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Design of novel injectable cationic microspheres based on aminated gelatin for prolonged insulin action

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Abstract

The aim of this study was to prepare two types of injectable cationized microspheres based on a native gelatin (NGMS) and aminated gelatin with ethylenediamine (CGMS) to prolong the action of insulin. Release of rhodamin B isothiocyanate insulin from CGMS was compared with that from NGMS under in-vitro and in-vivo conditions. Lower release of insulin from CGMS compared with that from NGMS was caused by the suppression of initial release. The disappearance of ^{125}I-insulin from the injection site after intramuscular administration by NGMS and CGMS had a biphasic profile in mice. Almost all the ^{125}I-insulin had disappeared from the injection site one day after administration by NGMS. The remaining insulin at the injection site after administration by CGMS was prolonged, with approximately 59% remaining after one day and 16% after 14 days. The disappearance of CGMS from the injection site was lower than that of NGMS. However, the difference in these disappearance rates was not great compared with those of ^{125}I-insulin from the injection site by NGMS and CGMS. The time course of disappearance of ^{125}I-CGMS from the injection site was similar to that of ^{125}I-insulin by CGMS. The initial hypoglycaemic effect was observed 1 h after administration of insulin by NGMS, thereafter its effect rapidly disappeared. The hypoglycaemic effect was observed 2–4 h after administration by CGMS and continued to be exhibited for 7 days. The prolonged hypoglycaemic action by CGMS depended on the time profiles of the disappearance of insulin from muscular tissues, which occurs due to the enzymatic degradation of CGMS.

Introduction

Gelatin obtained from collagen has biocompatible and biodegradable characteristics and is an excellent material for medical use. It has been used clinically as an excipient for drugs and implanted formulations (Tabata & Ikada 1998). Furthermore, it has an advantage as a material in that its physicochemical characteristics can be modified by chemical treatment. Acidic and basic gelatins are denatured by the alkaline and acidic treatment of collagen, respectively. This treatment affects the electrical nature of the collagen, yielding a series of gelatins with different isoelectric points (IEPs). Anionic and cationic gelatin microspheres were prepared using acidic gelatin (IEP = 5.0) and basic gelatin (IEP = 9.0), respectively.

Peptide and protein drugs have a relatively short half-life ($t_{1/2}$) following injection and a poor bioavailability following oral administration. We have been investigating drug delivery systems to improve the bioavailability and controlled release of peptide and protein drugs using microspheres based on acidic gelatin and basic gelatin for a number of years (Tabata et al 1999; Morimoto et al 2000, 2001). We previously reported that the release profiles of protein and peptide drugs from anionic and cationic gelatin microspheres can be explained by an ionic interaction (Tabata et al 1999). An incorporated acidic peptide drug, such as bovine insulin (IEP = 5.3), with an IEP < 7.0, is released in a sustained manner from positively charged gelatin microspheres, whereas basic peptides, such as b-FGF (IEP = 9.6) and salmon calcitonin (IEP = 8.2), with an IEP > 7.0, are released initially from positively charged gelatin microspheres (Tabata et al 1999; Morimoto et al 2001). We also reported that gelatin microspheres enhance the bioavailability of calcitonin after pulmonary, nasal and

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intramuscular administration in rats (Morimoto et al 2000, 2001) and that the release rate of insulin from positively charged gelatin microspheres can be sustained by increasing the cross-linking density of gelatin under both in-vitro and in-vivo conditions (Iwanaga et al 2003). Furthermore, we recently reported the controlled release of ^{125}I-plasmid DNA from a cationized hydrogel based on aminated gelatin with ethylenediamine (Fukunaka et al 2002). When cationized hydrogels incorporating ^{125}I-plasmid DNA were implanted into the femoral muscle of mice, the plasmid DNA radioactivity remaining decreased with time and the retention period was prolonged.

In the present study, we prepared two different types of injectable cationized microspheres based on a native cationic gelatin (NGMS) and aminated gelatin with ethylenediamine (CGMS) to prolong the action of insulin. The release profiles of insulin from the two different types of microspheres were compared in vitro. Furthermore, the dispositions of insulin and the microspheres in muscle tissue and the hypoglycaemic effects after intramuscular administration of insulin incorporated in NGMS and CGMS were compared in mice.

