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Optimum formulation for sustained-release insulin

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Abstract

Our aim was to prepare an optimum formulation for a sustained-release preparation of insulin using biodegradable polymer composed of co-poly(D,L-lactic/glycolic) acids (PLGA)(L/G ratio: 50/50). Various kinds of PLGA microcapsules containing 3% insulin were administered subcutaneously (250 U/kg) as a single dose to rats with streptozotocin-induced diabetes, and plasma insulin levels were monitored. The following results were obtained. (1) Glycerin and water were suitable additives to prepare a reproducible injectable formulation. (2) Addition of zinc compounds was essential to diminish rapid insulin release and six-fold molar excess of ZnO to insulin was desirable. (3) The size of insulin particles showed the following order: human insulin > lyophilized human insulin > Zn-free human insulin. Zn-free insulin was similar to lyophilized insulin with respect to control of rapid release, so a smaller particle size was essential. (4) The size of the microcapsules also affected the release of insulin. With larger microcapsules (~30 μm), there was gradual release and a significant second phase of insulin release, while smaller microcapsules did not allow sustained release. Some variation in microcapsule size contributed to more constant and sustained release. (5) Based on the insulin release profile in vivo, a suitable molecular weight for PLGA was around 6000. The biological activity of insulin extracted from the formulation was similar to that of normal insulin. These experiments allowed us to prepare a desirable sustained-release insulin formulation.

Keywords: Controlled release; Diabetes mellitus; Co-poly(D,L-lactic/glycolic) acid (PLGA); Insulin; Zinc

1. Introduction

There is a continuing search for improved insulin formulations in order to reproduce as closely as possible the physiological pattern of insulin secretion and thereby minimize the complications of diabetes mellitus (DM). Patients with insulin-dependent DM (IDDM) usually have to boost the basal insulin supply once or twice a day, in addition to doses at mealtimes, because they need a relatively constant basal level of insulin to achieve a near physiological pattern of insulin secretion. Such therapy has been shown not only to improve their general condition, but also to reduce the incidence of diabetic complications (Kawamori, 1994; Haak, 1999). If an insulin formulation was available that could release the drug in a controlled fashion for longer periods without any burst of rapid release, patients would be freed from the need to administer multiple doses.

We have investigated the use of microcapsules to make a preparation that releases insulin in a controlled fashion for a long period without any rapid release phase. In order to achieve this purpose, we have employed co-poly(D,L-lactic/glycolic) acid (PLGA). This

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is a biodegradable injectable polymer that is useful for sustained-release preparation of drugs. As previously reported (Takenaga et al., 2002), addition of the appropriate amount of glycerin significantly improves the turbidity of a mixture of insulin and PLGA, and the resultant formulation shows dramatic reduction of the initial rapid release phase. PLGA microcapsules containing insulin without any additives increased the plasma insulin level to 2440.5 μU/ml (6 h) when administered to streptozotocin-induced diabetic rats at 250 U/kg, but the level was only 84.9 μU/ml in rats treated with the best formulation. Further study revealed that little insulin was localized at the surface of the microcapsules when glycerin was added during the preparation of a microcapsule formulation (Yamaguchi et al., 2002). This raised the possibility that insulin and PLGA formed a complex and showed close interaction through addition of glycerin to the oil phase, with this relationship being maintained after addition of the water phase. The appropriate amount of glycerin, ethanol, or water was added in an attempt to improve the turbidity of the oil phase. In each case, homogenization of the insulin/PLGA mixture into compact particles was confirmed (Yamaguchi et al., 2002), but there was still a possibility that other compounds would make a better insulin formulation. Other factors also needed to be assessed, such as the molecular weight of PLGA and the size of the microcapsules.

In this study, we aimed to prepare an optimum formulation of insulin in microcapsules for controlled release over a much longer period. In order to evaluate the insulin-release profile, various formulations were tested in rats with streptozotocin-induced diabetes.

2. Materials and methods

2.1. Reagents

PLGA (L/G ratio: 50/50, mean molecular weight: 4100 (4k), 5800 (5k), 6600 (6k), and 7900 (8k)) was synthesized and kindly provided by Wako Pure Chemical Industries Ltd. (Osaka, Japan). The characteristics of the PLGAs tested are shown in Table 1.

Recombinant human insulin, streptozotocin, and other chemicals were purchased from Wako. For cell culture, Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Company, St. Louis, MO, USA) was supplemented with fetal bovine serum (FBS, Sanko Junyaku Co., Ltd., Tokyo, Japan) and antibiotics (Gibco, Grand Island, NY, USA).

