ELSEVIER

International Journal of Pharmaceutics 211 (2000) 57–65

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Polyphosphazene microspheres for insulin delivery

Paolo Caliceti [a,*], Francesco M. Veronese [a], Silvano Lora [b]

[a] *Department of Pharmaceutical Sciences, University of Padua, Via F. Marzolo, 5, 35131 Padova, Italy*
[b] *Institute of Photochemistry and High Energy Radiations, C.N.R., Via Romea, 4, 35020 Legnaro, Padova, Italy*

Received 6 September 2000; received in revised form 18 September 2000; accepted 20 September 2000

Abstract

Polyphosphazene based microspheres for insulin delivery were prepared following three different procedures: (A) suspension-solvent evaporation; (B) double emulsion-solvent evaporation; (C) suspension/double emulsion-solvent evaporation. Methods A and C allowed for higher protein loading than procedure B. Scanning electron microscopy showed that all preparation procedures achieve microparticles with spherical shape, porous surface and internal honeycomb structure. In all cases insulin was released 'in vitro' by a bi-modal behaviour: fast release during the first 2 hours followed by a slow release. However, both the physical properties and the 'in vitro' release profiles were found to depend upon the preparation conditions. Subcutaneous administration to diabetic mice of microspheres obtained with methods A and C rapidly reduced the glucose levels of about 80% but most of activity was lost in 100 hours. Both preparations B induced a remarkable decrease in glucose levels and the activity was maintained throughout 1000 h. Finally all preparations stimulated anti-insulin antibody production that constantly increased over a period of 8 weeks.

Keywords: Polyphosphazenes; Microspheres; Insulin; Drug delivery

1. Introduction

Polyphosphazenes bearing amino acid derivatives and amino acid derivatives/imidazole as substituents at the inorganic nitrogen/phosphorus backbone are good candidates as materials for preparation of prolonged drug delivery systems (Allcock et al., 1990; Allcock, 1999; Caliceti et al., 1994). Indeed, these polymers were found to be biocompatible and to degrade to harmless low molecular weight products at a rate that depends upon the physico-chemical character of the side groups (Crommen et al., 1992; Caliceti et al., 1993; Ruiz et al., 1993).

Although to date polyphosphazenes have been largely investigated for low molecular weight drug release, only few examples are reported in the literature for the formulation of proteins and peptides (Laurencin et al., 1987; Caliceti et al., 1997; Andrianov and Payne, 1998a). Nevertheless protein delivery is an interesting challenge in pharmaceutical technology and polymeric matrixes can represent a satisfactory solution in overcoming their poor biopharmaceutical and

* Corresponding author. Tel.: +39-49-8275695; fax: +39-49-8275366.
E-mail address: caliceti@dsfarm.unipd.it (P. Caliceti).

0378-5173/00/$ - see front matter
PII: S0378-5173(00)00588-3

physico-chemical properties (Langer, 1990; Wearley, 1991).

In order to exploit the advantages of polyphosphazenes in pharmaceutical technology their use in the preparation of protein delivery systems was investigated. In particular the development of formulations for sustained insulin delivery, a hormone of high therapeutic relevance, was studied. Indeed, systems designed to maintain the basal glucose level for a long time are usually required to allow for a proper managing of diabetes (Chetty and Chien, 1998; Trehan and Ali, 1998).

In this paper the preparation of insulin loaded microspheres obtained with a polyphosphazene derivatised with phenylalanine ethyl ester and imidazole at a molar ratio of 80/20 is reported. This polymer was selected, among the ones available in the laboratory, because of its high local and systemic biocompatibility and suitable degradation profile (Caliceti et al., 1994). Furthermore this polymer was successfully used for preparation of naproxen sustained release systems (Conforti et al., 1995; Veronese et al., 1998).

Microspheres were prepared following different preparation techniques and characterised for drug loading, morphological properties and in vitro drug release. In vivo studies were carried out by subcutaneous administration of the microparticles to diabetic rats and the glucose in blood as well as the anti insulin antibody levels were estimated.