Materials and Methods

Materials

Gelatin (IEP = 9.0; 100 kDa) was supplied by Nitta Gelatin Co. Ltd (Osaka, Japan). Recombinant human insulin (insulin; 26.0 IU mg^{-1}) and ^{125}I recombinant human insulin (^{125}I-insulin) were purchased from Wako Pure Chem. Ind. Ltd (Osaka, Japan) and Amersham Bioscience (Piscataway, NJ), respectively. 1,2-Ethylenediamine and glycine were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Glutaraldehyde (25% in water solution) and 2,4,6-trinitrobenzenesulfonic acid sodium salt (TNBS) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride was purchased from Wako Pure Chem. Ind. Ltd (Osaka, Japan). All other chemicals were of reagent grade and used as received.

Synthesis of aminated gelatin

Aminated gelatin was synthesized by the reaction of native cationic (basic) gelatin (IEP = 9.0) with 1,2-ethylenediamine (Wang et al 2000). Briefly, native cationic gelatin was dissolved in phosphate buffer (pH 5.3) and 1,2-ethylenediamine was added under stirring. After adjusting the pH to 5.0 with 5 M HCl, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride was added. The final concentrations of native gelatin, ethylenediamine and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride in the resultant solution were 20, 56 and 10.7 mg mL^{-1}, respectively. The resultant solution was incubated at 37°C for 18 h, followed by dialysis against purified water for 48 h, and then freeze-dried to obtain the aminated gelatin. The numbers of primary amino groups introduced into the native cationic and aminated gelatin were measured by the TNBS method (Snyder & Sobocinski 1975). The amino group contents of native cationic gelatin and aminated gelatin were 0.28 and 0.88 mmol g^{-1}, respectively.

Preparation of native cationic and aminated gelatin microspheres containing insulin

NGMS and CGMS were prepared by glutaraldehyde cross-linking in the dispersed state of an aqueous gelatin solution in the oil phase by a modification of the method of Tabata & Ikada (1989). Briefly, 1 mL native cationic or aminated gelatin aqueous solution (10 wt%, preheated to 40°C) was added dropwise to 25 mL olive oil and then emulsified by vortex mixing to yield a water-in-oil emulsion. The emulsion temperature was reduced to 4°C then 7.5 mL cold acetone was added to the emulsion and stirring was continued for 1 h to induce gel formation. The resulting microspheres were collected by centrifugation (2500 × g, 4°C, 5 min) and washed three times with acetone. The cross-linking of microspheres was conducted using 0.06% glutaraldehyde in acetone/0.01 M HCl solution (7:3) at 4°C for 48 h. Following collection by centrifugation (2500 × g, 4°C, 5 min), the microspheres were agitated in 10 mM aqueous glycine solution at room temperature for 1 h to block the residual aldehyde groups in the unreacted glutaraldehyde. The resulting microspheres were finally washed three times with purified water by centrifugation and then freeze-dried.

For incorporation of insulin, insulin solution (pH 7.4 phosphate-buffered saline; PBS) was added to the dried microspheres, which were then left at 4°C for 3 h to obtain microspheres containing insulin. The insulin contents of the NGMS and CGMS were 1.28 ± 0.05 and 12.6 ± 0.10 IU mg^{-1}, respectively. The NGMS and CGMS had a water content of approximately 90%. The mean diameters of the microspheres were determined by photomicroscopy. The diameters of NGMS and CGMS containing insulin were 32.3 ± 1.56 and 29.7 ± 1.51 μm, respectively.

Release profiles of rhodamin B isothiocyanate insulin from gelatin microspheres

The release profiles of insulin from microspheres were determined by the following method. The microspheres were prepared by the same method as described above. Insulin was labelled with rhodamin B isothiocyanate (RITC-insulin) by the method reported by Mossberg & Ericsson (1990) and incorporated into microspheres. Briefly, 20 μL RITC-insulin aqueous solution (2.0 mg mL^{-1}) was added to 2.0 mg microspheres. They were left at 4°C for 3 h to allow the solution to penetrate into the microspheres. In this study, approximately 100% of the RITC-insulin aqueous solution was allowed to impregnate the microspheres during the swelling process because the solution volume was less than that which theoretically would impregnate the microspheres. The microspheres containing RITC-insulin were added to 1.5 mL PBS (pH 7.4) and this suspension was incubated at

37°C for up to 24 h. The suspension was centrifuged at a predetermined time for 10 000 rpm and 1 mL supernatant was taken as a sample. The fluorescence intensity of each sample was determined by fluorospectrophotometry (Hitachi F-2000, Tokyo, Japan). The excitation and emission wavelengths were set at 554 and 578 nm, respectively.

