Pure Chemicals Co., Ltd., Osaka, Japan).

Results and Discussion

Effect of Preparative Conditions on Insulin Loading of AS-HG Microspheres We selected hydroxypropylmethylcellulose acetate succinate (AS-HG type) (AS-HG) as a model enteric polymer based on its solubility in an organic solvent such as methylene chloride or acetone for use in the preparative process. Microencapsulation was performed according to the previous w/o/w emulsion solvent evaporation method.[8] Throughout the pilot study, we found that the critical factor for successful preparation of enteric microspheres was the choice of acidic condition as an external aqueous phase, such as pH 3.0 citrate buffer containing 0.5% of PVA solution. For example, when we used 0.5% of PVA solution (pH 5.5) as an external aqueous phase for microencapsulation, the prepared enteric microspheres were very fragile and ruptured by centrifugation in the recovery process (data not shown). Therefore, in the present study we used pH 3.0 citrate buffer containing 0.5% PVA solution.

The table summarizes the effect of preparative conditions on insulin loading efficiency in AS-HG microspheres, theoretically loaded with 10% of insulin. Formulations 1—4 summarize the effect of internal aqueous phase component on loading efficiency. The mixture of methylene chloride and acetone (4 : 1) as an organic phase was employed in the preparative process since the mixed solvent has high solubility to AS-HG. The choice of 400 μl of 30% acetic acid solution (bovine insulin was completely dissolved in this solution) as an internal aqueous phase resulted in quite high loading efficiency (formulation 3). When water was selected as an internal aqueous phase, loading efficiency was comparatively low (55.5 and 65.5% of loading for each internal aqueous phase volume; formulations 1, 2). The pH of internal aqueous phase seems to be important because the AS-HG membrane bound to this phase may form a rigid membrane structure in an acidic condition. To prove this theory, AS-HG microspheres were prepared by changing the pH of the internal aqueous phase. A small amount of brilliant blue as a marker substance was incorporated and, as shown in Fig. 1, with an increase in pH of the internal aqueous phase from 1.2 up to 5.0, instability of the w/o/w emulsion structure also increased. When pH 1.2 of buffer solution was used as an internal aqueous phase (Fig. 1 left), the appearance of this phase remained unchanged after the second emulsification process, confirming the stability of the prepared w/o/w emulsion. Incorporated dye was not released during this preparative process, whereas with the usage of pH 3.0 or 5.0 buffer solution as an internal aqueous phase, the phase volume expanded with time, dye gradually leaked to the external aqueous phase during the preparative process.

The optimized organic solvent was then determined even though a mixture of methylene chloride and acetone (4 : 1) was employed in the above experiment. In the table, the ef-

Table 1. Effect of Three Phases of Preparative Conditions on Loading Efficiency of Insulin in AS-HG Microspheres Loaded 10% Insulin Theoretically

Formulation No.	(w) Bovine Insulin 20 mg		(o) AS-HG 180 mg/solvent 5 ml	(w) PVA/pH 3.0 citrate buffer 200 ml		Loading efficiency (%)	S.D.
	Solvent	Volume (μl)	Solvent ratio (CH_2Cl_2 : acetone)	PVA conc. (%)	PVA M.W., hydrolyzed (%)		
1	Water	60	4 : 1	0.5	85000—146000, +87—89%	55.5	11.7
2		400	4 : 1	0.5	85000—146000, +87—89%	65.5	6.6
3	30% Acetic acid	400	4 : 1	0.5	85000—146000, +87—89%	87.7	9.6
4		800	4 : 1	0.5	85000—146000, +87—89%	87.0	6.8
5	30% Acetic acid	400	5 : 0	1	85000—146000, +87—89%	88.4	12.4
6		400	4 : 1	0.1	85000—146000, +87—89%	77.9	6.9
7		400	4 : 1	0.5	31000— 50000, +87—89%	75.9	4.6
8		400	4 : 1	0.5	31000— 50000, +98—99%	73.8	5.3
9		400	4 : 1	0.5	85000—146000, +87—89%	87.7	9.6
10		400	4 : 1	0.5	85000—146000, +99%	73.9	7.1
11		400	4 : 1	1	85000—146000, +87—89%	89.3	4.0
12		400	3 : 2	1	85000—146000, +87—89%	89.0	7.3
13		400	1 : 1	1	85000—146000, +87—89%	84.4	3.9

Each value represents the mean of 3—7 experiments.