2. Materials and methods

Bovine insulin, streptozotocin, standard glucose solution, glucose Trinder Kit and phosphatase conjugated goat anti insulin IgG + IgM-Sigma (St. Louis, MO). The analytical Reverse Phase C-4 column-Vydac (Hesperia, USA). All the other reagents of analytical grade-FLUKA (Steinheim, Germany). Male Sprague Dawley rats weighing 200–250 g used for in vivo studies — Charles River (Calco, Italy). Animal treatments were performed according to the Italian law (DL n. 116/92) and the institutional European guidelines (EEC n. 86/609).

The polyphosphazene substituted at the phosphorus atoms with phenylalanine ethyl ester and imidazole at a molar ratio of 80/20 was synthesised according to the method reported in literature (Caliceti et al., 1994).

2.1. Insulin loaded microsphere preparation

Insulin loaded polyphosphazene microspheres were obtained using the apparatus described by Goedemoed et al. (1991). The preparation was carried out following three different procedures:

(A) *Suspension-solvent evaporation*. Twenty milligrams of insulin were suspended under stirring in 2 ml of polymer solution (200 mg/ml of polyphosphazene in dichloromethane). The suspension was sonicated for 5 min and then injected at a rate of 1.25 ml/min into the vessel of the apparatus containing 200 ml of 0.01 M of CH_3COONH_4, 2.5% Tween 80, pH 5.4, maintained under stirring at 800 rpm. After injection, a vacuum was applied to the apparatus and stirring was maintained at 1500 rpm for 5 min and at 500 rpm for further 5 min. The suspension was decanted and the precipitate was collected, filtered, washed with 10 ml of 0.01 M CH_3COONH_4, pH 5.4 and centrifuged at 2500 rpm for 2 min. The washing procedure was repeated three times. Finally the microspheres were lyophilised for 20 h.

(B) *Double emulsion-solvent evaporation*. Twenty milligrams or 80 mg of insulin were dissolved in 200 μl of 0.01 M CH_3COOH pH 3.0 and added under stirring to 2 ml of polymer solution (200 mg/ml of polyphosphazene in dichloromethane). The emulsion was sonicated for 5 min and the microspheres were prepared following the procedure reported in A.

(C) *Suspension/double emulsion-solvent evaporation*. Twenty milligrams of insulin were suspended in 200 μl of 0.01 M CH_3COONH_4, pH 5.4 under stirring. The suspension was sonicated for 5 min and then added under stirring to 2 ml of polymer solution (200 mg/ml of polyphosphazene in dichloromethane). The emulsion was sonicated for 5 min and microspheres were obtained following the procedure reported in A.

Each preparation was repeated five times.

2.2. Microsphere characterisation

Scanning electron microscopy was performed on the intact lyophilised microspheres and on the microspheres after fracture in liquid nitrogen.

The particle size distribution analysis was carried out using an optical microscope (Laborlux, Sleitz, Germany) and a computer aided system (software by Casti Imaging, Venice, Italy). The statistical elaboration was performed using the computer programs Lotus and S.A.S. on the basis of 800 measurements of each sample randomly obtained by 100 images.

2.3. Insulin loading determination

Ten milligrams of microspheres were dissolved in 1 ml of CH_2Cl_2 and insulin was extracted with 1 ml of H_2O, 0.05% TFA. The amount of insulin in the aqueous phase was determined by HPLC using a reverse phase C-4 column isocratically eluted with 27.5:72.5 acetonitrile/0.05% TFA in water. The protein was detected at 220 nm and the protein concentration was calculated on the basis of the peak area using a titration standard curve.

The reliability of the method used for insulin determination in the microspheres was verified as follows: 100 μl CH_2Cl_2 containing 20 mg of polymer and 10 μl of H_2O/0.05% TFA containing insulin in the range of 0.2–1 mg were mixed and lyophilised. The dry samples were added of 100 μl of CH_2Cl_2 and 100 μl of H_2O/0.05% TFA and insulin was extracted as described above and analysed by HPLC. The insulin recovery was found to be $91.4 \pm 10.6\%$ with respect to the theoretical value.