Table 1
The four PLGAs used in this study

PLGA	Mw	Mn	Terminal –OH ratio	
			LA	GA
4k	4100	3000	36.11	63.89
5k	5800	3316	48.84	51.16
6k	6600	3480	56.82	43.18
8k	7900	3780	38.36	61.64

Mw: weight average molecular weight, Mn: number average molecular weight Mw and Mn were measured by GPC (gel permeation chromatography).
–OH ratio; terminal –OH ratio of LA (lactate) and GA (glycolate). The LA/GA ratio and –OH ratio were both determined by NMR.

2.2. Insulin-containing PLGA microcapsules

Zn-free insulin was used, as reported previously (Takenaga et al., 2002). Zn-free insulin was obtained by lyophilization after dialysis. Human insulin and lyophilized insulin were also tested.

Insulin (3%)-containing PLGA microcapsules were prepared by the solvent evaporation method, as reported previously (Takenaga et al., 2002). For example, 240 mg of insulin, 39.9 mg of zinc oxide, and 7.84 g of PLGA dissolved in 8 ml of CH_2Cl_2 were mixed and agitated vigorously to form an S/O suspension. Then the appropriate amount of one of the test additives was added to improve the turbidity of the suspension. This solution was added to 1.6 l of a 1.0% (w/v) polyvinyl alcohol (PVA, GOHSENOL:EG-25, average molecular weight: 45,000, Nippon Gosei Kagaku Inc., Tokyo, Japan) solution while stirring. The resulting emulsion was stirred at 1200 rpm for 3 h to evaporate the organic solvent and obtain microcapsules. The microcapsules were washed three times with distilled water by centrifugation and then sieved with a 125 μm screen to remove larger particles. Next, mannitol was added to prevent aggregation of the microcapsules, which were then lyophilized. When the particle size was determined using a particle analyzer (Multisizer IIE, Beckman Coulter, Tokyo, Japan), the mean diameter was 23.45 ± 13.61 μm (mean ± S.D., $N = 8$). All of the prepared formulations were stored at 4 °C until use. A list of the formulations tested in this study is shown in Table 2.

Table 2
Insulin-containing PLGA microcapsule formulations tested in this study

Formulation	Insulin	PLGA	ZnO	Additive	*
		Mean Mw	Fold mole to insulin		
A	Zn-free insulin	6600 (6k)	1.2	Glycerin	
B	Zn-free insulin	6600	0	Glycerin water	Fig. 1
C	Zn-free insulin	6600	1.2	Glycerin water	Figs. 1 and 2
D	Zn-free insulin	6600	6	Glycerin water	Fig. 1
E	Zn-free insulin	6600	12	Glycerin water	Figs. 1–5
F	Zn-free insulin	4100 (4k)	1.2	Glycerin water	Fig. 2
G	Zn-free insulin	4100	12	Glycerin water	Fig. 2
H	Zn-free insulin	7900 (8k)	1.2	Glycerin water	Fig. 2
I	Zn-free insulin	7900	12	Glycerin water	Fig. 2
J	Human insulin	6600	12	Glycerin water	Fig. 3
K	Lyophilized human insulin	6600	12	Glycerin water	Fig. 3
L	Zn-free insulin	5800 (5k)	12	Glycerin water	Fig. 5

The insulin content of each microcapsule formulation was determined after extraction with CH_2Cl_2 and 0.01N HCl according to the method of Lowry et al. (1951), using the insulin as a standard. It was confirmed that insulin could be extracted from the microcapsules with a high recovery rate (>98%). The efficiency of encapsulation was >90% for each formulation.

2.3. Electron microscopy

Insulin-containing microcapsules and normal insulin were examined under a SEM 4300 scanning electron microscope (Hitachi, Ibaraki).

2.4. Animals

Seven-week-old male Wistar rats (220–240 g) were purchased from SLC Experimental Animals (Shizuoka, Japan). The animals were housed at a constant temperature ($23 \pm 1\,^{\circ}C$) and humidity (50–60%) with free access to a standard diet and water. The animal room had a 12-h light/dark cycle (lights on from 6:30 to 18:30 h). The study protocol was approved by the Animal Experimentation Committee of St. Marianna University.

