Preparation and Characterization of Polylactic Acid 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 polylactic acid (PLA) microspheres containing bovine insulin as a sparingly water soluble model drug using a water-in-oil-in-water (w/o/w) emulsion solvent evaporation method. The preparative conditions were optimized. Employment of smaller internal aqueous phase volume (50 μl or 100 μl) in the manufacturing process, resulted in the high loading efficiency (over 95% of theoretical insulin loading efficiency). The addition of 10% (w/v) NaCl to the external aqueous phase (0.5% polyvinyl alcohol solution) reduced loading efficiency compared to the case where no NaCl was added to the external phase. The mean volume diameter for prepared PLA microspheres was in the region of 15—25 μm in all cases. PLA microspheres containing 5% and 10% insulin theoretically exhibited burst release in the initial stage. After a three week dissolution test, the surface of the microspheres became more porous due to the degradable characteristics of PLA polymer itself. Nevertheless, about 80% of the insulin still remained undegraded in PLA microspheres. Finally, insulin-loaded PLA microspheres (corresponding to 4 I.U. insulin) were administered to normal rats subcutaneously, and the pharmacological effect (a decrease in serum glucose level) was demonstrated.

Key words microsphere; polylactic acid; bovine insulin; burst release; sustained-release; serum glucose level

The sustained delivery of proteins, peptides, or active reagents which are difficult or costly to handle repetitively, is advantageous for administration to humans or animals. Scientists have been tested various methods of sustaining the release of those compounds. One method available to extend the delivery of those active drugs is the production of microspheres consisting of a biodegradable polymer[1] and an active agent.[2] A once a month injectable poly-(lactide-co-glycolide) (PLGA) microsphere of leuprolide acetate prepared by a w/o/w emulsion solvent evaporation method was recently developed as a commercial product.[3] This microsphere system has not only advantages of biocompatibility and biodegradation of the polyester polymer, but also the capability of controlling particle size, and might be attractive for sustained delivery of active compounds. This w/o/w emulsion solvent evaporation method have been applied to highly water soluble drugs in general,[4] but few studies have been undertaken to microencapsulate the sparingly water soluble active drug into this polyester matrix. In the present study, therefore, an attempt was made to produce polylactic acid (PLA) microspheres containing bovine insulin as a sparingly water soluble model drug using a w/o/w emulsion solvent evaporation method, and *in vitro* preparative conditions were optimized. The pharmacological effect of insulin-loaded PLA microspheres was also evaluated.

Experimental

Materials Polylactic acid (PLA; Mw = 58000) was purchased from Medisorb Technologies (Cincinnati, OH, U.S.A.). Polyvinyl alcohol (PVA; 87—89% hydrolyzed, Mw = 85000—146000) was supplied by Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Bovine insulin was purchased from Sigma Co., Ltd., (St.Louis, MO, U.S.A.). The diameter of powdered bovine insulin was confirmed to be under 10 μm by electron microscopic observation. Other reagents were all of special grade.

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Preparation of PLA Microspheres Containing Bovine Insulin A w/o/w emulsion solvent evaporation method was adopted; the procedure was essentially the same as used previously.[5] First of all, 11.1 and 22.2 mg of bovine insulin (corresponding to 5 and 10% of theoretical loading, respectively) was dispersed in 50—800 μl purified water. This suspension as the internal aqueous phase was emulsified with 5 ml of methylene chloride containing 200 mg of PLA for 1 min using an ultrasonic disruptor (UD-200; Tomy Seiko Co., Ltd., Tokyo). This w/o emulsion was poured into 200 ml of 0.5% (w/v) PVA solution. Emulsification was continued using a homogenizer (NS-60, Nichion Irikakikai Co., Ltd. (Tokyo, Japan) at 3000 rpm for 1 min. Zero or 10% (w/v) of NaCl was added into the 0.5% (w/v) PVA solution as the external aqueous phase. This dispersion was gently agitated in a 500 μl beaker on a stirring plate containing a 3.75 cm stirring bar for 4 h at room temperature. The microspheres were collected by centrifugation at 3000 rpm for 10 min. The obtained microspheres were washed with water and freeze dried (FD-1, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) for at least 12 h.

The microsphere yield was determined as the percentage of weight of the recovered microspheres after drying divided by the initial amount of PLA and the drug employed. Morphology study was performed using a scanning electron microscope (SEM) (Akashi WS 250, Tokyo).