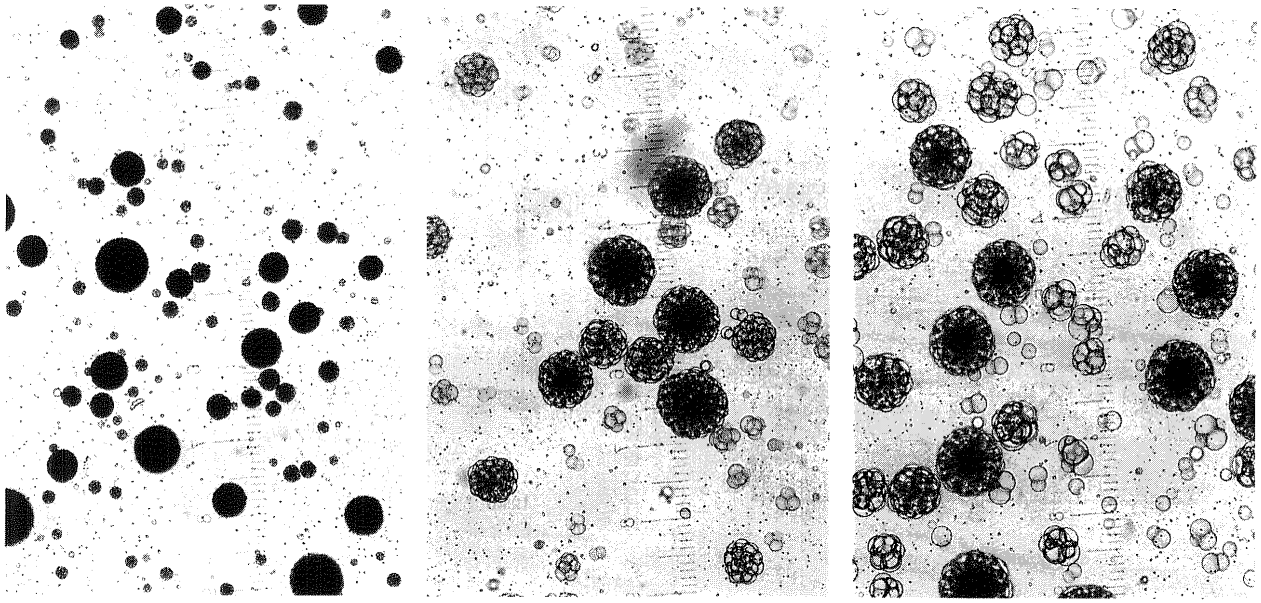

Fig. 1. Optical Microphotographs of AS-HG Microspheres Prepared Using Different pH Buffers as an Internal Aqueous Phase

The smallest scale in the photograph represents 10 μm.