2.4. In vitro release study

Twenty milligrams of microspheres were suspended in 2 ml of 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.2 and maintained under gentle shaking at 37°C. At scheduled times the microspheres were centrifuged and 1 ml of supernatant was removed and replaced with 1 ml of fresh buffer. The samples were lyophilised and dissolved in 100 μl of 27.5:72.5 acetonitrile/0.05% TFA in water and the insulin content was determined by HPLC as reported above.

2.5. In vivo release evaluations

Sprague Dawley male rats weighting 200–250 g were intraperitoneously treated with 65 mg/kg of streptozotocin (40 mg/ml in saline solution). After 5 days the animals were anaesthetised with ethyl ether and 50 μl of blood was taken by intracardiac puncture. Five microliters of serum, obtained by blood centrifugation for 2 min at 3500 rpm, were added to 45 μl of 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2 and 20 μl of the diluted serum were added to 200 μl of Trinder Kit. The samples were prepared in duplicate and the O.D. at 505 nm was determined after 30 min using an UV-VIS spectrophotometer.

The animals were randomly divided into four groups of ten rats each: A, B1, B2 and C.

Appropriate amounts of microspheres prepared by method A were suspended in 500 μl saline solution and subcutaneously injected to the animals of group A to administer a dose of 3 mg of insulin/kg. Similarly, microspheres prepared by method B using 5% insulin/polymer were administered to animals of group B1, microspheres prepared by method B using 20% insulin/polymer were administered to animals of group B2 and microspheres prepared by method C were administered to animals of group C. At scheduled times 50 μl of blood were taken by intracardiac puncture and the glucose concentration in serum were estimated.

The accuracy of the Trinder Kit based method used for estimation of the glucose levels in the serum was verified by analysis of mouse serum containing increasing glucose amounts. Each point was repeated five times and the mean values as well as the relative standard deviations (RSD) were calculated. The RSD was found to be of 1.2–2.3% of the mean values and the O.D. values reported as function of the glucose concentrations in the samples were found to fit a

linear plot with a linear correlation of R^2 of 0.998.

2.6. Immunological characterisations

Ten microlitres of serum obtained from the animals treated with microspheres for the in vivo release studies were used for estimation of anti insulin antibodies.

ELISA microplates were incubated overnight at 4°C with 100 μl/well of a 1 μg/ml insulin coating solution in 0.1 M $NaHCO_3$, pH 9.5. The wells were washed three times with 200 μl of PBS, 0.05% Tween 20, pH 7.2 (PBS-T) and then incubated for 2 h at room temperature with 200 μl of a 50 μg/ml bovine serum albumin solution in 0.1 M $NaHCO_3$, pH 9.5. The wells were washed and then incubated for 2 h at room temperature with 100 μl of serum samples serially diluted in PBS-T and, after washing, for 2 h with 100 μl of alkaline phophatase conjugated goat anti mouse IgG + IgM properly diluted in PBS-T. The wells were washed and 100 μl of a 1 mg/ml of p-nitrophenol phosphate (SIGMA[104]) solution in 1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8 were added. The enzymatic reaction was stopped after 1 h by addition of 50 μl of 3 N NaOH and the O.D. at 405 nm was determined.

3. Results and discussion

3.1. Microsphere preparation and characterisation

Three different preparation procedures were followed for obtaining insulin loaded polyphosphazene microspheres. Table 1 shows the microparticle preparation conditions and their main physical properties. All the preparation procedures were found to be reproducible where both product yield and microparticle physical properties are concerned.

The incomplete drug loading obtained by all the preparation procedures may be ascribed to the rapid insulin diffusion to the continuous phase during the microparticle formation. Indeed the adjustment of the external phase pH at 5.4 do not prevent the rapid diffusion of the protein to the continuous buffer since at this pH the insulin solubility is still very high (about 2 mg/ml). However when insulin in aggregate state was used during the preparation (methods A and C) the massive drug diffusion to the external phase was partially prevented probably because the drug dissolution takes place at a lower rate than microparticle hardening. On the other hand, solubilised insulin used in method B is available to diffusion to the continuous phase and consequently a low loading yield is obtained.