2.5. Animal experiments

Animal experiments were conducted using rats with streptozotocin (STZ)-induced diabetes. Three days after the rats received an intravenous injection of 60 mg/kg of STZ dissolved in 10 mM citrate–citrate Na buffer (pH 4.5), they were given a single subcutaneous injection of insulin-containing PLGA microcapsules. Just before administration, the microcapsule insulin formulation was dispersed (20%, w/v) with 5% mannitol (pH 6.5) containing 0.5% carboxymethylcellulose and 0.1% Tween 80. Blood samples were collected from the inferior ophthalmic vein before and after treatment to measure the blood glucose and plasma insulin concentrations. All samples were taken in the morning (9:00–11:30 h) until the 14th day, except for that at 6 h after dosing. All animals were housed with free access to a standard diet and water. The plasma insulin level was determined using an RIA kit (Shionogi Seiyaku Co. Ltd., Osaka, Japan), and the blood glucose level was determined by the glucose oxidase method using a glucose analyzer (Glucoster-M, Sankyo Co., Ltd., Tokyo, Japan) immediately after blood collection.

The STZ-treated rats were all hyperglycemic (a morning blood glucose concentration of more than 290 mg/dl) and their plasma insulin levels ranged from 3.0 to 5.6 μU/ml. The insulin level in normal untreated animals was between 20.4 and 51.4 μU/ml, and their blood glucose levels were between 98 and 150 mg/dl (morning nonfasting level).

2.6. Cell growth assay

NIH 3T3 cells ($1 \times 10^3\,ml^{-1}$) were suspended in culture medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics)

and seeded into a 24-well tissue culture plate (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Insulin extracted from microcapsules or unencapsulated insulin was added to the cells, and culture was done for 24 h in a humidified atmosphere of 5% CO_2 in air at 37 °C. Then cell growth was assessed by the [^{3}H]thymidine ([methyl-1′,2′-^{3}H]thymidine (1.40 TBq/mmol)], Amersham, UK) incorporation assay. Radioactivity was counted with a scintillation counter (MicroBeta TRILUX, Pharmacia, Sweden).

Insulin was extracted from microcapsules by addition of an excess of acid and was neutralized by NaOH. Then the extracted insulin was subjected to further lyophilization.

2.7. Statistical analysis

Results are represented as the mean (±S.D.). Determination of the significance of differences was done using the Mann–Whitney *U*-test, and $P < 0.05$ was taken as indicating significance.

3. Results and discussion

3.1. Additive

Glycerin was found to be an effective additive. However, a formulation prepared with glycerin as the only additive (Formulation A, Table 2) required a large volume of vehicle for suspension. The presence of glycerin may have caused the microcapsules to swell soon after coming into contact with the aqueous medium. In turn, this might have contributed to the suppression of rapid release. It was also difficult to subcutaneously administer the formulation to diabetic animals under the same conditions compared with other formulations and this resulted in a large standard deviation of insulin release in vivo. When eight diabetic animals were tested (Formulation A; 250 U/kg s.c.), the plasma insulin level was 86.7 ± 54.0 μU/ml, 108.4 ± 91.4 μU/ml, and 84.9 ± 85.1 μU/ml at 1, 2, and 6 h after injection, respectively (mean ± S.D.).

Water was also an effective additive for improving the turbidity of the oil phase containing insulin and PLGA. However, the later peak of insulin on day 6 was increased to 300.1 μU/ml, which was high enough to have a significant hypoglycemic effect. Insulin might have been concentrated more centrally in the microcapsules compared with glycerin alone as the additive. However, an advantage of water is that it can be evaporated by lyophilization, and another is that it reduces the swelling of microcapsules seen after the addition of glycerin alone. Use of both additives enabled us to prepare a reproducible injectable formulation of insulin.

3.2. Addition of zinc

However, this formulation still allowed significant early release of insulin (Fig. 1). When a dose of 250 U/kg of insulin in microcapsules (Formulation C) was given subcutaneously to diabetic rats, the mean plasma insulin level was 237.1 μU/ml at 1 h, while

