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Development and in vivo evaluation of an oral insulin–PEG delivery system

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Abstract

Insulin-monomethoxypoly(ethylene glycol) derivatives were obtained by preparation of mono- and di-terbutyl carbonate insulin derivatives, reaction of available protein amino groups with activated 750 Da PEG and, finally, amino group de-protection. This procedure allowed for obtaining high yield of insulin–1PEG and insulin–2PEG. In vivo studies carried out by subcutaneous injection into diabetic mice demonstrated that the two bioconjugates maintained the native biological activity. In vitro, PEGylation was found to enhance the hormone stability towards proteases. After 1 h incubation with elastase, native insulin, insulin–1PEG and insulin–2PEG undergo about 70, 30 and 10% degradation, respectively, while in the presence of pepsin protein degradation was 100, 70 and 50%, respectively. The attachment of low molecular weight PEG did not significantly ($P > 0.05$) alter insulin permeation behavior across the intestinal mucosa. Insulin–1PEG was formulated into mucoadhesive tablets constituted by the thiolated polymer poly(acrylic acid)–cysteine. The therapeutic agent was sustained released from these tablets within 5 h. In vivo, by oral administration to diabetic mice, the glucose levels were found to decrease of about 40% since the third hour from administration and the biological activity was maintained up to 30 h. According to these results, the combination of PEGylated insulin with a thiolated polymer used as drug carrier matrix might be a promising strategy for oral insulin administration.

Keywords: Oral delivery; Insulin; Mucoadhesive tablets; PEGylation; Thiolated polymers; Thiomers

1. Introduction

The recent advances in DNA technology has prompted the interest for pharmaceutical application of many proteins and peptides. The development of 'invasive-to-oral-conversions' for protein delivery, however, remains a great challenge in modern pharmaceutical technology. Indeed, due to the high structural fragility, hydrophilicty and molecular weight, proteins undergo unsatisfactory absorption through the mucosa and rapid elimination from circulation, which compromise seriously their therapeutic applicability and performance (Cleland et al., 2001; Drews, 2003). In addition, during the transit through the intestinal tract they are chemically and enzymatically inactivated by the high acidity of the stomach and the presence of nutrients and secreted enzymes. Nevertheless, oral delivery is a very attractive option for administration of many proteins such as hormones, vaccines and other biomodulators (Sastry et al., 2000; Lee, 2002; Shein, 2003).

In order to benefit from the advantages of oral delivery, a number of studies has been carried out to develop oral insulin formulations. Actually, orally administered insulin is delivered first to the liver through the portal circulation, similarly to the physiological route of insulin secretion in non-diabetic individuals. Furthermore, potential benefits from this route include improved disease management, enhanced patient compliance and reduction of long-term complications of diabetes (Ray and Sharma, 2003; Gordon-Still, 2002).

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To improve oral insulin delivery, different strategies have been pursued such as the use of protease inhibitors, buffers, permeation enhancers, mucoadhesive excipients, smart polymers, colon release or micro- and nano-particles (Gordon-Still, 2002; Marschütz et al., 2000; Damagè et al., 1997; Lowman et al., 1999; Haupt and Rubinstein, 2002). Modification of protein structure by attachment of proper moieties which alter the biopharmaceutical properties of proteins has also been investigated. The conjugation technology was applied to several proteins including insulin, which was derivatized with lipophylic or glycosilic molecules or synthetic oligomers (Ashada et al., 1995; Hinds and Kim, 2002; Baudys et al., 1995; Caliceti and Veronese, 1999; Still, 2002). Fatty acid conjugation, for example, was found to enhance the protein absorption through biological membranes while conjugation of poly(ethylene glycol) improved the protein solubility and protected from enzymatic degradation. Recently, a promising protein modification was generated by attachment of a short chain PEG linked to an alkyl group to Lys-29 of the B-chain. Biological activity is retained and this compound is readily absorbed from the gastrointestinal tract (Ashada et al., 1995).

In view of currently available literature data it seems clear that a combination of both strategies, protein structure modification together with a proper solid dosage formulation, can succeed in the generation of effective protein delivery systems for oral administration.

In this paper we report about a formulation study for oral insulin delivery based on hormone bioconjugation and loading into mucoadhesive tablets. Insulin was modified by attachment of one or two low molecular weight PEG to convey to the hormone enhanced stability towards proteases: pepsin and elastase. Pepsin and elastase were selected because of their low specificity and because they are secreted in the gastric and intestinal sites, respectively. Biological stability of insulin–PEG bioconjugates towards pepsin and elastase were evaluated as thiolated poly(acrylic acid) was chosen as drug carrier matrix, which provides a protective effect towards most other secreted gastrointestinal exo- and endo-peptidases (Bernkop-Schnürch and Thaler, 2000; Luessen et al., 1995). The derivative bearing 1PEG molecule was formulated into a mucoadhesive tablet and in vivo studies were undertaken.

2. Materials and methods

Bovine insulin, monomethoxypoly(ethylene glycol) 750 Da, elastase from porcine pancreas (4 IU/mg), pepsin from porcine gastric mucosa (3200 IU/mg), streptozotocin and the Trinder glucose kit were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were furnished from Fluka (Steinheim, Germany).

Monomethoxypoly(ethylene glycol) was activated as *p*-nitrophenylchloroformate according to the procedure described previously (Veronese et al., 1985). Purification steps of the polymer were carried out by precipitation from ethyl ether at −20 °C.