Insulin Loading The actual insulin loading percentage in PLA microspheres was determined in the following way. About 10 mg of microspheres were precisely weighed and dissolved in 2 ml of acetonitrile for further dissolution of PLA in a glass of vial. The polymer solution containing suspended insulin was centrifuged at 3000 rpm for 5 min. The acetonitrile was decanted and replaced with fresh acetonitrile; this procedure was repeated three times. The remaining pellet was dissolved in 5 ml of pH 7.4 Tris buffer containing 0.1% TFA (trifluoroacetic acid). The concentration of the bovine insulin solution 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-R6A) and reversed phase column (Asahipak ODP-50 6D, 6.0 × 150 mm, Asahi Chemical Industry Co., Ltd., Tokyo, Japan). The mobile phase was a mixture of acetonitrile: 0.3% (v/v) triethanol amine solution (adjusted to pH 2.0 by adding phosphoric acid) = 28 : 72. The flow rate was 2.0 ml/min; the wavelength was set at 220 nm and the column was operated at 40 °C. Loading was calculated from the weight of the initial microspheres and the amount of drug incorporated.

***In Vitro* Insulin Release Test** The *in vitro* release profile of insulin from PLA microspheres was determined as follows. Microspheres cor-

responding to 1 mg of insulin were suspended in 5 ml of Tris buffer (pH 7.4) containing 0.02% (w/v) of Tween 80 and sodium azide, and shaken horizontally at 75 rpm at 37 °C. At predetermined intervals, 200 μl of the suspension was taken as a sample, centrifuged (3000 rpm, 5 min) and the concentration of the supernatant was analyzed by the HPLC method described above.

Stability of Insulin in PLA Microspheres The stability of bovine insulin in PLA microspheres was determined as follows: after release test (0, 5, 10 and 20 d), microspheres were filtered, and dried. Then bovine insulin remaining in the microspheres was extracted and confirmed using the HPLC method as described in the "Insulin loading" in the experimental section. Mass balance characteristics were also examined.

***In Vivo* Experiment** Normal male Wistar rats (7—8 weeks of age) were given with free access to water and fasted for 20 h. After insulin-loaded PLA microspheres (corresponding to 4 I.U. insulin) were suspended in 0.3 ml of saline and administered to normal rats subcutaneously, the glucose levels were also measured periodically. Assay of glucose levels in plasma was performed using a Glucose-test kit (Wako Pure Chemicals, Osaka, Japan).

Results and Discussion

Effect of the Internal Aqueous Phase Volume and NaCl Added in the External Aqueous Phase on Insulin Loading of PLA Microspheres We previously reported that smaller internal aqueous phase volume (50 or 100 μl) and the addition of 5 or 10% (w/v) of NaCl into the PVA solution as the external aqueous phase, significantly enhanced the loading efficiency of water soluble dyes[6] or sodium salt anesthetics[7] into PLA microspheres. Therefore, we first examined the effect of the internal aqueous phase volume on insulin loading efficiency in the microspheres in the presence or absence of NaCl in the external aqueous phase. Tables 1 and 2 show the effect of the internal aqueous phase volume on loading efficiency of insulin in PLA microspheres theoretically loaded with 5 and 10% of insulin, respectively. In the internal aqueous phase volume, less volume was found to be advantageous in obtaining high loading efficiencies. In particular, high loading efficiencies over 95% were obtained when 50 μl or 100 μl of the internal aqueous phase volume was employed in the absence of NaCl in the external aqueous phase. This phenomenon did not coincide with our previous papers,[6,7] in which the addition of 5 or 10% of NaCl to the external aqueous phase (0.5% polyvinyl alcohol solution) was essential for obtaining high drug loading efficiencies even though smaller internal aqueous phase volume was also advantageous for high loading efficiencies. In this study, the addition of 10% (w/v) NaCl to the external PVA aqueous solution phase instead reduced loading efficiencies compared to the case in which NaCl was not added to the external aqueous phase. The situation of the internal aqueous phase state was different from our previous cases in which incorporated drugs were present as a solution in the internal aqueous phase. In fact, in the present study, most bovine insulin powders were suspended in the internal aqueous phase (pH 6.5) as fine crystals as shown in Fig. 1a, since the insulin solubility at neutral pH is comparatively low (pK_a of bovine insulin is 4.5). The molecular weight of insulin was also much larger than other drugs (soluble dyes or anesthetics) employed in previous studies.[6,7] Therefore, the osmotic pressure of the internal aqueous phase containing bovine insulin powders as a suspension seemed to be much lower than in a case in which bovine insulin powders were present as a perfect solution. This comparatively lower osmotic pressure in the internal aqueous phase was apparently one reason for lower loading since the osmotic pressure caused by 5 or 10% (w/v) of the NaCl in the external aqueous phase would be much larger than that of the internal aqueous phase. This osmotic pressure discrepancy between the