Table 1
Preparation procedures and main properties of microspheres: insulin loading yield and mean particle size[a]

Preparation	Method of preparation	Insulin/polymer mixture used in the preparation (w/w) (%)	Insulin loading yield (%)	Loaded insulin (mg of insulin/100 mg of polymer)	Mean particle size (μm)
A	Suspension-solvent evaporation	5	61.0 ± 4.6	3.06 ± 0.23	55.44 ± 17.78
B1	Double emulsion-solvent evaporation	5	35.0 ± 3.8	1.75 ± 0.19	43.15 ± 18.34
B2	Double emulsion-solvent evaporation	20	43.5 ± 4.2	8.72 ± 0.84	60.17 ± 30.08
C	Suspension/double emulsion-solvent evaporation	5	66.9 ± 5.8	3.34 ± 0.29	43.3 ± 18.61

[a] The data were obtained by five different preparation lots that were examined in double.

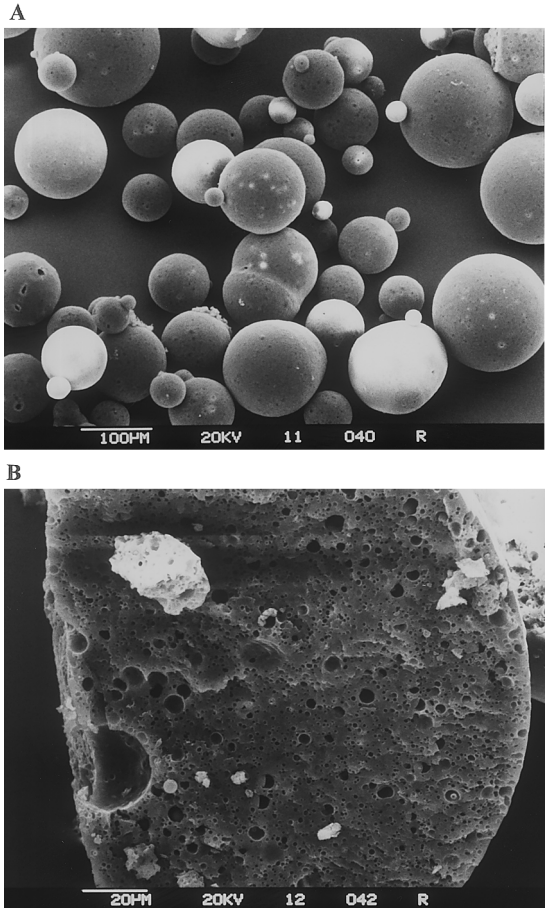

Fig. 1. Scanning electron microscopy of intact (A) and (B) fractured microspheres obtained by double emulsion-solvent evaporation using 20% insulin/polymer (preparation B2).

Scanning electron microscopy analysis demonstrated that all the preparation procedures achieve spherical microparticles with a porous surface and honeycomb internal structure (Fig. 1). The high porosity of preparations B and C is due to the elimination of the water microdrops of the double emulsion inner phase during the hardening of the microparticles. However, also preparation A presents high porosity indicating that microdrops of the continuous phase can be entrapped into the polymeric matrix during the microparticle formation, a behaviour already observed in preparations by emulsion-solvent evaporation (Veronese et al., 1998).

The data reported in Table 1 indicate that the preparation method slightly affects both the mean particle size and the particle distribution profiles. Preparation A presents a Gaussian like particle distribution profile with over 90% of microparticles in the range of 30–90 μm; preparation B1 presents a main population with 20–30 μm diameter and over 90% of microparticles in the range of 10–60 μm; preparation C displays similar fractions in the range of 10–50 μm and over 90% of the microparticles in the range of 0–60 μm. Remarkable differences were instead found between preparations B1 and B2 indicating that the insulin/polymer ratio used in the preparation plays an important role in obtaining microparticles with defined morphological properties. Microparticles of preparation B2 present, in fact, higher mean particle size and broader distribution profiles with respect to preparation B1. This behaviour could be due to an inefficient dispersion of the discontinuous phase during the microparticle formation because of the high density of the double emulsion inner phase.