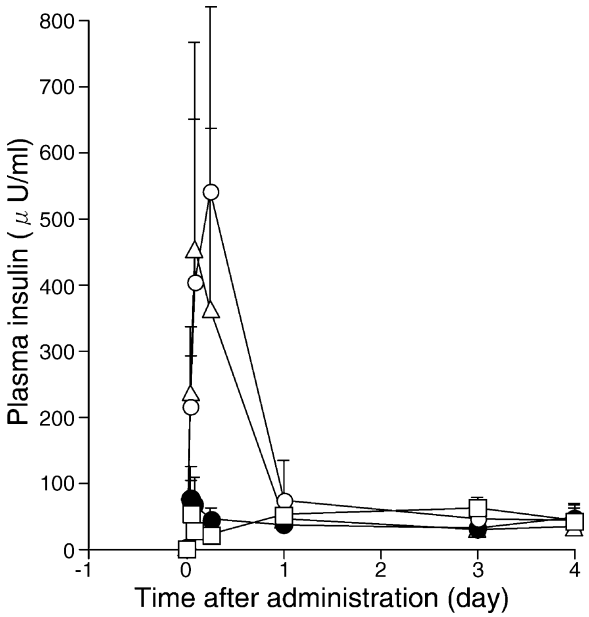

Fig. 1. Effect of ZnO on the initial profile of insulin release from the microcapsules in vivo. Insulin-containing PLGA microcapsules were prepared with various amounts of zinc oxide (ZnO) in the oil phase. Glycerin (30 mg) and water (13.5 mg) were both added to the oil phase (1 ml) containing PLGA (0.98 g) and insulin (30 mg) in order to improve its turbidity. The amount of ZnO was as follows: (○) no ZnO (Formulation B), (△) 1.2-fold molar excess relative to insulin (Formulation C), (□) six-fold molar excess (Formulation D), (●) 12-fold molar excess (Formulation E). The formulations are all listed in Table 2. Each insulin formulation (250 U/kg) was given subcutaneously as a single dose to STZ-induced diabetic rats, and plasma insulin levels were monitored. Data up to the 4th day are shown (mean ± S.D., $N = 4$–7). The mean molecular weight of PLGA was 6600.