Table 1. Effect of the Internal Aqueous Phase Volume and NaCl Concentration in the External Aqueous Phase on Loading Efficiency of Insulin in PLA Microspheres Loaded with 5% of Insulin, Theoretically

Treatment number	Theoretical loading of insulin (%)	Internal aqueous phase volume (μl)	NaCl concentration (%)	Experimental loading of insulin (%)	Loading efficiency (%)
1	5	50	0	4.85	97.0
2	5	50	10	4.32	86.4
3	5	100	0	3.94	78.8
4	5	100	10	3.68	73.6
5	5	200	0	3.47	69.4
6	5	400	0	3.25	65.0

Each value represents the mean of three experiments.

Table 2. Effect of the Internal Aqueous Phase Volume and NaCl Concentration in the External Aqueous Phase on Loading Efficiency of Insulin in PLA Microspheres Loaded with 10% of Insulin, Theoretically

Treatment number	Theoretical loading of insulin (%)	Internal aqueous phase volume (μl)	NaCl concentration (%)	Experimental loading of insulin (%)	Loading efficiency (%)
1	10	100	0	9.50	95.0
2	10	100	10	7.91	79.1
3	10	200	0	8.06	80.6
4	10	200	10	5.33	53.3
5	10	400	0	7.36	73.6
6	10	400	10	4.77	47.7
7	10	800	0	4.13	41.3
8	10	800	10	3.79	37.9

Each value represents the mean of three experiments.

internal and external aqueous phases might be the major reason for the decrease in insulin loading efficiencies caused by the addition of NaCl to the external aqueous phase. Figures 1b and 1c show optical photographs containing 10% of bovine insulin theoretically prepared using 400 μl of the internal aqueous phase volume in the absence or presence of 10% of NaCl, respectively. A large quantity of internal aqueous droplet phases was observed in the absence of NaCl in the external aqueous phase (Fig. 1b), while clear internal aqueous droplet phases were not observed (Fig. 1c). This phenomenon may suggest that the internal aqueous droplet phases containing insulin powders were expelled by the excess osmotic pressure caused by NaCl, thereby decreasing insulin loading as described in the above paragraph.