Animal experiments

Male ICR mice, 6 weeks old, were used as experimental animals (Japan SLC Co. Ltd, Shizuoka, Japan) and were housed in a room at a controlled temperature (24°C) and humidity (55%). Animal experiments were carried out in accordance with the Guiding Principles for the Care and Use of Experimental Animals, Hokkaido Pharmaceutical University (1998).

Disappearance of insulin and microspheres from dosing site after intramuscular injection

Microspheres containing ^{125}I-insulin were prepared by the method described. Separately, gelatin microspheres (^{125}I-gelatin microspheres) were radiolabelled with ^{125}I using Bolton & Hunter reagent (Amersham Bioscience, Piscataway, NJ) and unlabelled insulin was incorporated into the ^{125}I-gelatin microspheres (^{125}I-NGMS and ^{125}I-CGMS) for the in-vivo animal experiment. A total of 20 mg of either gelatin microspheres containing ^{125}I-insulin or ^{125}I-gelatin microspheres containing cold unlabelled insulin was suspended in 1 mL PBS (pH 7.4). These suspensions were used as test formulations. Under ether anaesthesia, 50 μL of each test solution was injected into the femoral muscle of the mice. The mice were then sacrificed and the leg injected with the test solution was removed at designated times (1, 3, 5, 7, 10 and 14 days) after injection. The remaining radioactivity of either ^{125}I-insulin or ^{125}I-gelatin microspheres in the injected site was determined by gamma-scintillation counting (ARC-301B, Aloka, Tokyo, Japan).

Hypoglycaemic effects after intramuscular injection of microspheres containing insulin

Just before administration, the microspheres containing insulin were dispersed in PBS (pH 7.4). The preparations were given as a single injection into the femoral muscle of the mice (insulin dose 26 IU kg^{-1}; dosage volume 50 μL). Blood samples were taken from the tail vein just before administration (0 h) and at designated times (1, 2, 3, 4, 6 and 8 h) after administration. The mice were fasted from 16 h before administration until the last sample had been taken.

Separately, blood glucose levels were determined until the 14th day after administration. The preparations were injected as described above. The mice were fasted from 6:00 pm of the day before administration until 9:00 am following the final administration. Blood samples were taken at 8:50 am, just before administration (0 day) and at 9:00 am on the designated days (1st, 3rd, 5th, 7th, 10th and 14th day) after administration. Every day during the experimental period mice were fasted from 6:00 pm to 9:00 am. Blood glucose levels were determined using a blood glucose monitoring system (Nipro Free Style, Nipro, Osaka, Japan).

Statistical analysis

The mean values and their standard errors (SE) were calculated in each experiment. Differences between NGMS and CGMS in the in-vitro release of RITC-insulin, and ^{125}I-insulin and ^{125}I-gelatin remaining in-vivo were evaluated by the Mann–Whitney U-test. The effect of insulin after application as NGMS and CGMS on the plasma glucose levels was evaluated by the Kruskal–Wallis test. Statistical significance was taken as $P < 0.05$.

Results

Release profiles of RITC-insulin from gelatin microspheres

Figure 1 shows the release profiles of RITC-insulin from CGMS and NGMS in pH 7.4 PBS at 37°C in vitro. The time profiles of the release of RITC-insulin from CGMS and NGMS were biphasic. RITC-insulin was rapidly released from NGMS within 3 h; the amount released was approximately 80%. In contrast, the release of RITC-insulin from CGMS was significantly lower than that from NGMS; the amount released was approximately 40% within 6 h and then levelled off. The cationization of the microspheres based on aminated gelatin therefore