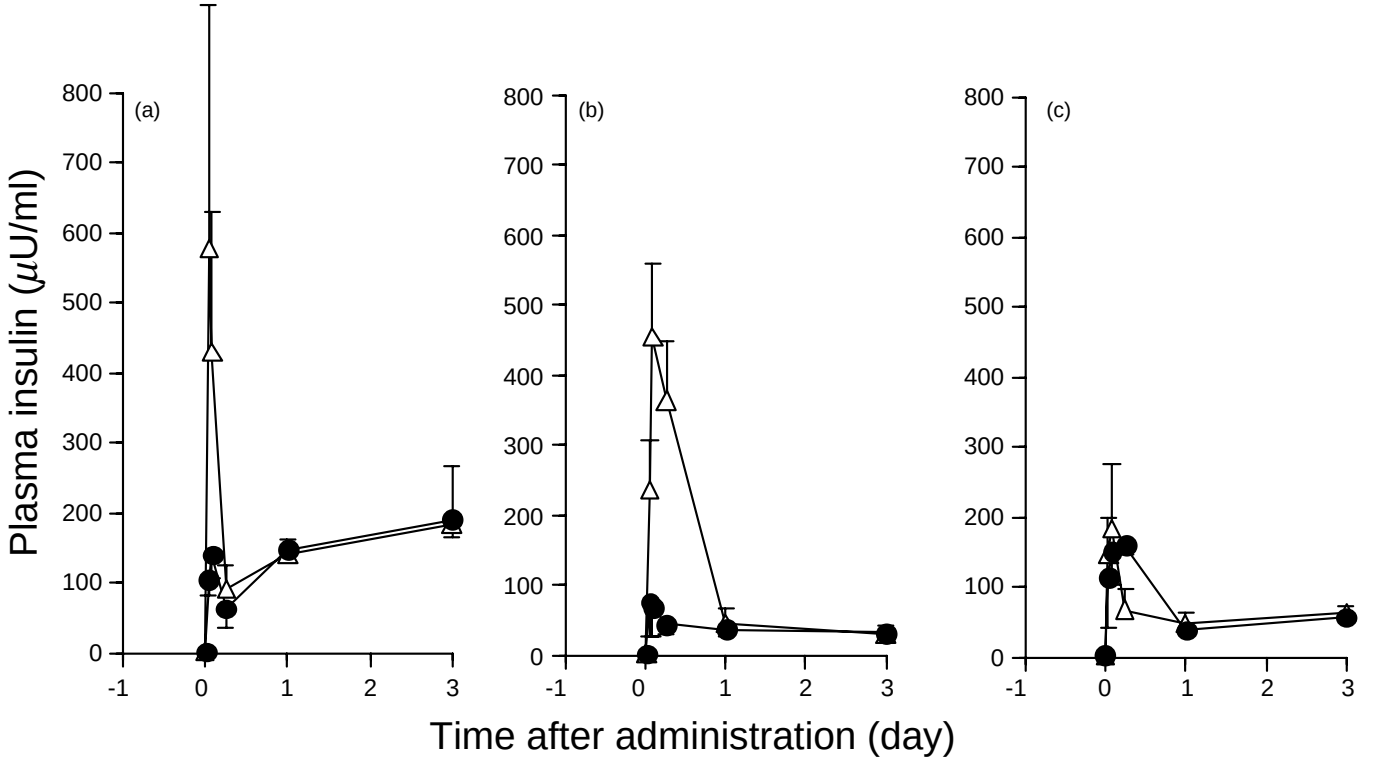

Fig. 2. Effect of ZnO content and type of PLGA on the initial insulin release profile in vivo. Insulin-containing PLGA microcapsules were prepared with ZnO at a 1.2-fold (△) or 12-fold (●) molar excess relative to insulin in the oil phase. Glycerin (30 mg) and water (13.8 mg) were both added to the oil phase (1 ml) containing PLGA (1 g) and insulin (30 mg) in order to improve its turbidity. The mean molecular weight of the PLGAs used in this experiment was 4k(4100) (a), 6k (6600) (b), and 8k (8000) (c). Each formulation is listed in Table 2. Plasma insulin levels were monitored following a single subcutaneous injection of each formulation (250 U/kg). Data up to the 3rd day are shown (mean ± S.D., $N = 4$–7).

the levels at 2 and 6 h were 455.2 and 363.9 μU/ml, respectively ($n = 7$).

A formulation prepared with water as the only additive to oil phase showed significantly less early release of insulin (Takenaga et al., 2002), indicating that improved turbidity of the oil phase containing insulin and PLGA would not necessarily have the same effect. Smaller amounts of glycerin and water could separately improve the oil phase turbidity, but did not fully alter the localization of insulin in the microcapsules.

This problem was overcome by the addition of zinc oxide (ZnO) to the oil phase. A formulation prepared without ZnO (Formulation B) caused much greater early release, with an insulin level of 541.8 μU/ml at 6 h ($n = 4$). Surprisingly, when a 12-fold molar excess of ZnO relative to insulin was added, the formulation thus obtained (Formulation E) achieved a reduction of early release (insulin levels at 1, 2, and 6 h were 76.9, 68.2, and 45.5 μU/ml, respectively ($n = 7$)).

A formulation prepared with a six-fold molar excess of ZnO relative to insulin (Formulation D) also showed similar insulin release ($n = 4$). These findings clearly indicated that ZnO was important for reducing early release. ZnO itself is insoluble in the oil phase in the presence of glycerin and water. When insulin and PLGA were also added, the emulsion became almost clear. This suggested that some interaction between insulin, PLGA, and ZnO might be important to alter the localization of insulin in the microcapsules.

ZnO can increase viscosity, and it is widely used in products such as dental and film materials (DuBois et al., 2000). Addition of glycerin makes the oil phase more viscous (Yamaguchi et al., 2002). Therefore, addition of both ZnO and glycerin may have led to an augmented interaction among them and further increased the viscosity. If less glycerin or water was added, ZnO would be needed to maintain the viscosity and alter the localization of insulin to reduce early release from the microcapsules.

The effect of ZnO was more obvious when the type of PLGA was changed, as shown in Fig. 2. When 4k PLGA (molecular weight: 4100) was used, the formulation prepared with a high content of ZnO showed a reduction in the rapid release of insulin. When insulin-containing microcapsules were prepared with a 1.2-fold molar excess of ZnO over insulin (Formulation F) and were administered subcutaneously to diabetic rats, the plasma insulin level increased rapidly. The mean plasma insulin level was 597.3 μU/ml at 1 h and 430.4 μU/ml at 2 h. In contrast, a formulation prepared with a 12-fold molar excess of ZnO (Formulation G) produced a plasma insulin level of 104.9 μU/ml at 1 h and 141.6 μU/ml at 2 h, which was one-fourth to one-fifth of the former level, respectively.

When 6k PLGA (molecular weight: 6600) was used with a 1.2-fold molar excess of ZnO (Formulation C), the plasma insulin level increased to 455.2 μU/ml, while a 12-fold molar excess of ZnO (Formulation E) significantly reduced the plasma insulin level (68.2 μU/ml).

On the other hand, little effect of ZnO was observed in the case of 8k PLGA (molecular weight: 7900), probably because the higher molecular weight PLGAs have a higher intrinsic viscosity.