The analytical and preparative C18 RP columns were furnished by Vydac (Hesperia, CA, USA). The sample purification was carried out by eluting with a H_2O/acetonitrile (0.1% trifluoroacetic acid) gradient: 5% acetonitrile (0–5 min), 5–70% acetonitrile (5–45 min). The analytical chromatography was carried out by C18 column elution with a H_2O/acetonitrile (0.1% trifluoroacetic acid): 10% acetonitrile (0–5 min), 20–80% acetonitrile (5–35 min).

The gel permeation chromatography was carried out using a Superdex 75 column (Pharmacia, Uppsala, Sweden) eluted isocratically with 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2.

The male Balb/C mice weighing 23–25 g used for in vivo studies were furnished from the Department of Pharmaceutical Sciences (University of Padua). Animal treatments were performed according to the Italian law (DL no. 116/92) and the Institutional European Guidelines (EEC no. 86/609).

2.1. Insulin–PEG synthesis

Insulin–1BOC and insulin–2BOC were prepared according to the method described previously (Markussen et al., 1991). Briefly, bovine insulin (100 mg) was dissolved in 5 ml of DMSO and pH was adjusted at 8.5 by addition of triethylamine. The solution was added to 268 μl of 0.05 M di-terbutylcarbonate in dimethylsulfoxide to reach a 3:2 protein amino group/di-terbutylcarbonate molar ratio. The solution was maintained at room temperature for 3 h and then added to 10 ml of demineralized water, congealed in liquid nitrogen and lyophilized. The dry product was dissolved in 1 ml of acetonitrile/water/TFA (60/40/1) and the reaction products were purified by preparative reverse phase-C18 chromatography. The peaks corresponding to insulin–BOC (insulin–1BOC, insulin–2BOC and insulin–3BOC) were collected, concentrated under vacuum and characterized by mass spectrometry using a Kratos Kompact MALDI TOF and spectrophotometric analysis for determination of derivatized amino groups (Habeeb, 1966).

Twenty milligrams of insulin–1BOC or insulin–2BOC were dissolved in 1 ml DMSO and added to 23.5 mg of activated 750 Da PEG to achieve 3:9 protein amino group/PEG molar ratio. The reaction mixture was maintained under stirring overnight and then dropped into 500 ml of ethyl ether. The precipitate was desiccated and the dry product was dissolved in 1 ml of 95% TFA and maintained at room temperature for 12 h. TFA was eliminated under vacuum and the residue was dissolved in 1 ml of 20 mM phosphate buffer, 0.15 M NaCl, pH 7.4. The insulin–PEG conjugates were purified by gel filtration using a Superdex 75 column operated on a FPLC and eluted with the same phosphate buffer. The peaks corresponding to insulin–1PEG and insulin–2PEG were collected and analyzed by UV-Vis, circular dichroism, mass spectrometry, C18 RP-chromatography, colorimetric assay for amino group determination (Habeeb, 1966) and io-

dine test for PEG determination (Sims and Snape, 1980). The isomer composition of insulin–1PEG and insulin–2PEG was examined by the insulin–PEG treatment with tributylphosphine/iodoacetamide and RP-HPLC analysis of the reaction products. The eluted peaks were identified by amino acid sequence analysis (Caliceti and Veronese, 1999).

2.2. Proteolytic studies

Native insulin (400 μg) or equimolar amounts of insulin–1PEG and insulin–2PEG were dissolved in 988 μl 0.1 M Tris HCl, 5 mM EDTA, pH 8.5, and added to 12 μl of a 152 μg/ml elastase solution in the same buffer. The hormone/enzyme solutions were maintained at 25 °C. At scheduled times (0, 5, 20, 35, 60, 90, 120, 150, 180 min), 50 μl samples were taken and added to 50 μl of H_2O containing 0.1% of TFA to stop the enzyme activity. The samples were lyophilized and then re-dissolved in 50 μl of H_2O/acetonitrile (50:50). The solution was analyzed by RP-HPLC and the area under the peak corresponding to the undegraded protein was referred to the standard curve in order to obtain the undegraded protein concentration in the starting solution.

Native insulin (400 μg) or equimolar amounts of insulin–1PEG 750 and insulin–2PEG 750 were dissolved in 986 μl of 0.01 M HCl and added to 14 μl of a 100 μg/ml pepsin solution in 0.01 M HCl. The hormone/enzyme solutions were maintained at 25 °C. At scheduled times (0, 5, 20, 35, 60, 90, 120 min), 50 μl samples were drawn and added to 50 μl of 0.1 M borate buffer, pH 8.0 to stop the degradation reaction. The samples were lyophilized and processed as reported above for determination of undegraded protein concentration.

2.3. Synthesis and characterization of poly(acrylic acid)–cysteine

The poly(acrylic acid)–cysteine conjugate was synthesized as described previously (Marschütz and Bernkop-Schnürch, 2002). In brief, L-cysteine was covalently attached to poly(acrylic acid) of 450 kDa molecular mass. Mediated by a carbodiimide amide bonds between the primary amino group of the amino acid and the carboxylic acid group of the polymer were formed. The resulting conjugate was isolated by exhaustive dialysis followed by lyophilization at −30 °C and 0.01 mbar.

The amount of covalently attached thiol groups was determined using Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid) according to a method described previously (Bernkop-Schnürch et al., 1999).