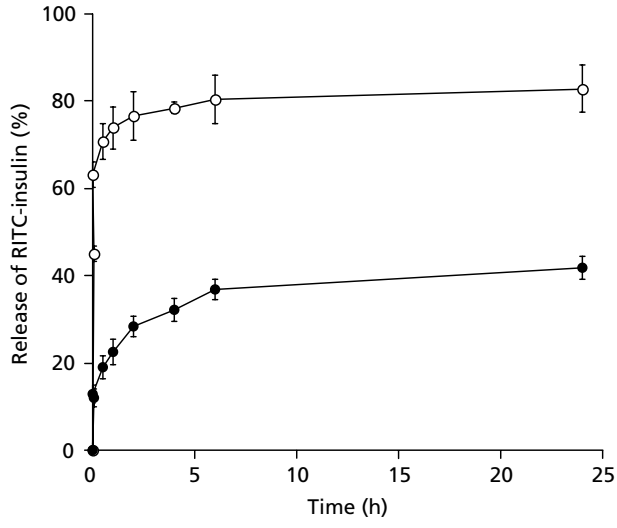

Figure 1 Release profiles of RITC-labelled insulin from NGMS and CGMS in pH 7.4 PBS at 37°C. ○, NGMS; ●, CGMS. Each value represents the mean ± s.e.m. of three experiments. A significant difference was shown by the Mann–Whitney U-test ($P < 0.05$) for each time point.

produced a sustained release of RITC-insulin from the gelatin microspheres.

Disappearance of ^{125}I-insulin and ^{125}I-gelatin microspheres from muscle tissues after injection in mice

Figure 2A and B shows the time courses of the remaining ratio of radioactivity in the injection site after intramuscular administration of gelatin microspheres (NGMS and CGMS) containing ^{125}I-insulin (A) and ^{125}I-gelatin microspheres (NGMS and CGMS) containing insulin (B) in mice, respectively. The disappearance of ^{125}I-insulin from the injection site after intramuscular administration of both types of NGMS and CGMS showed biphasic profiles. Almost all the ^{125}I-insulin (approximately 99%) rapidly disappeared from the injection site within 1 day after administration of NGMS. In contrast, the time that ^{125}I-insulin remained at the injection site after administration of CGMS was significantly prolonged; the amount remaining was approximately 60% after 1 day and approximately 4% after 14 days.

The time profiles of the remaining radioactivity at the injection site after intramuscular administration of ^{125}I-gelatin microspheres (NGMS and CGMS) containing insulin are shown in Figure 2B. The disappearance of radioactivity from the injection site after intramuscular administration of both types of ^{125}I-NGMS and ^{125}I-CGMS showed a biphasic profile. The disappearance of ^{125}I-CGMS from the injection site after administration was significantly slower than that of ^{125}I-NGMS. However, the difference between these rates was not great compared with the disappearance rate of ^{125}I-insulin from the injection site after administration of NGMS and CGMS. The time course of the remaining ^{125}I-CGMS from the injection site was very similar to that of ^{125}I-insulin after intramuscular administration of CGMS.