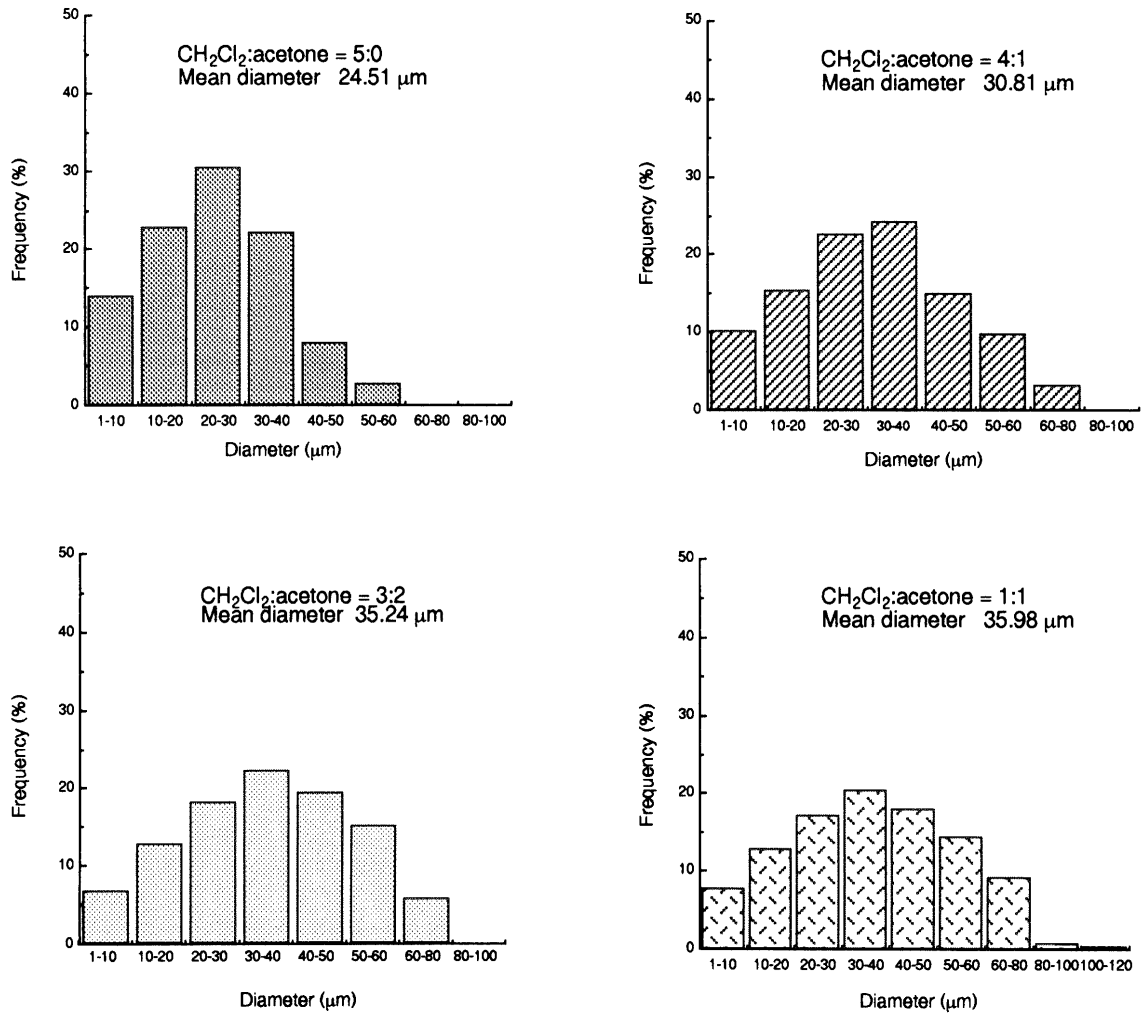

Fig. 2. Size Distributions for AS-HG Microspheres Prepared Using Different Solvent Ratios as an Oleaginous Phase