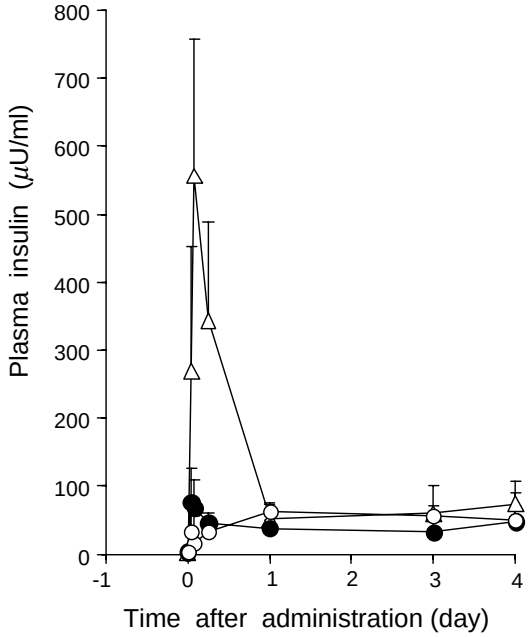

Fig. 3. Effect of insulin particle size. Insulin-containing PLGA microcapsules were prepared using human insulin (△) (Formulation J, Table 2), lyophilized human insulin (○) (Formulation K), or Zn-free insulin (●) (Formulation E). ZnO was added at a 12-fold molar excess over insulin, and glycerin and water were used as the other additives. The mean molecular weight of PLGA was 6600. Plasma insulin levels were monitored after a single subcutaneous injection of each insulin formulation (250 U/kg). Data up to the 3rd day are shown (mean ± S.D., $N = 3$–7). The mean molecular weight of PLGA was 6600.

3.3. Size of drug particles

Smaller drug particles have been reported to be more suitable (Jhonson et al., 1996; Tracy, 1998), so lyophilized insulin obtained after dialysis (Zn-free insulin) was used for all of the above formulations and its particle size was predominantly 2–3 μm. Since zinc has been reported to stabilize proteins (Jhonson et al., 1996; Tracy, 1998), ZnO and $Zn(CH_3COO)_2$ were respectively added to the oil phase and the water phase.

It was still necessary to confirm that Zn-free insulin is suitable for the desired pattern of release. Not only human insulin but also lyophilized insulin was used for preparation of the test formulations. Lyophilized insulin with a particle size of around 2–10 μm was obtained after human insulin was dissolved in water and frozen in nitrogen gas.

Formulation K (lyophilized insulin) and Formulation E (Zn-free insulin) caused little early release of insulin (Fig. 3) and there was no significant difference between these two formulations. The human insulin (20–30 μm particles)-used formulation (Formulation J) allowed early release, indicating that smaller particles were essential.

3.4. Size of PLGA microcapsules

PLGA microcapsules measuring 15–30 μm were obtained by our solvent evaporation method. We separated smaller and larger particles by sedimentation of Formulation E. Sedimentation was performed by letting a suspension of Formulation E in distilled water stand for 10 min. The diameter of the particles was 33.67 ± 13.61 μm and 10.98 ± 11.21 μm, respectively (mean ± S.D.). Fig. 4 shows the plasma insulin profile after a single injection of each formulation. The initial release of insulin from smaller microcapsules was significantly greater compared

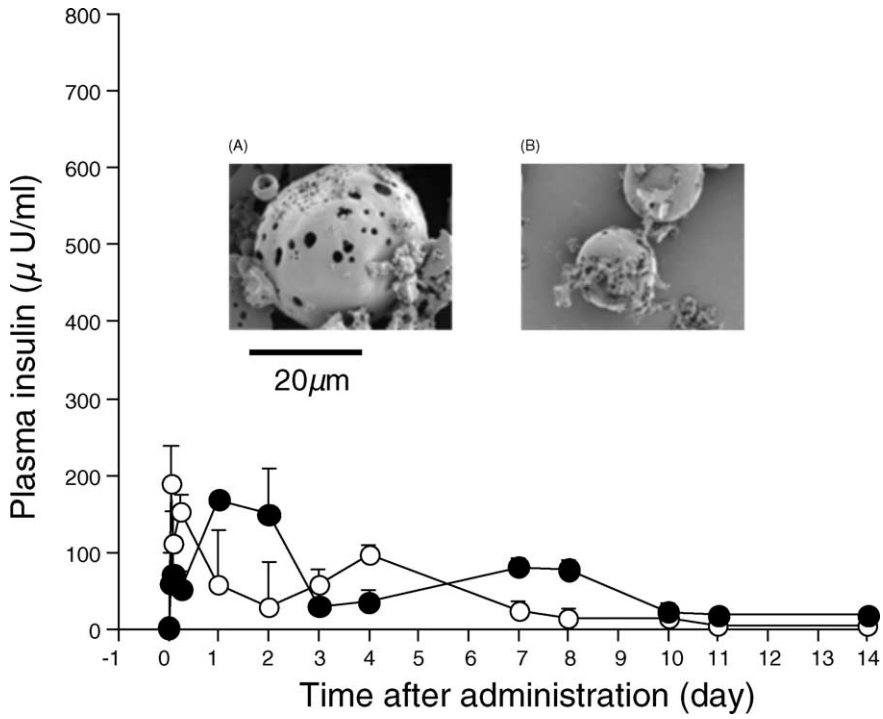

Fig. 4. Effect of microcapsule size. Insulin-containing PLGA microcapsules (Formulation E) were divided into larger and smaller ones by centrifugation. The respective microcapsules are shown (A: larger (●), B: smaller (○)). Each formulation was given subcutaneously as a single dose (250 U/kg) to STZ-induced diabetic rats, and plasma insulin levels were monitored until the 14th day. The insulin content (%) was 2.5% (larger, ●), or 2.6% (smaller, ○). Data up to the 14th day are shown (mean ± S.D., $N = 3$–7). The mean molecular weight of PLGA was 6600.