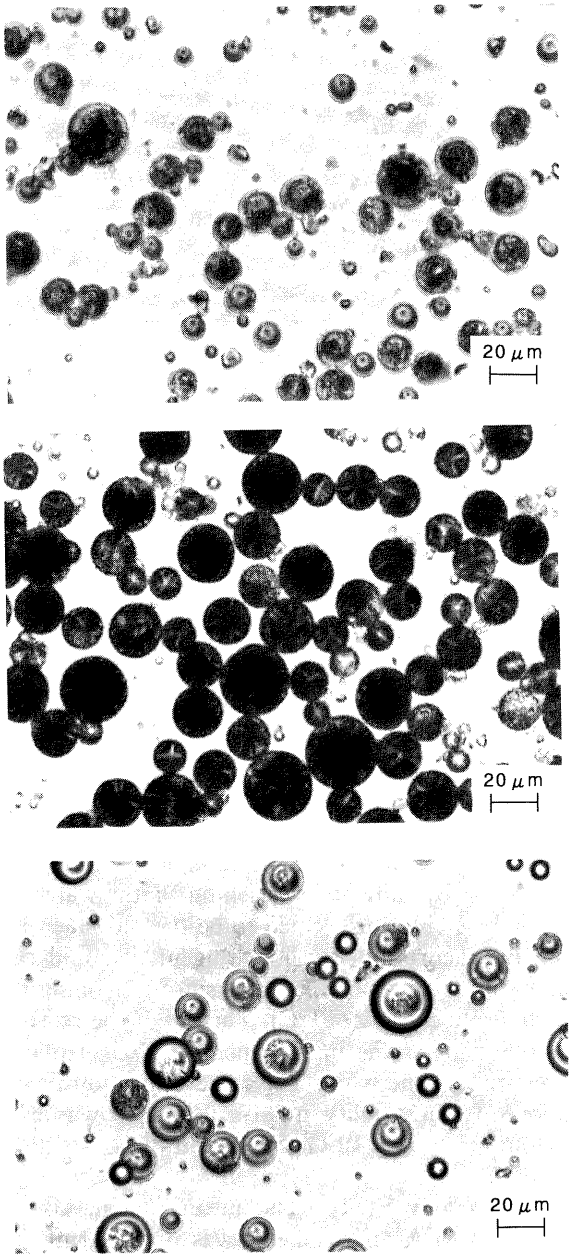

Fig. 1. Optical Micrographs of PLA Microspheres Loaded with 10% of Insulin, Theoretically, Prepared Using 50 μl of the Internal Aqueous Phase in the Absence of NaCl (a), and Using 400 μl of the Internal Aqueous Phase in the Absence (b) or Presence (c) of NaCl in the External Aqueous Phase, Respectively

On the contrary, in our previous studies, soluble dyes[6] and sodium salt anesthetics[7] were present in the internal aqueous phase as a perfect solution state, which gave comparatively high osmotic pressure of the internal aqueous phase. Therefore, comparatively high osmotic pressure provided by the addition of NaCl to the external aqueous phase might balance against a comparatively high osmotic pressure of the internal drug solution phase. The osmotic balance seemed to be very important for obtaining stable water-in-oil-in-water (w/o/w) emulsion, and resulting high loading efficiency.

Another reason for the high loading efficiency in the present study was probably the small diameter particles used for insulin powder. As described in the experimental section, the smaller size of insulin particles is essential for high loading since the particle sizes of the drug powder must be smaller than that of the targetted size of prepared microspheres.

Therefore, the method employed in the study could be applicable to other sparingly water soluble drugs if drugs were well micronized, and their size was much smaller than those of targetted microspheres.

Release of Bovine Insulin from Prepared PLA Microspheres Figures 2a and 2b show the release profiles

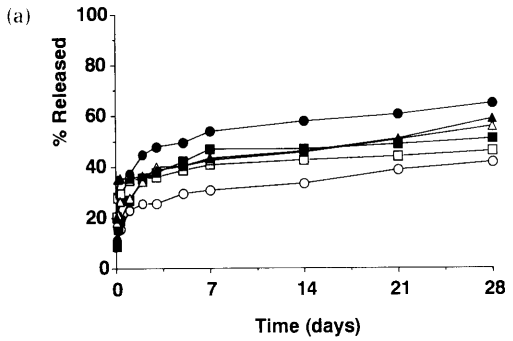

Fig. 2a. Effect of the Internal Aqueous Phase Volume and NaCl Concentration in the External Aqueous Phase on Release Profiles of Insulin from PLA Microspheres Loaded with 5% of Insulin, Theoretically

(○) 50 μl, 0%, 4.85%, (●) 50 μl, 10%, 4.32%, (□) 100 μl, 0%, 3.94%, (■) 100 μl, 10%, 3.68%, (△) 200 μl, 0%, 3.47%, (▲) 400 μl, 0%, 3.25%. For example, 50 μl, 0%, 4.85% means that the product prepared when the internal aqueous phase volume was 50 μl and 0% (w/v) of NaCl, had actual loading of 4.85% of insulin. Each point represents the mean of three experiments.