2.4. Preparation of the delivery system

First, 20 mg of poly(acrylic acid)–cysteine conjugate, 2.5 mg of insulin–1PEG, 0.5 mg of the permeation mediator glutathione and 2 mg of mannitol were hydrated in 2 ml of demineralized water. The mixture was lyophilized at −30 °C and 0.01 mbar and 2 mg micro-tablets (diameter: 1.5 mm; thickness: 1.0 mm) were compressed. The compaction force was kept constant during the preparation of all micro-tablets. The dosage form was then enteric coated with Eudragit L100-55 according to a method described previously (Marschütz et al., 2000).

2.5. Drug release studies

As the efficacy of the enteric coating has already been demonstrated previously (Marschütz et al., 2000), drug release studies were focused on the dissolution behavior at intestinal pH conditions. The in vitro release rate of PEGylated insulin from the oral delivery system was determined by a method which is not conform to United States Pharmacopeia (USP) due to the small size of the tablets. The dosage forms were each placed into 1.5-ml vials containing 1 ml release medium (100 mM phosphate buffer, pH 7.1). The closed tubes were then placed on an oscillating waterbath (GFL 1092; 60 rpm) and incubated at 37 ± 0.5 °C. Aliquots of 100 μl were withdrawn at predetermined time points and replaced with an equal volume of fresh release medium equilibrated at 37 °C. Sink conditions were maintained throughout the study. The withdrawn volumes were filtered with 0.22 μm filters and released insulin was determined by reversed phase HPLC chromatography according to a method described previously (Marschütz et al., 2000). Released insulin concentrations were quantified from integrated peak areas and calculated by interpolation from an according standard curve. Cumulative corrections were made for previously removed samples.

2.6. FITC labeling of (PEGylated) insulin

The fluorescence labeling of insulin and PEGylated insulins was performed according to a method described previously. In this previous study it was shown that FITC labeled insulin remains stable throughout such permeation studies (Clausen and Bernkop-Schnürch, 2001).

2.7. Permeation studies

Permeation studies were carried out in Ussing type chambers displaying a volume of 1 ml (= 1 cm^3) in the donor- and acceptor-chamber and a permeation area of 0.64 cm^2. The incubation medium contained 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose and 50 mM $NaHCO_3$ buffered with 40 mM HEPES, pH 7.4.

Immediately after sacrificing the guinea pig (line: Duncan Hardley) 15 cm of the small intestine (duodenum) were excised and mounted in the Ussing chamber. All experiments were performed at least three times in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. After 15–20 min of preincubation with the buffer solution, the incubation medium of the donor compartment was substituted by the same medium but

containing 0.05% (m/v) FITC labeled insulin, insulin–1PEG or insulin–2PEG, respectively. Samples of 100 μl were withdrawn from the acceptor compartment every 30 min over a time period of 2 h. Samples were immediately replaced by 100 μl buffer solution equilibrated at 37 °C. The amount of permeated drug was determined using a fluorimeter (SLT, Spectra Fluor, Tecan, Austria). Cumulative corrections were made for the previously removed samples. The apparent permeability coefficient (P_{app}) for insulin and its derivatives was calculated according to the following equation:

$$P_{app} = \frac{Q}{A \times c \times t}$$

where P_{app} is the apparent permeability coefficient (cm/s), Q is the total amount permeated within the incubation time (μg), A is the diffusion area of the Ussing chamber (cm^2), c is the initial concentration of the marker in the donor compartment ($\mu g/cm^3$), and t is the total time of the experiment (s).

After permeation studies the medium was removed from the donor chamber and 1 ml trypan blue dye was added and the mucosa was incubated for 30 min. Microscopic investigations demonstrated, that the mucus is still present and that the viability of the intestinal membrane was guaranteed, as no blue color was detectable within the cells.

2.8. Biological activity evaluation

A 65 mg/kg dose of streptozotocin (30 mg/ml in 10 mM phosphate buffer, 0.15 N NaCl, pH 7.2) was intraperitoneously administered to Balb/C mice. After 3 days from the first administration the mice were treated with the same dose of streptozotocin. One week later, 30 μl of blood samples were taken from the retrobulbar site, centrifuged and the glucose levels in the serum were estimated using the Trinder glucose kit. The glucose was determined two times per day for 3 consecutive days in order to verify the diabetic state of mice. Animals with glycaemic levels of 450 ± 50 mg/dl were used for the study. Three groups of five mice each were subcutaneously treated with 2 μg/mouse of native insulin (group A) or equimolar amount of insulin–1PEG (group B) and insulin–2PEG (group C). At scheduled times the glucose content in serum was determined as reported above.

Insulin–1PEG loaded tablets were administered orally to 10 diabetic mice (group D). The formulations were placed very deeply into the throat in order to initiate the swallowing reflex mechanism. Additionally, 50 μl of a 0.2% (w/v) aqueous ascorbic acid solution were administered. As control, 200 μl solution of insulin–1PEG and insulin–2PEG in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.2 (1 mg insulin equivalent/ml) was administered to mice (group E and group F, respectively).

One group of five diabetic animals was used as negative control. The animals were not treated with insulin preparations and the glycaemic levels were determined throughout 2 days.

2.9. Statistical data analysis

Statistical data analysis was performed using the Student's t-test with $P < 0.05$ as the minimal level of significance unless indicated otherwise.