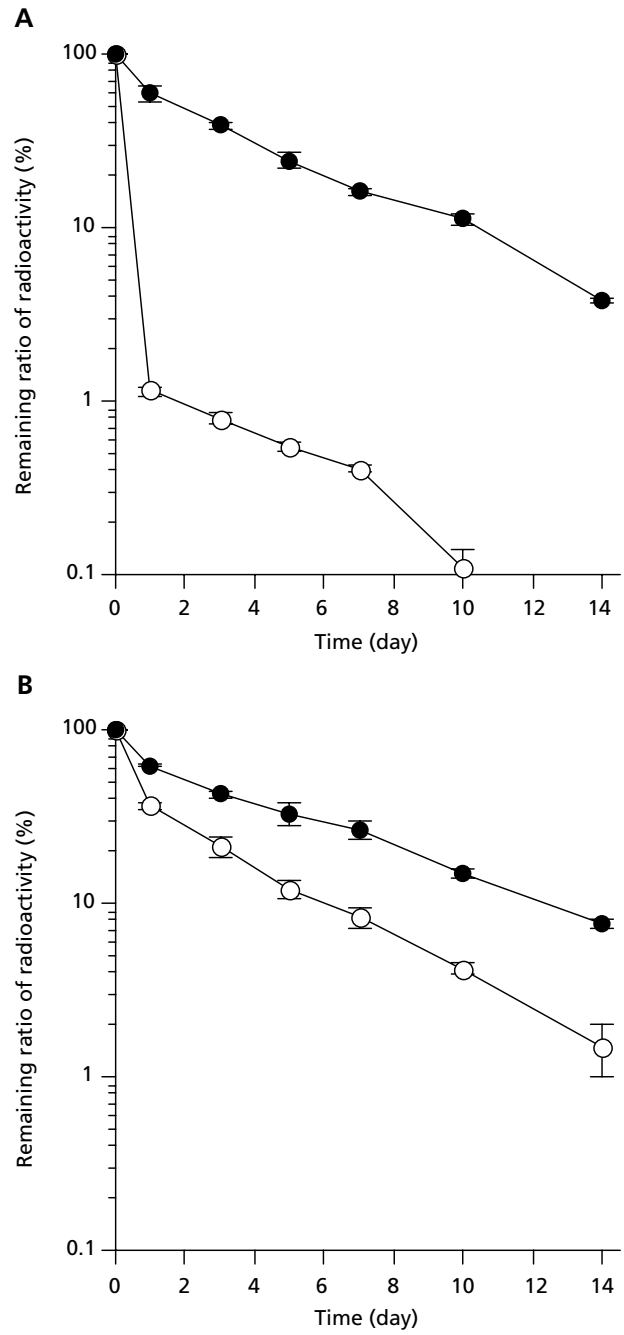

Figure 2 Ratios of remaining radioactivity in the injection site after intramuscular administration of gelatin microspheres (NGMS and CGMS) containing ^{125}I-insulin (A) and ^{125}I-gelatin microspheres (NGMS and CGMS) containing insulin (B) in mice. ○, NGMS; ●, CGMS. Each point represents the mean ± s.e.m. of four experiments. A significant difference was shown by the Mann–Whitney U-test ($P < 0.05$) for each time point.

Hypoglycaemic effects after intramuscular injection of microspheres containing insulin

Figure 3A and B shows the changes in blood glucose levels until 8 h (short period) and 14 days (long period) after intramuscular administration of gelatin microspheres (NGMS and CGMS) containing insulin, respectively. The maximum hypoglycaemic effect was observed 1 h after intramuscular administration of NGMS containing insulin, thereafter its hypoglycaemic effect rapidly decreased. In contrast, the hypoglycaemic effect was observed 4 h after intramuscular administration of CGMS containing insulin, and a long period of hypoglycaemia was exhibited over 5 days.

Discussion

Recently, microspheres composed of various kinds of materials have been prepared and used as carriers for the controlled release of peptide and protein drugs (Hahn et al 2004, Leitner et al 2004). Although the drug-release rate from microspheres is generally difficult to control, it must be strictly controlled for safe therapy when microspheres are used as carriers for highly potent peptide and protein