3.2. In vitro insulin release

The in vitro insulin release time courses depicted in Fig. 2 indicate that the hormone is released from all preparations with a two-phase behaviour: fast release in the range of 0–2 h and

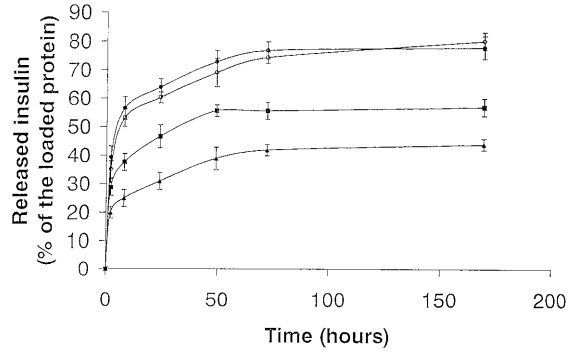

Fig. 2. In vitro insulin release time courses of microspheres obtained by: suspension-solvent evaporation (▲); double emulsion-solvent evaporation using 5% insulin/polymer mixture (○); double emulsion-solvent evaporation using 20% insulin/polymer mixture (●); suspension/double emulsion-solvent evaporation (■). The mean value and the S.D. were calculated on the basis of ten experiments carried out using five different preparation lots.

slow release in the range of 2–70 h. Furthermore the complete hormone release was never observed.

The lower burst effect and a lower amount of released insulin obtained with preparations A and C as compared to preparations B1 and B2 can be ascribed to the uneven distribution of insulin in the polymeric matrices. Indeed, it is reasonable to think that in the case of preparations B1 and B2 most of the insulin is molecularly dispersed in the pores that are rapidly perfused by the releasing buffer. On the other hand, in the case of preparation A most of insulin is present in solid state in the matrix network and therefore it is not easily available to the releasing buffer. Of interest are the results obtained with preparation C where insulin should be localised in the pores partially as molecular dispersion and partially in the solid state. The significantly lower burst effect obtained with this preparation as compared to the preparations B1 and B2 seems to indicate that the drug release is determined by the protein dissolution process.

It is worth noting that the hormone physically entrapped in the polymeric network is probably released by matrix erosion mechanism that takes place at a very slow rate and is reflected in the apparent absence of drug release (plateau) because undetectable amounts of drug are released over the time.

3.3. In vivo studies

Fig. 3 describes the in vivo hypoglycaemic profiles obtained by subcutaneous injection of the microspheres obtained by the various techniques to diabetic rats. The results point out the key role played by the physical state of the protein in the matrix on the therapeutic performance of the formulation.

The short hypoglycaemic activity displayed by preparations A and C seems in good agreement with the in vitro release profile. The maximal activity was observed after 2 h from administration according to the burst effect observed in vitro. After this period of time, the glucose level slowly increased to recover the initial value in 300–400 h.

The rapid decrease in the glucose concentration in serum obtained with preparations A and C can be ascribed to a little amount of promptly available protein molecularly dispersed on the microparticle surface. Instead, the release of the main protein amount, entrapped in the aggregate state into the polymeric network or in the matrix channels, takes place by slower processes, namely polymer degradation, that reflect in a poor hormone availability as already observed in the in vitro studies.

Interesting results were obtained with the microspheres prepared by procedure B which displayed a prolonged hypoglycaemic activity (Fig. 3B1 and B2). After 1 h from administration, preparation B1 induced a remarkable reduction of glycaemia (80% of the initial glucose level). The glucose concentration was maintained in the range of 80 and 50% of the starting value for about 500 h and then the glycaemia slowly increased to achieve the starting hyperglycaemic value in 1000 h. Microspheres prepared by method B using 20% of insulin/polymer mixture (preparation B2) showed a reduction to 20% of the glucose level after 1 h from administration but the starting glycaemia was recovered in 48 h. After this time the glucose level decreased to 40% in the following 24 h and then to 20% in 400 h. Finally the initial glycaemia was slowly recovered in 1000 h.