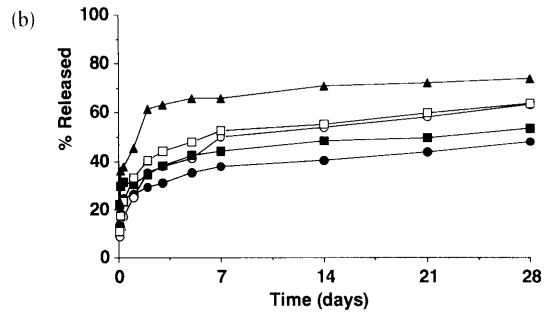

Fig. 2b. Effect of the Internal Aqueous Phase Volume and NaCl Concentration in the External Aqueous Phase on Release Profiles of Insulin from PLA Microspheres Loaded with 10% of Insulin, Theoretically

(●) 100 μl, 0%, 9.50%, (○) 100 μl, 10%, 7.91%, (■) 200 μl, 0%, 8.06%, (□) 200 μl, 10%, 5.33%, (▲) 400 μl, 0%, 7.36%. For example, 100 μl, 0%, 9.5% means that the product prepared when the internal aqueous phase volume was 50 μl and 0% (w/v) of NaCl, had actual loading of 9.50% of insulin. Each point represents the mean of three experiments.

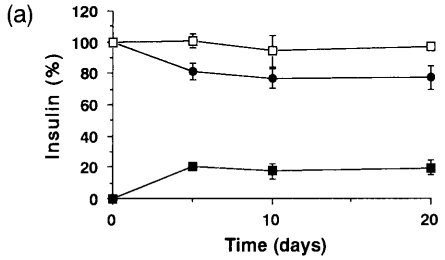

Fig. 3a. Time Courses of Residual % of Insulin in PLA Microspheres (●), % of insulin released from microspheres (■) and total % of insulin (□). Each point represents the mean ± S.D. of three experiments.

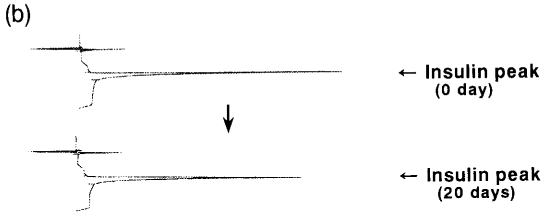

Fig. 3b. Chromatograms of Insulin Extracted from PLA Microspheres

from PLA microspheres, theoretically loaded with 5 and 10% insulin, respectively. Burst releases were observed to some extent with all PLA microspheres, following which, there was an additional slow release phase. This fact was similar to that in our previous report.[5] Smaller internal aqueous phase volume seemed to be advantageous for controlling the initial burst release. The largest burst releases were shown in both 5 and 10% of loading in those PLA microspheres prepared with 400 μl of the internal aqueous phase. This faster release of insulin might have been due to the relatively thinner thickness of PLA matrix membrane, if we assume obtained microspheres to be a perfect monolayer type microcapsule. Another possible cause of the burst release may be the fast release of insulin crystal embedded near the surface.

Stability of Bovine Insulin in PLA Microspheres We recently reported a preparative method for PLA microspheres containing bovine insulin by an oil-in-oil (o/o) emulsion solvent evaporation method.[8] High loading efficiencies were demonstrated in that article. Nevertheless, the degradation of bovine insulin was observed during a 7 d dissolution test. Therefore, the stability of bovine insulin incorporated in the microspheres obtained by the w/o/w emulsion solvent evaporation method seems very important and interesting. Figure 3a represents the result of a mass balance study related to PLA microspheres containing 9.5% of insulin. Almost 20% of bovine insulin was released into the buffer medium while almost 80% remained in the microspheres after a 20 d dissolution test.

Bovine insulin incorporated into PLA microspheres prepared by the w/o/w emulsion solvent evaporation method were found to be stable since no degraded peak was observed after the 20 d dissolution test (Fig. 3b). The chromatographic profile for extracted insulin was exactly the same as that of intact insulin powder solution. Further, the chromatographic profile of intact insulin solution was not also changed by 1 min sonication (data not shown).