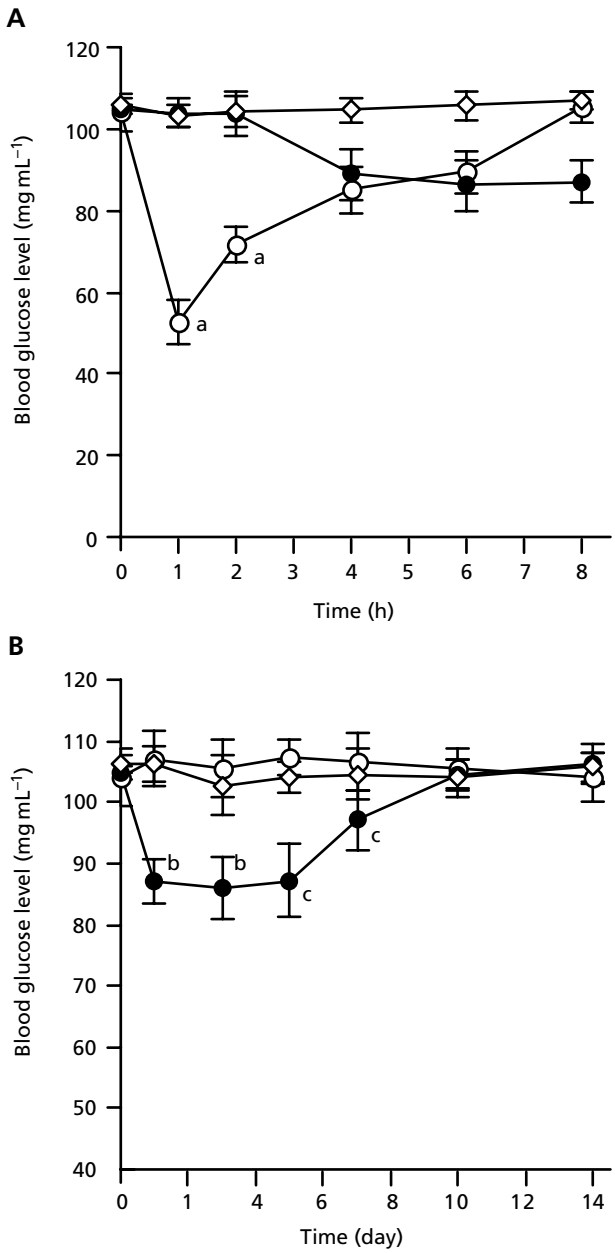

Figure 3 Changes in blood glucose levels up to 8 h (short period (A)) and up to 14 days (long period (B)) after intramuscular administration of gelatin microspheres (NGMS and CGMS) containing insulin in mice. ○, NGMS containing insulin; ●, CGMS containing insulin; ◇, PBS. Each point represents the mean ± s.e.m. of five or seven experiments. a, $P < 0.05$ vs PBS and CGMS; b, $P < 0.05$ vs PBS and NGMS; c, $P < 0.05$ vs NGMS: by the Kruskal–Wallis test.

drugs. Among these carriers, gelatin microspheres prepared with gelatin hydrogel are expected to be useful drug carriers because they are biodegradable as well as biocompatible (Nakaoka el al 1996; Tabata & Ikada 1998). The cross-linking density, molecular weight, electrical charge and biodegradation rate of gelatin affects the release rate of peptide and protein drugs from gelatin microspheres (Tabata et al 1999; Morimoto et al 2001).

In this study, the slower release of insulin from CGMS compared with that from NGMS was caused by the suppression of burst release during the initial phase under in-vitro non-degradation conditions without enzyme. These results suggest that the release of insulin from CGMS occurred through the simple diffusion of insulin in the microspheres, and this simple diffusion of insulin in the microspheres may depend on the electrostatic relationship between insulin and the aminated gelatin in CGMS. Since human recombinant insulin, which has an IEP of 5.3, is negatively charged at physiological pH, the initial release of insulin from CGMS is suppressed by the formation of a polyion complex. Furthermore, the cross-linking density in CGMS based on aminated gelatin with an amino group content of $0.88\ \text{mmol g}^{-1}$ may be higher than that in NGMS based on native cationic gelatin with an amino group content of $0.28\ \text{mmol g}^{-1}$. The higher cross-linking density of CGMS may cause the prolonged release of insulin.

The disappearance of insulin from the injection site after intramuscular administration of CGMS containing ^{125}I-insulin was significantly slower than that of NGMS. The disappearance of insulin from the injection site showed a biphasic profile for both types of microspheres. The difference between CGMS and NGMS in terms of the remaining amount of insulin in the injection site during the initial phase one day after administration was related to the initial release profiles of insulin from CGMS and NGMS.

The disappearance of CGMS from the injection site after intramuscular administration of CGMS containing insulin was significantly slower than that of NGMS. The higher cross-linking density of CGMS may cause prolonged degradation of microspheres in the muscle tissue after intramuscular injection. The time profile of the insulin remaining in the injection site after administration of NGMS was not similar to that of NGMS after its administration. However, the time profiles of the ^{125}I-insulin remaining and CGMS in the injection site after administration of CGMS were almost identical. It is well known that gelatin hydrogels of the microsphere type are slowly degraded by enzymatic (trypsin, pepsin and collagenase) hydrolysis (Nakaoka et al 1996). The release of insulin from CGMS was zero after the initial release (until 6 h) in the release experiments without enzyme. These results therefore suggest that the disappearance of insulin from muscle tissues 6 h after administration may have occurred by degradation of CGMS by enzymes in muscle tissue.