3. Results

3.1. Insulin–PEG preparation

The chromatographic pattern relative to purification of insulin reacted with di-terbutylcarbonate showed the presence of four main peaks. The free amino group determination by colorimetric analysis and the mass spectrometry analysis allowed for identification of the four insulin forms which were: native insulin, insulin–1BOC, insulin–2BOC and insulin–3BOC. The relative abundance of the insulin derivatives and the main physical–chemical properties of the derivatives is reported in Table 1. The reverse phase C18 chromatographic analysis evidenced heterogeneous composition of both purified insulin–1BOC and insulin–2BOC indicating that these products were composed by a mixture of isomers: insulin–Gly_1–BOC, insulin–Phe_1–BOC and insulin–Lys_{29}–BOC in the case of the mono-BOC derivative and insulin–Phe_1,Lys_{29}–BOC, insulin–Gly_1,Lys_{29}–BOC and insulin–Phe_1,Gly_1–BOC in the case of the di-BOC derivative. However, the quantitative separation of the isomers compromised seriously the production yield.

Table 1
Identification, composition and main physico-chemical properties of the various insulin forms: native insulin, relative abundance, molecular mass and free amino groups

	RP-18 HPLC analysis	Relative abundance (%)	Molecular mass (Da)	Free amino groups (%)
Native insulin	First peak	16.1	5734	100
Insulin–1BOC	Second peak	50.4	5834	66
Insulin–2BOC	Third peak	27.9	5934	33
Insulin–3BOC	Fourth peak	5.5	6034	0
Insulin–1PEG	–	90[a]	6484	66
Insulin–2PEG	–	90[a]	7234	39

[a] Approximate value.

Therefore, insulin–1BOC and insulin–2BOC were not further purified before reaction with activated PEG.

The use of high available protein amino group/activated PEG molar ratio (3:9) yielded for extensive derivatization of the free protein amino groups. After de-protection by TFA, insulin–1BOC–PEG was found to generate insulin–2PEG while insulin–1PEG was obtained from insulin–2BOC–PEG. The purification step of insulin–1PEG by gel filtration showed that the PEGylation reaction was not complete, since a small amount of native insulin (about 10% of the total protein) remained. The gel filtration profile of insulin–2PEG did not show the presence of native insulin, but the peak corresponding to insulin–2PEG presented a small shoulder indicating that a small amount of insulin–1PEG was remaining in the final product. The presence of insulin–1PEG was confirmed by the colorimetric determination of the free amino groups which was slightly higher than that expected (33%). On the basis of the spectrophotometric data, the insulin–1PEG content in the final product was calculated to be 18% (insulin–1PEG/Pegylated, moles/moles%).

The RP-C18 chromatographic analysis of insulin–1PEG and insulin–2PEG treated with tributylphosphine/iodoacetamide demonstrated the heterogeneous composition of the purified bioconjugates. Indeed, the disulphide bridge reduction of PEGylated insulin showed the simultaneous presence of peaks corresponding to unmodified A and B chains, PEG derivatized A chain and mono- or di-PEG derivatized B chain. This result indicates that PEG was randomly bound to Gly_1 (chain A), Phe_1 (B chain) and Lys_{29} (B chain). By the analysis of the peaks obtained after insulin β–mercaptoethanol/iodoacetamide reaction and according to the reverse-phase analysis of the BOC-derivatives, insulin–1PEG was found to be mainly constituted by equimolar amounts of insulin–Lys_{29}–1PEG and insulin–Gly_1–1PEG. In the case of insulin–2PEG the product was mainly constituted by insulin–Gly_1, Lys_{29}–2PEG.

3.2. Proteolytic characterization

The proteolytic profiles of native insulin, insulin–1PEG and insulin–2PEG after incubation with elastase and pepsin are reported in Figs. 1 and 2, respectively. Although the concentrations of elastase and pepsin with 7.3 mIU/ml and 1.4 μg/ml were below the simulated physiological concentrations being determined to be 189–836 mIU/ml and 1.25 mg/ml, respectively (Bernkop-Schnürch, 1998; Wissenschaftliche Tabellen Geigy, 1977), in both cases native insulin is rapidly degraded (100% degradation in 180 and 120 min with elastase and pepsin, respectively). Both PEGylated insulins (insulin–1PEG and insulin–2PEG) displayed a significantly higher resistance to proteolysis. In particular, insulin–2PEG maintained higher structural integrity compared to insulin–1PEG.

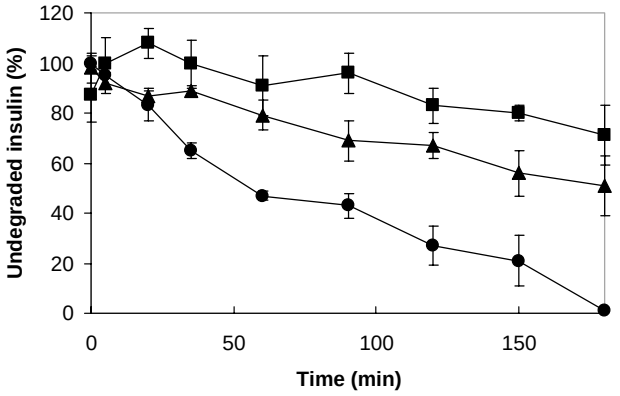

Fig. 1. Degradation profiles of native insulin (●), insulin–1PEG (▲) and insulin–2PEG (■) incubated with elastase (7.3 μIU/ml). The mean values and standard deviations were calculated on the basis of four experiments.