It is worth noting that the insulin molecular dispersion into the matrix pores obtained in preparations B1 and B2 permits a protein release kinetic that allows for a sustained pharmacological performance. However, the prolonged activity obtained with both preparations B does not reflect the rapid in vitro release probably because in vivo a slower insulin diffusion throughout the embedded pores takes place. Furthermore the results obtained with preparations B1 and B2 underline the effect of the protein/polymer ratio on the therapeutic performance of the preparations. Preparation B2 displays a pharmacological burst followed by a prolonged hypoglycaemic effect indicating that insulin is partially promptly and partially slowly available. This behaviour is probably due to the high protein concentration used in the microparticle preparation that on side promote the localisation of the protein on the microparticle surface (promptly available insulin) and on the other induces the partial protein pre-

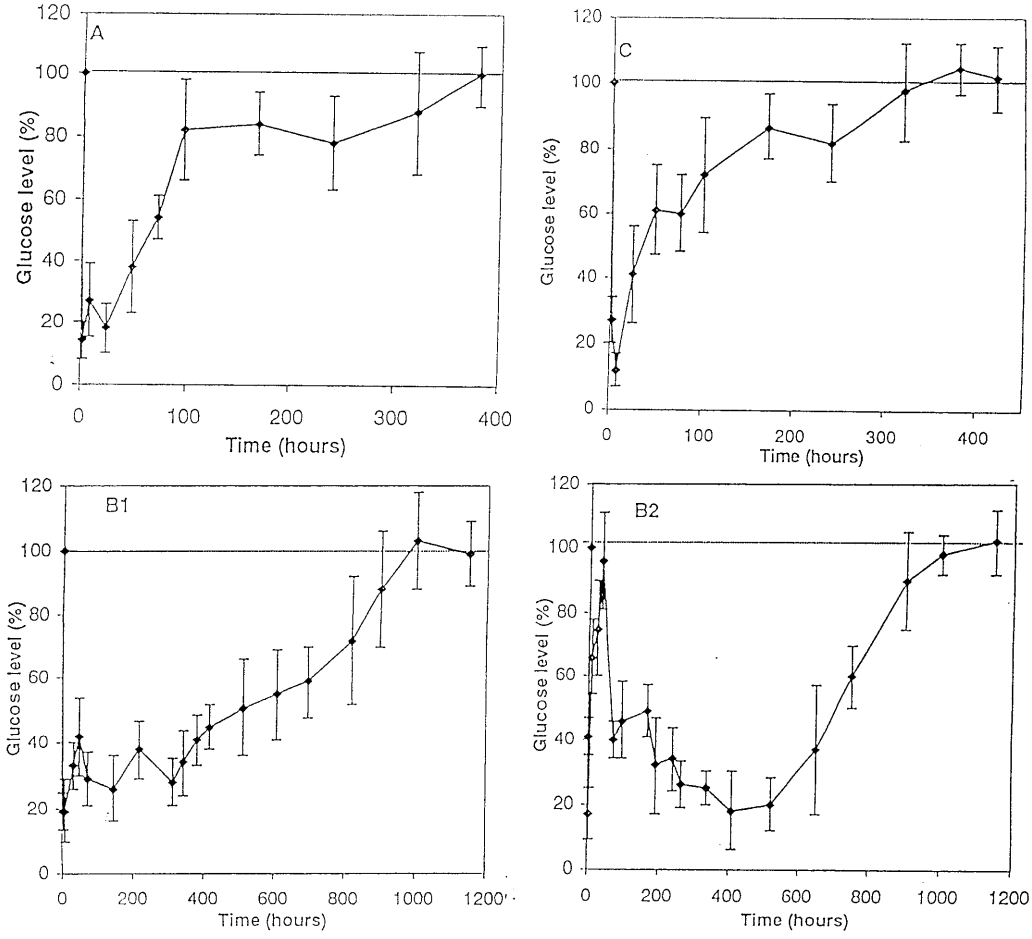

Fig. 3. Hypoglycaemic time courses after subcutaneous administration to diabetic rats of microspheres obtained by: suspension-solvent evaporation (A); double emulsion-solvent evaporation using 5% insulin/polymer mixture (B1); double emulsion-solvent evaporation using 20% insulin/polymer mixture (B1); suspension/double emulsion-solvent evaporation (C). The mean value and the S.D. were calculated on the basis of ten experiments.

cipitation (slow insulin release). Instead, using lower protein/polymer ratios more constant glycaemic levels are maintained over the first 400 h indicating that in this case a homogeneous dispersion of the protein in the polymeric system has been obtained.