The initial hypoglycaemic effect was observed 1 h after intramuscular administration of NGMS containing insulin, thereafter its hypoglycaemic effect rapidly disappeared. In contrast, the hypoglycaemic effect was observed 2–4 h after intramuscular administration of CGMS containing insulin, and a long period of hypoglycaemia continued over 7 days. These time profiles of the initial hypoglycaemic effects of NGMS and CGMS are related to the initial release profiles (until 6 h) of NGMS and CGMS in the release experiment without enzymes. The prolonged hypoglycaemic effects of CGMS depend on the time profiles of the disappearance of insulin from the injection site, which may be due to enzymatic degradation of CGMS.

Conclusion

Intramuscular administration of CGMS containing insulin results in a sustained hypoglycaemic effect. This prolonged hypoglycaemic effect of CGMS may be due to suppression of the initial release of insulin from CGMS over a short period and continued enzymatic degradation of CGMS in the injection site. CGMS is therefore a useful injectable vehicle for acidic peptides such as insulin.

References

Fukunaka, Y., Iwanaga, K., Morimoto, K., Kakemi, M., Tabata, Y. (2002) Controlled release of plasmid DNA from cationized gelatin hydrogel based on hydrigel degradation. *J. Control. Release* **80**: 333–343

Hahn, S. K., Kim, S. J., Kim, M. J., Kim, D. H. (2004) Characterization and in vivo study of sustained-release formulation of human growth hormone using sodium hyaluronate. *Pharm. Res.* **21**: 1374–1381

Iwanaga, K., Yabuta, T., Kakemi, M., Morimoto, K., Tabata, Y., Ikada, Y. (2003) Usefulness of microspheres composed of gelatin with various cross-linking density. *J. Microencapsul.* **20**: 767–776

Leitner, V. M., Guggi, D., Krauland, A. H., Bernkop-Schnurch, A., Andreas, B. S. (2004) Nasal delivery of human growth hormone: in vitro and in vivo evaluation of a thiomer/glutathione microparticulate delivery system. *J. Control. Release* **100**: 87–95

Morimoto, K., Kstsumata, H., Yabuta, T., Iwanaga, K., Kakemi, M., Tabata, Y., Ikada, Y. (2000) Gelatin microspheres as a pulmonary delivery system: evaluation of salmon calcitonin absorption. *J. Pharm. Pharmacol.* **52**: 611–617

Morimoto, K., Kstsumata, H., Yabuta, T., Iwanaga, K., Kakemi, M., Tabata, Y., Ikada, Y. (2001) Evaluation of gelatin microspheres for nasal and intramuscular administrations of salmon calcitonin. *Eur. J. Pharm. Sci.* **13**: 179–185

Mossberg, K., Ericsson, M. (1990) Detection of doubly stained fluorescent specimens using confocal microscopy. *J. Microsc.* **159**: 215–224

Nakaoka, R., Inoue, Y., Tabata, Y., Ikada, Y. (1996) Size effect on the antibody production induced by biodegradable microspheres containing antigen. *Vaccine* **13**: 1251–1256

Snyder, S. L., Sobocinski, P. Z. (1975) An improved 2,4,6-trinitro-benzensulfonic acid method for the determination of amines. *Anal. Biochem.* **64**: 284–288

Tabata, Y., Ikada, Y. (1989) Synthesis of gelatin microspheres containing interferon. *Pharm. Res.* **6**: 422–427

Tabata, Y., Ikada, Y. (1998) Protein release from gelatin matrices. *Adv. Drug Deliv. Rev.* **31**: 287–301

Tabata, Y., Ikada, Y., Morimoto, K., Kstsumata, H., Yabuta, T., Iwanaga, K., Kakemi, T. (1999) Surfactant-free preparation of biodegradable hydrogel micro-spheres for protein release. *J. Bioact. Compat. Polymers* **14**: 371–384

Wang, J., Tauchi, Y., Deguchi, Y., Morimoto, K., Tabata, Y. (2000) Positively charged gelatin microsphere as gastric mucoadhesive drug delivery system for clearance of H. pylori. *Drug Deliv.* **7**: 237–243