3.3. Polymer characterization

The poly(acrylic acid)–cysteine conjugate displayed 556±98 μmol thiol groups per gram polymer (mean±S.D.l, $n = 3$). Features of the polymer such as swelling behavior and mucoadhesive properties (data not shown) were in good correlation to the previously synthesized and characterized polymer (Marschütz and Bernkop-Schnürch, 2002).

3.4. Permeation studies

Permeation studies were carried out to verify the influence of PEG on insulin transport through the intestinal mucosa. The attachment of PEG to insulin did not significantly alter the permeation behavior of this therapeutic peptide through the small intestinal mucosa. The P_{app} values for unmodified insulin, insulin–1PEG conjugate and insulin–2PEG conjugate were determined to be 2.3×10^{-6}, 2.7×10^{-6} and 2.3×10^{-6} cm/s with a standard deviation in no case exceeding $\pm 0.4 \times 10^{-6}$ cm/s, respectively.

In contrast, recent studies demonstrated that the conjugation of low molecular weight PEGs to drugs can enhance

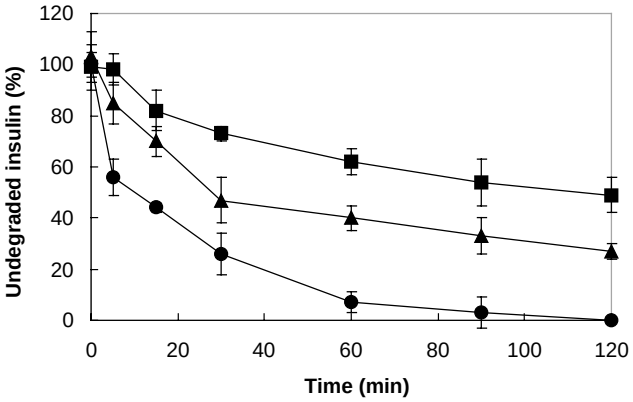

Fig. 2. Degradation profiles of native insulin (●), insulin–1PEG (▲) and insulin–2PEG (■) incubated with pepsin (7.7 IU/ml). The mean values and standard deviations were calculated on the basis of four experiments.

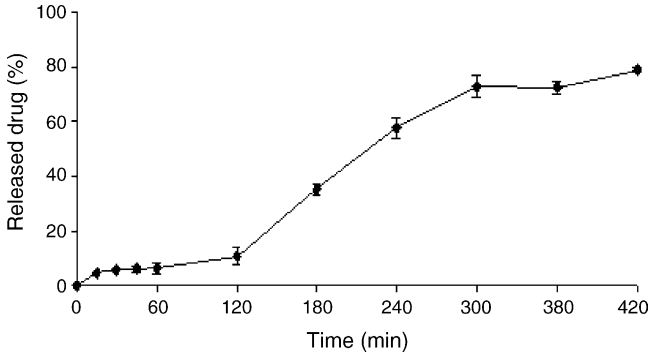

Fig. 3. Release profile of insulin–1PEG from tablets based on poly(acrylic acid)–cysteine conjugate. Studies were carried out in 100 mM phosphate buffer, pH 7.1 on an oscillating waterbath (60 rpm) at 37±0.5 °C. Indicated values are mean ± S.D. of at least three experiments.

the absorption by promoting the paracellular transport (Yeh et al., 2000). The effect of PEG on drug permeability, however, is strictly related to the polymer size and, in the case of a multi-point conjugation, of the polymer mass of the conjugate.

3.5. Drug release studies

The drug release rate was directly determined in a buffer solution. Results as shown in Fig. 3 demonstrated a sustained release of PEGylated insulin out of the polymeric carrier matrix. The relatively lower release rate within the first 2 h can be explained by the hydration process of the enteric coating material. Thereafter, an almost zero-order release kinetic could be observed for 3 h followed by a plateau phase where approximately three quarters of the drug were released. An explanation for reaching a plateau phase already when three quarters of the drug are released might be given by a partially absorptive binding of the drug to the plastic vessel and/or the polymeric carrier matrix.

3.6. Biological activity

In vivo studies were carried out by subcutaneous and oral administration of native insulin, insulin–1PEG and insulin–2PEG solutions and oral administration of tablets containing insulin–1PEG to diabetic mice.

Fig. 4 reports the hypoglycaemic profiles obtained by subcutaneous injection of equimolar amounts of native insulin, insulin–1PEG and insulin–2PEG to diabetic mice. The glycaemic profiles indicate that all the insulin forms display similar biological and biopharmaceutical behavior either in term of maximal activity or pharmacological time course. In particular, insulin–1PEG and insulin–2PEG showed overlapped profiles. The higher mean activity observed after 180 min with the two PEGylated forms as compared to the native hormone was not statistically significant.

Oral administration of insulin–1PEG and insulin–2PEG solutions did not affect significantly the glucose levels which were similar to the ones obtained with untreated animals (negative control). On the contrary, Fig. 5 shows that insulin–1PEG formulated into the poly(acrylic acid)–cysteine tablets administered orally to diabetic mice could induce a significant reduction of the blood glucose level in the range of 40% within 3 h from administration. The low glucose levels were constantly maintained throughout 24 h.