3.4. Immunogenic profiles

The bovine insulin used in the present study possesses a potential immunogenic character when administered to rats because of its significant structural differences as compared to rat insulin (Randle et al., 1964). Furthermore polymeric systems providing for slow and prolonged drug release have been often observed to possess adjuvant properties in immunostimulation (Ohagan et al., 1998). Polyphosphazenes, in particular, have been demonstrated to act as adjuvant materials useful in preparation of vaccines (Andrianov and Payne, 1998b; Gupta et al., 1998). For such a reason anti insulin antibody levels were evaluated in the animals treated with the microspheres.

The anti insulin immunoresponse was tested in the serum of the rats treated with the various

preparations by ELISA (Fig. 4). All formulations were found to induce anti insulin immunoresponse from the 1st week of administration and the antibody levels increased constantly throughout the 8-week experiment. It is interesting to note that formulations A and C display a longer immunogenic activity with respect to the pharmacological one. Indeed anti insulin immunoresponse is continuously stimulated for 8 weeks, while hypoglycaemic activity terminated after 3 weeks. This demonstrates that, although insulin is released at an unsuitable rate for displaying the pharmacological activity, a slow release is maintained for a long time. In this respect it is, in fact, important to note that the antigen amount necessary to stimulate the immunoresponse is much lower than that required to maintain a prolonged pharmacological activity.

4. Conclusions

Although biodegradable polyphosphazenes are largely investigated for the preparation of low molecular weight drug release systems, they can be also successfully used to develop polymeric matrixes for protein delivery providing for a suitable alternative to polyesters.

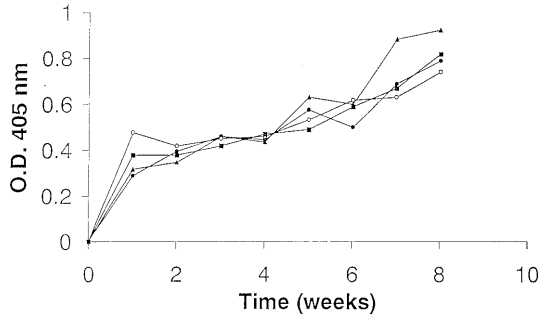

Fig. 4. Anti insulin immunoresponse elicited in diabetic rats after subcutaneous administration of microspheres obtained by: suspension-solvent evaporation (▲); double emulsion-solvent evaporation using 5% insulin/polymer mixture (○); double emulsion-solvent evaporation using 20% insulin/polymer mixture (●); suspension/double emulsion-solvent evaporation (■). The anti-insulin IgG + IgM levels are expressed as OD at 405 nm determined by ELISA using serum samples diluted at the same level. The mean value and the S.D. were calculated on the basis of ten experiments.

In the present investigation it was demonstrated that it is possible to prepare insulin loaded microspheres by using a biodegradable and biocompatible polyphosphazene allowing for the exploitation of new insulin formulations useful in diabetes management. In particular impressive in vivo results were obtained with the microspheres prepared by double emulsion that permitted the glucose level control for over 1 month. However, the manufacturing process was found to be crucial in the preparation of microspheres with the desired requisites, namely high drug loading yield and suitable physical properties. Furthermore the preparation conditions dictate the protein physical state and localisation into the polymeric matrix that deeply affect the pharmacological performance of the formulation.

It is also important to remember that the physical properties as well as the therapeutic performance of these systems depend strictly also upon the polymer chemical composition. In this respect a broad variety of polyphosphazenes with different physico-chemical properties, namely degradation rate and hydrophilic/hydrophobic balance, can be synthesised by careful choice of the substituents at the inorganic backbone. It is therefore possible to prepare tailored systems that can satisfy the specific requirements for a proper diabetes control on the basis of the individual disease condition.

Finally of interest is also the anti protein immunoresponse obtained with these formulations that, aside any consideration on the adjuvant effect of these polymers, confirm the potential application of these materials in the development for one shot vaccines as already pointed out by other researches.

Acknowledgements

This research was partially supported by MURST and C.N.R. grant programs.

References

Allcock, H.R., 1999. Recent developments with an impact on the future of polyphosphazene science and technology.

Phosphorus sulphur and silicon and related elements 146, 61–64.