These findings in the present study were quite different

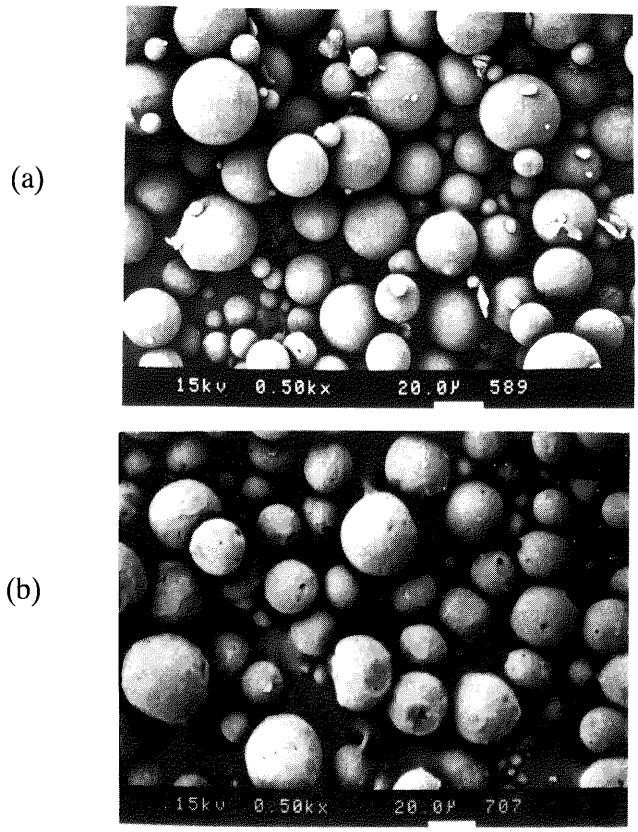

Fig. 4. Scanning Electron Micrographs of PLA Microspheres Containing 9.5% of Insulin after Release Test
(a) 0 d, (b) 20 d.

from our previous report[8] in which bovine insulin degradation was observed during a 7 d dissolution test related to PLA microspheres prepared by an oil-in-oil (o/o) emulsion solvent evaporation method. The discrepancy probably comes from differences in the method of preparation. This instability of insulin in PLA microspheres prepared by o/o method might be caused by many of the components employed in the preparation process: aluminum tristearate as a dispersing agent, mineral oil, hexane, or acetonitrile, although we did not ascertain the precise reason. In contrast, in the present experiment using a w/o/w emulsion solvent evaporation method, PVA solution used in the external aqueous phase could be removed by adequate washing with water. Though methylene chloride seemed to be the only factor destroying the incorporated reactive agent, it could be removed easily at room temperature since its boiling point was comparatively low (39 °C). This might be reason for the stability of bovine insulin incorporated in microspheres prepared by the w/o/w solvent evaporation process. The above insulin stability in polyester matrix was also proved in another type of PLGA85/15 and PLGA50/50 (data not shown).

Morphology Study The mean volume diameter for prepared PLA microspheres was obtained in the region of 15—25 μm. Figure 4 shows scanning electron micrographs of PLA microspheres containing 9.5% of bovine insulin. After the 20 d dissolution test, the surface of the microspheres had become slightly more porous (Fig. 4b) than just after the dissolution test begun (Fig. 4

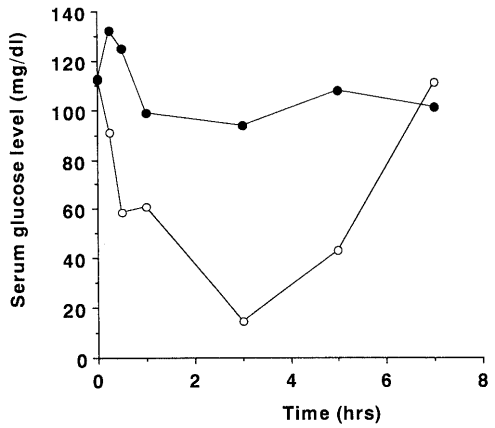

Fig. 5. Serum Glucose Levels following Subcutaneous Administration of PLA Microspheres Corresponding to 4 I.U. Insulin

(●) control level, (○) PLA microspheres. Each point represents the mean of two experiments.

a). The pores may be formed by the faster release of insulin crystal embedded near the surface.

***In Vivo* Study** After insulin-loaded PLA microspheres (corresponding to 4 I.U. bovine insulin) were administered to normal rats subcutaneously, the serum glucose level became minimum level at 3 h after administration and gradually rose up to normal level (Fig. 5). This suggested that the bovine insulin incorporated in PLA microspheres actually showed a pharmacological effect (hypoglycemic response) even though the present experiment was just a brief pilot study.

Conclusion

In conclusion, we were successful in optimizing a method to efficiently entrap bovine insulin into PLA microspheres using a w/o/w emulsion solvent evaporation method. The insulin incorporated in the microspheres was stable for at least three weeks. This preparation method and the preparative condition used will be applied to other sparingly water soluble drugs or proteins in the near future.

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