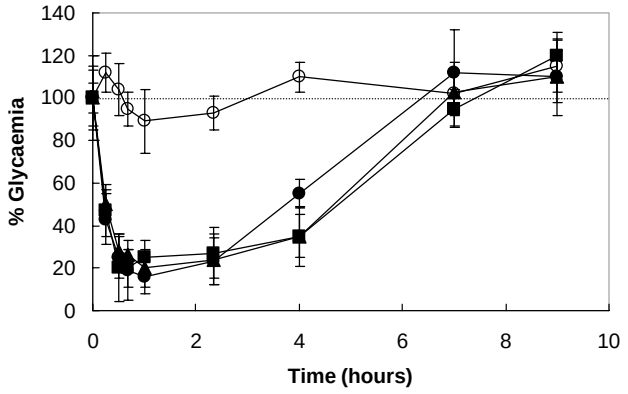

Fig. 4. Glycaemic profiles in diabetic mice after subcutaneous administration of equimolar amounts of native insulin (2 μg) (●), insulin–1PEG (▲), insulin–2PEG (■), negative control (untreated mice) (○). Each point represents the mean ± S.D. of five experiments.

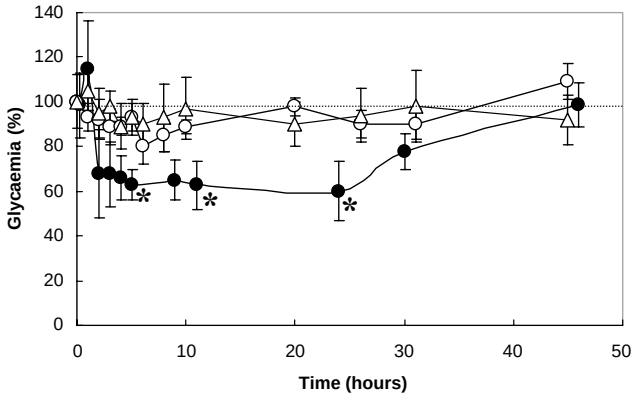

Fig. 5. Glycaemic profiles in diabetic mice after oral administration of insulin–1PEG (200 μg insulin equivalent) loaded tablets (2 mg microtablets comprising poly(acrylic acid)–cysteine conjugate, insulin–1PEG, glutathione and mannitol, 20+2.5+0.5+2, coated with Eudragit L100-55) (●); control (200 μg insulin equivalent of insulin–1PEG in 200 μl of phosphate buffered saline, pH 7.2) (○); negative control (untreated diabetic mice) (△). Each point represents the mean ± S.D. of 10 experiments. *: $P < 0.001$.

4. Discussion

In order to develop an effective formulation for oral insulin delivery, hormone derivatives were prepared by conjugation of one or two 750 Da PEG molecules and then formulated into mucoadhesive tablets.

PEGylation is known to improve protein stability towards proteases, reduce immunogenicity and antigenicity and

prolong the protein permanence in the blood (Veronese, 2001). However, alteration of these properties is related to both polymer molecular mass and number of polymer chains attached on the protein surface (Caliceti and Veronese, 2003; Knauff et al., 1988). Previous studies demonstrated that conjugation of 5 kDa PEG reduced the insulin biological activity and delayed the protein absorption (Caliceti and Veronese, 1999). Activity reduction and retardation in protein absorption increased as the PEG molecular mass as well as the number of polymer chains bound on the protein surface increased. Therefore, to balance the positive and negative effects of PEG, the conjugation was carried out using the 750 Da oligomer.

Preparation of conjugates by a multi-step procedure, which involves the partial protein amino group protection, was found to be more convenient than the direct polymer conjugation usually adopted in protein PEGylation. Orientating studies demonstrated, in fact, that the direct reaction of insulin with low molecular mass PEGs (350 and 750 Da) yields a mixture of derivatives at different degree and position of modification, although reaction was carried out under controlled conditions. Separation of conjugates was difficult either by preparative ion exchange chromatography or reverse-phase chromatography. Also, because of the small size difference among conjugates and native insulin, gel permeation chromatography or size exclusion techniques did not allow for quantitative separation of mono-, di- and tri-PEGylated insulin derivatives. The insulin-terbutylcarbonate derivatives (insulin bearing 1, 2 or 3 BOC residues) were quantitatively isolated by reverse phase preparative chromatography and easily identified by mass spectrometry, colorimetric assays and chromatographic analysis. Analytical reverse phase C18 chromatography showed that both insulin–1BOC and insulin–2BOC were composed by isomers. However, since the isomer separation lowered significantly the final product recovery, isomer mixtures were used in the following step. The use of isomer mixtures reflected in obtaining insulin–PEG isomers. Insulin–2PEG was composed by a mixture of insulin–Phe_1,Lys_{29}–2PEG, insulin–Gly_1,Lys_{29}–2PEG and insulin–Phe_1,Gly_1–2PEG, while insulin–1PEG was composed by insulin–Gly_1–1PEG, insulin–Phe_1–1PEG and insulin–Lys_{29}–1PEG. The isomers were not further separated although studies reported in the literature demonstrated that they can display different biological activity. Indeed, Gly_1 is directly involved in receptor recognition while Phe_1 and Lys_{29} play a minor role in insulin activity (Blundell et al., 1972). Nevertheless, in virtue to their p*K*a value and steric availability, the amino group reactivity was in the rank order $Gly_1 > Lys_{29} > Phe_1$.