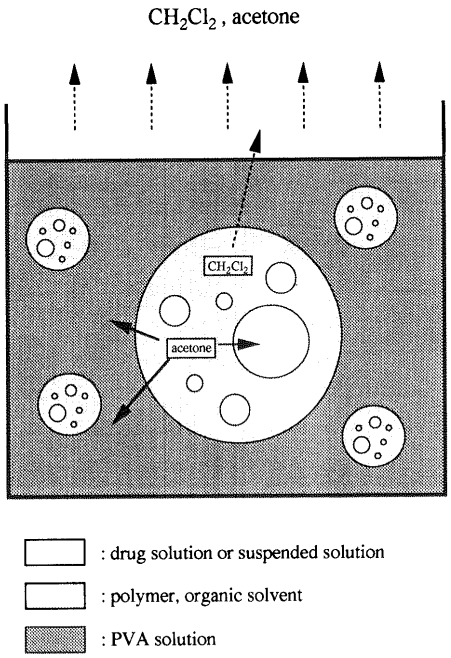

Fig. 3. Scheme of Solvent Evaporation in the Present Encapsulation Method

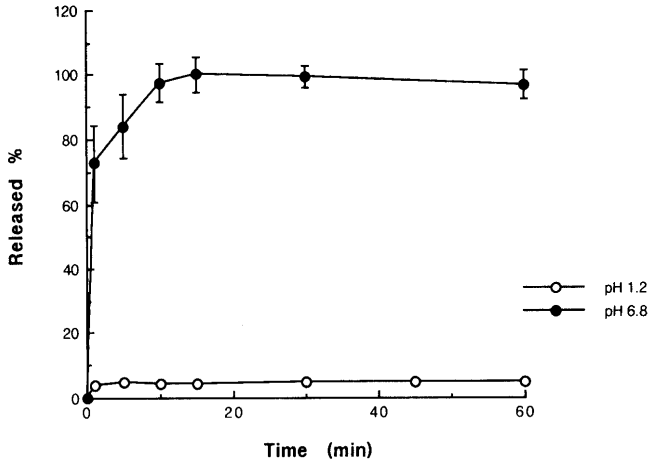

Fig. 4. Insulin Released Profile from AS-HG Microspheres

Each value represents the mean of 2—3 experiments. Microspheres contained 8.9% insulin.

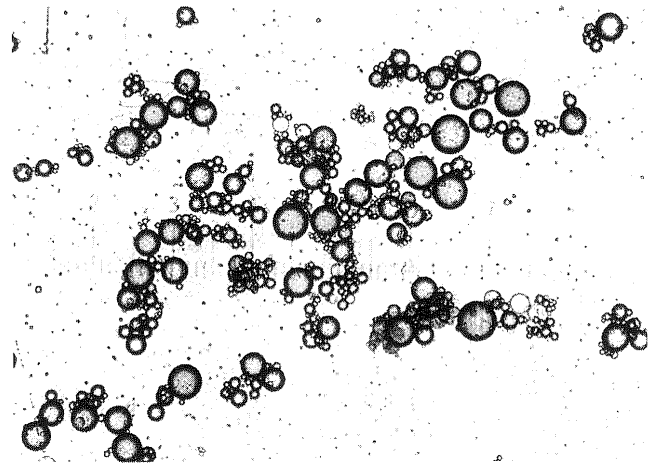

Fig. 5. Optical Microphotographs of AS-HG Microspheres after 3 h Dissolution Test (pH 1.2)

The smallest scale in the photograph represents 10 μm.

fect of solvent component on loading efficiency was examined in formulations 5, 11—13. The ratio of methylene chloride and acetone was changed. In relation to loading efficiency, every batch showed comparatively high loading, but formulation 11 showed the most reproducible yield (90%) or loading. Typical microsphere size distributions for corresponding batches are shown in Fig. 2. As acetone ratio increased, mean diameter also increased. In general, in the microspheres using o/w solvent evaporation method,[10,11] the addition of methanol or acetone to methylene chloride phase as organic solvent phase gave rise to the reduction of tensile strength of o/w at the boundary, thereby reducing the diameter of prepared microspheres. In the present study, on the contrary, the addition of acetone increased the mean diameter of the product. We do not know the precise reason for this phenomenon, one possible reason is the instability of the first (w/o) emulsion in the preparative process. As illustrated in Fig. 3, the acetone easily diffuses not only to the external aqueous phase but also to the internal aqueous phase under these conditions. In fact, when the mixture of acetone and methylene chloride (1 : 1) was sonicated with bovine insulin solution for 1 min to form the first emulsion, its turbidity was much less than that obtained after sonication of bovine solution with methylene chloride. In formulaion 5, the loading efficiencies were comparatively high (88.4%), but less reproducibility was demonstrated (S.D. of loading efficiency was 12.4%). This variability in loading efficiency might be due to poor solubility of the polymer in methylene chloride.