with that from larger microcapsules. The plasma insulin level was 189.3, 112.4, and 154.0 μU/ml at 1, 2, and 6 h, respectively, after injection of a formulation with smaller microcapsules. In diabetic rats injected with larger ones, the plasma insulin level at 1 h, 1 day, and 2 days was 60.3, 168.4, and 150.0 μU/ml, respectively. The longer disintegration time seemed to allow gradual release of insulin from the larger microcapsules. It was interesting that the second release phase was significant with larger microcapsules. This could be explained by the possibility that rapid degradation of PLGA led to rapid insulin release. PLGA microcapsules have shown a similar second release profiles in vitro (Takenaga et al., 2002; Morita et al., 2000).

Before separation of the microcapsules by sedimentation, the in vivo insulin release profile was flatter, as shown in the next experiment. This led to the conclusion that some variation in the size of the microcapsules was desirable for obtaining the targeted release profile.

3.5. Optimum formulation

There are numerous PLGAs that vary in molecular weight, LA/GA ratio, and terminal OH% among other factors. Biodegradable PLA and PGA have various characteristics that can markedly influence drug release from the resultant formulation. Because LA/GA ratio (50:50) was fixed in this study, PLGA with a molecular weight of around 6000 seemed to be desirable (Formulation E), as shown in Fig. 2. Formulation L (molecular weight: 5800) was also tested (Fig. 5). The second release peak produced by Formulation E and Formulation L was 122.2 μU/ml (on day 7) and 96.8 μU/ml (on day 8), respectively, with no significant difference between the two. The first peak was seen on the day of administration being 76.0 μU/ml

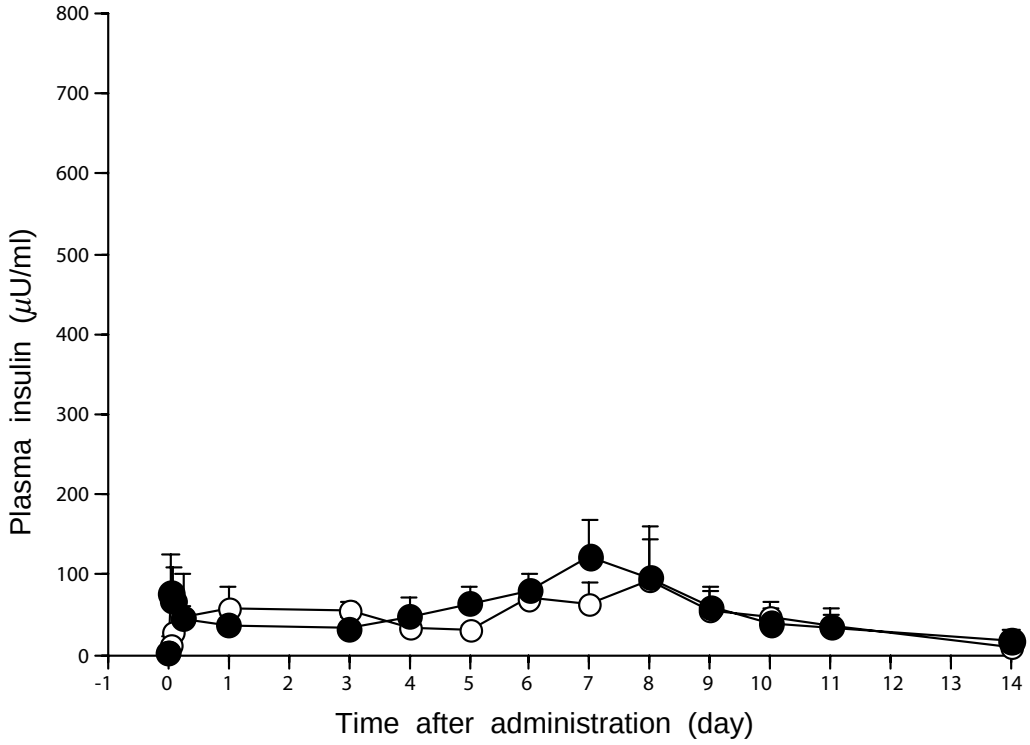

Fig. 5. Plasma insulin levels following a single subcutaneous dose of insulin-containing PLGA microcapsules. Insulin-containing PLGA microcapsules were subcutaneously administered (250 U/kg) as a single dose to STZ-induced diabetic rats and plasma insulin levels were monitored until the 14th day. The molecular weight of PLGA was 5800 (○) (Formulation L, Table 2) or 6600 (●) (Formulation E). Data up to the 14th day are shown (mean ± S.D., $N = 5$).