Allcock, H.R., 1990. Polyphosphazenes as new biomedical and bioactive material. In: Chasin, M., langer, R. (Eds.), Biodegradable Polymers as Drug Delivery Systems. Mercel Dekker, New York, pp. 163–193.

Andrianov, A.K., Payne, L.G., 1998a. Protein release from polyphosphazene matrices. Adv. Drug Del. Rev. 31, 185–196.

Andrianov, A.K., Payne, L.G., 1998b. Polymeric carriers for oral uptake delivery of microparticulates. Adv. Drug Del. Rev. 34, 155–170.

Caliceti, P., Veronese, F.M., Marsilio, F., Lora, S., Seraglia, R., Traldi, P., 1993. Fast atom bombardment in the structural identification of intermediates in the hydrolytic degradation of polyphosphazenes. Org. Mass Spectrom. 27, 153–161.

Caliceti, P., Lora, S., Marsilio, F., Guiotto, A., Veronese, F.M., 1994. Amino acid esters and imidazole derivatives of polyphosphazenes: influenece of release of drugs, degradability and swelling. Il Farmaco 49, 69–74.

Caliceti, P., Aldini, N.N., Fini, M., Rocca, M., Gnudi, S., Lora, S., Giavaresi, G., Monfardini, C., Giardino, R., Veronese, F.M., 1997. Bioabsorbable polyphosphazene matrices as systems for calcitonin release. Il Farmaco 52, 697–702.

Chetty, D.J., Chien, Y.W., 1998. Novel methods of insulin delivery: an update. Crit. Rev. Ther. Drug Carrier Syst. 15, 629–670.

Conforti, A., Bertani, S., Lussignoli, S., Grigolini, L., Terzi, M., Lora, S., Caliceti, P., Marsilio, F., Veronese, F.M., 1995. Anti-inflammatory activity of polyphosphazene-based naproxen slow-release systems. J. Pharm. Pharmacol. 48, 468–473.

Crommen, J.H.L., Schacht, E.H., Mense, E.H.G., 1992. Biodegradable polymers. II. Degradation characteristics of hydrolysis-sensitive poly[(organo)phosphazenes]. Biomaterials 13, 601–611.

Goedemoed, J.H., Mense, E.H.G., de Groot, K., Claessen, A.M.E., Scheper, R.J., 1991. Development of injectable antitumor microspheres based on polyphosphazene. J. Control. Rel. 170, 245–258.

Gupta, R.K., Chang, A.C., Siber, G.R., 1998. Biodegradable polymer microspheres as vaccine adjuvants and delivery systems. Dev. Biol. Stand. 92, 63–78.

Langer, R., 1990. New methods of drug delivery. Science 249, 1527–1533.

Laurencin, C.T., Koh, H.J., Neenan, T.X., Allcock, H.R., Langer, R., 1987. Controlled release using a new bioerodible polyphosphazene matrix system. J. Biomed. Mater. Res. 21, 1231–1246.

Ohagan, D.T., Singh, M., Gupta, R.K., 1998. Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines. Adv. Drug Del. Rev. 32, 225–246.

Randle, P.J., Insulins, G., Pincus, 1964. In: Thimann, K.V., Astwood, A.B. (Eds.), The Hormones, Academic Press, New York, pp. 481–530.

Ruiz, E.M., Ramirez, C.A., Aponte, M.A., Barbosa-Canovas, G.V., 1993. Degradation of poly[bis(glycine ethyl ester)phosphazene] in aqueous media. Biomaterials 14, 491–496.

Trehan, A., Ali, A., 1998. Recent approaches in insulin delivery. Drug Dev. Ind. Pharm. 24, 589–597.

Veronese, F.M., Marsilio, F., Caliceti, P., De Filippis, P., Giunchedi, P., Lora, S., 1998. Polyorganophosphazene microspheres for drug release: polymer synthesis, microsphere preparation, in vitro and in vivo naproxen release. J. Control. Rel. 52, 227–237.

Wearley, L.L., 1991. Recent progress in protein and peptide delivery by noninvasive routes. Crit. Rev. Ther. Drug Carrier Syst. 8, 331–394.