Native insulin, insulin–1PEG and insulin–2PEG subcutaneously administered to diabetic mice induced similar hypo-glycaemic profiles. Maximal activity, peak time and AUC were not significantly different indicating that the conjugation of one or two 750 Da PEG neither alter the protein structure nor interfere with the receptor interaction. To note that the maintenance of insulin structure by PEG conjugation was already observed with high molecular mass PEGs and confirmed in the present study. Indeed native insulin and insulin modified with one or two 750 Da PEG chains displayed identical circular dichroism patterns.

Although conjugation of one or two 750 Da PEG were not found to interfere with the receptor interaction, the polymeric cloud on the protein surface prevented partially the approach of proteolytic enzymes such as elastase and pepsin. Surprisingly, despite the low molecular mass of PEG, insulin–1PEG was shown to exhibit an appreciable higher stability in comparison to the native protein. The increase in hormone stability was very similar to the ones obtained with insulin modified with one 5 kDa PEG (Caliceti and Veronese, 1999). The attachment of two polymer chains was found to further increase the protein stability towards proteases, though the improvement was much lower than that already obtained by conjugation of 2PEG 5 kDa chains. However, this result is in agreement with many studies reported in literature, which highlight the correlation between polymer mass on the protein surface and stability towards proteases. The stability is, in fact, expected to increase as the polymer mass on the protein surface increases, either by increasing the polymer molecular mass or by increasing the number of polymer chains bound (Veronese et al., 1996).

In the contrast to former studies, where insulin was also embedded in thiolated poly(acrylic acid) and given orally (Marschütz et al., 2000) in this study no additional enzyme inhibitors were incorporated in the delivery system. As PEGylation, on the one hand, provides a protective effect toward enzymatic degradation by pepsin and elastase and the thiolated polyacrylate, on the other hand, inhibits all other intestinally secreted exo- and endo-peptidases (Bernkop-Schnürch and Thaler, 2000; Luessen et al., 1995), no enzyme inhibitors were needed. Although an enteric coating would not have been strictly necessary in order to avoid a pepsinic degradation of the therapeutic agent in the stomach, tablets were nevertheless coated with a polymetacrylate to enhance the hormone stability during its gastric transit. Furthermore, as orientating studies revealed that the delivery system adheres otherwise already to the gastric and not to the intestinal mucosa (data not shown).

In vivo studies demonstrated that the oral administration of the mucoadhesive tablets containing insulin–1PEG succeeded in decreasing the glucose levels in diabetic mice. The pharmacological time course showed a lag time of about 3 h, which was in fair agreement with the drug release from the tablets. However, the hypoglycaemic effect was maintained throughout 26 h. Apart from the PEGylation of the therapeutic agent, this long lasting activity may be attributable to the delivery system itself. Thiolated polymers—designated thiomer—were shown to exhibit strong mucoadhesive properties due to the formation of disulfide bonds between the intestinal mucus gel layer and the polymeric excipient (Leitner et al., 2003). Consequently the intestinal residence time of the delivery systems should be prolonged. In addition, thio-

lated polymers show strong permeation enhancing properties for the paracellular uptake of hydrophilic macromolecules (Clausen and Bernkop-Schnürch, 2001). Previous studies revealed that this permeation enhancing effect of thiomers can be even further improved by the addition of glutathione, which seems to mediate the opening process of the tight junctions caused by thiomers (Clausen et al., 2002). It is worth to note that the hypoglycaemic activity observed was comparable to the one already obtained by oral administration of native insulin formulated into mucoadhesive tablet (Marschütz et al., 2000). However, in this study a five-fold lower dose of insulin equivalent was administered, showing a more pronounced effect. The pharmacological efficacy calculated on the basis of the areas under the reduction in serum glucose levels of the oral formulation versus s.c. injection normalized by the administered dose was determined to be 7%. According to these results, the use of PEGylated insulin in combination with a thiolated polymeric carrier matrix represents a promising strategy for the oral administration of insulin.

5. Conclusions

Oral delivery of peptides and proteins for therapeutic treatment of diseases is feasible, if a proper formulation strategy is utilized. A promising option seems to involve the use of protein derivatives with enhanced intrinsic biopharmaceutical and physical/chemical properties, which are de facto new chemical entities and their formulation into mucoadhesive and enzyme inhibiting solid formulations.

The results reported in the present study underline the high potential of this strategy. Indeed, it is interesting to note that these formulations have great potential for improvements to make them flexible and adaptable to delivery of many proteins and peptides. In particular, the possibility of obtaining a wide array of derivatives together with the various modification of the physical/chemical properties of the solid dosage form, can allow of exploitation for delivery systems with high therapeutic performance.

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References

Ashada, H., Douen, T., Mizokoshi, Y., Fujita, T., Murakami, M., Yamamoto, A., Muranishi, S., 1995. Absorption characteristics of chemically modified insulin derivatives with various fatty acids in the small and large intestine. J. Pharm. Sci. 84, 682–687.

Baudys, M., Uchio, T., Hovgaard, L., Zhu, E.F., Avramaoglou, T., Jozefowics, M., Rihova, B., Park, J.Y., Lee, H.K., Kim, S.W., 1995. Glycosilated insulins. J. Control. Release 36, 151–157.

Bernkop-Schnürch, A., 1998. The use of inhibitory agents to overcome the enzymatic barrier to perorally administered therapeutic peptides and proteins. J. Control. Release 52, 1–16.

Bernkop-Schnürch, A., Thaler, S., 2000. Polycarbophil–cysteine conjugates as platforms for oral (poly)peptide delivery systems. J. Pharm. Sci. 89, 901–909.