The effect of PVA type on loading efficiency was simultaneously determined, and results are summarized in formulations 7—10. In the microencapsulation study on PLGA as a wall material, the molecular weight of PVA or hydrolyzed % affected *in vitro* characteristics of prepared microspheres. In the present study, molecular weight of 85000—146000, with 87—89% hydrolyzed PVA was the best even though the differences in loading efficiency were not large.

Preparative of Other Enteric Microspheres Regarding other types of hydroxypropylmethylcellulose acetate succinate (AS-MG, AS-LG type), the microencapsulation was performed simultaneously. The loading efficiency was almost the same or a little less than those obtained with AS-HG microspheres (detailed data not shown), although the choice of poly(methyl methacrylate), such as Eudragit L, S as a wall material caused aggregation or flocculation on preparative processes no matter what type of polymer was selected. This failure in preparation of poly(methyl methacrylate) is probably due to the hydrophilic characteristics of acrylic polymer itself and its poor solubilitiy in the organic solvents used in the microencapsulation preparative process.

Release of Bovine Insulin from AS-HG Microspheres Figure 4 shows the release profiles from AS-HG microspheres. The product contained 8.9% insulin and 4.2% lauric acid, respectively. In pH6.8 buffer, comparatively fast releases were observed, suggesting enteric characteristics of microspheres. On the other hand, no release fraction was observed in pH 1.2 buffer. Microspheres prepared using the solvent evaporation method sometimes show burst releases as reported in various articles;[12,13] this is due to the morphology of the drug crystal embedded in or located on the microsphere surface. As shown in Fig. 5, the surface of pre-

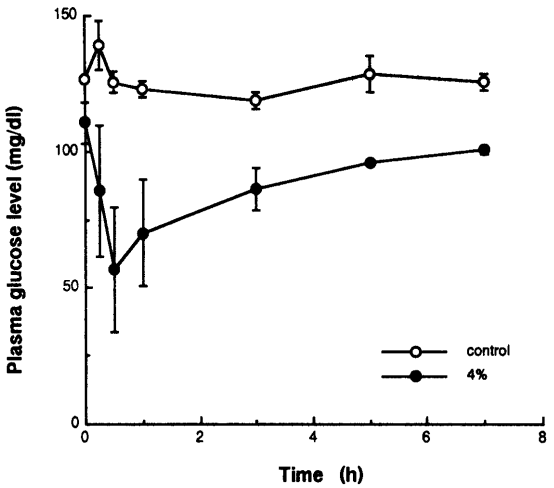

Fig. 6. Plasma Glucose Levels Following Rectal Administration of AS-HG Microspheres Corresponding to 50 I.U./kg Insulin

—○—, the administered saline. —●—, microspheres containing 4% lauric acid. Each value represents the mean of 2—3 experiments.

pared microspheres was monodispersed and smooth, and drug crystals were not observed on the surface at all. This phenomenon well coincided with the fact that no release fraction was observed in pH1.2 buffer. This information shows that enteric microspheres containing bovine insulin were successfully manufactured.

***In Vivo* Evaluation of AS-HG Microspheres Containing Insulin and Lauric Acid** Following administration of insulin (8.9%) and lauric acid (4.2%)-loaded enteric microspheres (corresponding to 50 I.U./kg insulin) to the normal rat rectum, the plasma glucose level became minimal level at 0.5 h and gradually rose to normal as shown in Fig. 6. This suggested that the bovine insulin was released from the microspheres and absorbed through the gastrointestinal tract with the aid of lauric acid in a comparatively short time, and thereby showing a pharmacological effect. Kreuter *et al.*[14] and Guiot and Couvreur[15] demonstrated that microspheres with small diameters administered to the gastrointestinal tract were later located in colon site. This information might suggest that enteric microspheres have the capability of specific delivery to the colon site which seems advantageous for peptide absorption. This microsphere system also has a capability of developing an oral peptide delivery system, and suggests the possibility of the enhancer amount might be minimized since an enhancer or drug was released as concentrated solution. We will study the enhancing mechanism of lauric acid used in the near future.

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