with Formulation E and 47.0 μU/ml with Formulation L. From these data, a molecular weight of around 6000 was concluded to be suitable for a sustained-release preparation. Since high insulin levels can induce hypoglycemia (Rizza et al., 1981), a lower peak/trough ratio is desirable. Formulation E produced a ratio of 2.8. Judging from this result, the present formulation was concluded to be close to optimum.

3.6. Biological activity of insulin

It was also important to determine whether insulin extracted from the microcapsules still maintained its biological activity or not. Insulin acts as a growth factor as well as controlling glucose levels (Kamide et al., 2000). NIH 3T3 mouse fibroblasts have few endogenous insulin receptors and are insensitive to insulins but express IGF-receptors and are responsive to IGF-1. Both insulin and IGF regulate certain cellular functions via both overlapping receptor and postreceptor signaling pathways (Hofmann et al., 1989). Therefore, we used these cells to test the biological activity of insulin extracted from microcapsules compared with that of normal insulin.

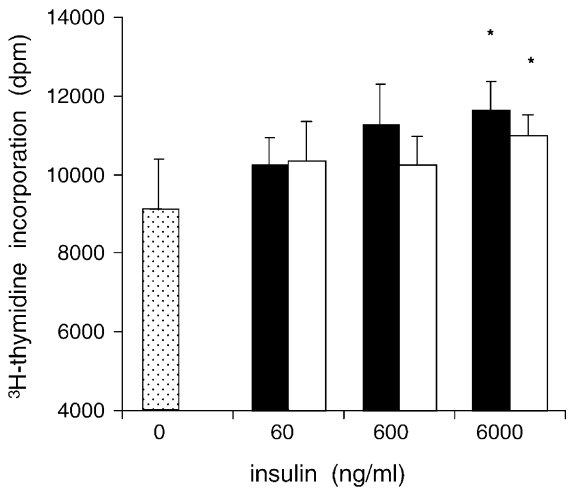

Fig. 6. Comparison of the biological activity of insulin extracted from microcapsules with that of normal insulin. The biological activity of insulin extracted from microcapsules was evaluated by a cell growth assay, and was compared with that of normal insulin (□). NIH3T3 cells (1×10^3 cells) were cultured in the presence of insulin from microcapsules (■) or normal insulin for 24 h and cell growth was determined by the ^{3}H-thymidine incorporation assay. Data are the mean ± S.D. (dpm) from four separate experiments ($N = 6$). $^*P < 0.05$ vs. control.

In control cells, [^{3}H]thymidine uptake was 9106 ± 1301.7 dpm (Fig. 6). Insulin dose-dependently increased the incorporation of [^{3}H]thymidine, as shown in Fig. 6. The radioactivity of cells treated at 6 μg/ml was 11654.3 ± 1715.8 dpm ($P < 0.05$ versus control). Insulin from microcapsules also enhanced the uptake of [^{3}H]thymidine (11013.7 ± 503.0 dpm, $P < 0.05$ versus control). The potency was similar, but slightly lower, than that of normal insulin. This might be partly because the extracted insulin contained NaCl produced by neutralization, but the extract and normal insulin were almost equal.

In vivo evaluation test was also carried out. When 1.0 mg/kg of either insulin was administered to STZ-induced diabetic rats, a similar hypoglycemic effect was obtained (data not shown).

4. Conclusion

This study demonstrated that glycerin plus water as additives could reduce the turbidity of the oil phase containing PLGA and insulin, enabling us to prepare a reproducible injectable formulation. To suppress initial rapid insulin release, addition of zinc (ZnO), and a smaller insulin particle size were essential. A suitable molecular weight for PLGA was around 6000. Some variation in the size of the microcapsules (10–30 μm) contributed to relatively constant insulin release. A low peak/trough ratio is desirable and that of the optimum formulation was only 2.8. In addition, the biological activity of insulin extracted from the microcapsules was confirmed to be similar to that of normal insulin. Thus, the present formulation was concluded to be nearly optimal.

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