Bernkop-Schnürch, A., Schwarz, V., Steininger, S., 1999. Polymers with thiol groups: a new generation of mucoadhesive polymers? Pharm. Res. 16, 876–881.

Blundell, T.L., Cutfield, J.F., Cutfield, S.M., Dodson, E.J., Dodson, G.G., Hodgkin, D.C., Mercola, D.A., 1972. Three-dimensional atomic structure of insulin and its relationship to activity. Diabetes 21, 492–505.

Caliceti, P., Veronese, F.M., 1999. Improvement of the physico-chemical and biopharmaceutical properties of insulin by poly(ethylene glycol) conjugation. STP Pharma Sci. 9, 107–113.

Caliceti, P., Veronese, F.M., 2003. Pharmacokinetic and biodistribution properties of poly(ethylene glicol)-protein conjugates. Adv. Drug Deliv. Rev. 55, 1261–1277.

Clausen, A.E., Bernkop-Schnürch, A., 2001. Thiolated Carboxymethylcellulose: in vitro evaluation of its permeation enhancing effect on peptide drugs. Eur. J. Parm. Biopharm. 51, 25–32.

Clausen, A.E., Kast, C.E., Bernkop-Schnürch, A., 2002. The role of glutathione in the permeation enhancing effect of thiolated polymers. Pharm. Res. 19, 602–608.

Cleland, J.L., Daugherty, A., Mrsny, R., 2001. Emerging protein delivery methods. Curr. Opin. Biotechnol. 12, 212–219.

Damagè, C., Vrankx, H., Balsschmidt, P., Couvreur, P., 1997. Poly(alkyl cianoacrylate) nanospheres for oral administration of insulin. J. Pharm. Sci. 86, 1403–1409.

Drews, J., 2003. Strategic trends in drug industry. Drug Discovery Today 8, 411–420.

Gordon-Still, J., 2002. Development of oral insulin: progress and current status. Diabetes Metab. Res. Rev. 18, S29–S37.

Habeeb, A.F.S.A., 1966. Determination of free amino groups in proteins by trinitrobenzensulfonic acid. Anal. Biochem. 14, 328–336.

Haupt, S., Rubinstein, A., 2002. The colon as a possible target for orally administered peptide and protein drugs. Crit. Rev. Ther. Drug Deliv. Syst. 19, 499–551.

Hinds, K.D., Kim, S.W., 2002. Effects of PEG conjugation on insulin properties. Adv. Drug Deliv. Rev. 54, 505–530.

Knauff, M.J., Bell, D.P., Hirtzer, P., Luo, Z.P., Young, J.D., Kartre, N.V., 1988. Relationship of effective molecular size to systemic clearance in rats of recombinant interleukin-2 chemically modified with water soluble polymers. J. Biol. Chem. 263, 15064–15070.

Lee, H.J., 2002. Protein drug oral delivery: the recent progress. Arch. Pharm. Res. 25, 572–584.

Leitner, V., Walker, G.F., Bernkop-Schnürch, A., 2003. Thiolated polymers: evidence for the formation of disulphide bonds with mucus glycoproteins. Eur. J. Pharm. Biopharm. 56, 207–214.

Lowman, A.M., Morishita, M., Kajita, M., Nagai, T., Peppas, N.A., 1999. Oral delivery of insulin using pH-responsive complexation gels. J. Pharm. Sci. 88, 933–937.

Luessen, H.L., Verhoef, J.C., Borchard, G., Lehr, C.M., de Boer, A.G., Junginger, H.E., 1995. Mucoadhesive polymers in peroral peptide drug delivery. II. Carbomer and polycarbophil are potent inhibitors of the intestinal proteolytic enzyme trypsin. Pharm Res. 12, 1293–1298.

Markussen, J., Halstrom, J., Wiberg, F.C., Schaffer, L., 1991. Immobilized insulin for high capacity affinity chromatography of insulin receptors. J. Biol. Chem. 266, 18814–18818.

Marschütz, M.K., Caliceti, P., Bernkop-Schnörch, A., 2000. Design and in vivo evaluation of an oral delivery system for insulin. Pharm. Res. 17, 1468–1474.

Marschütz, M.K., Bernkop-Schnürch, A., 2002. Thiolated polymers: advance in mucoadhesion by use of in situ crosslinking poly(acrylic acid)–cysteine conjugates. Eur. J. Pharm. Sci. 15, 387–394.

Ray, N.K., Sharma, C.P., 2003. Oral insulin—a perspective. J. Biomater. Appl. 17, 183–196.

Sastry, S.V., Nyshadham, J.R., Fix, J.A., 2000. Recent technological advances in oral drug delivery—a review. PSTT 3, 138–145.

Shein, W.C., 2003. Oral peptide and protein delivery: unfulfilled promises? Drug Discovery Today 8, 607–608.

Sims, G.E.C., Snape, T.J., 1980. A method for estimation of poly(ethylene glycol) in plasma protein fractions. Anal. Biochem. 107, 60–63.

Still, J.G., 2002. Development of oral insulin: progress and current status. Diabetes Met. Res. Rev. 18, S29–S37.

Veronese, F.M., 2001. Peptide and protein PEGylation: a review of problems and solutions. Biomaterials 22, 405–417.

Veronese, F.M., Largajolli, R., Boccù, E., Benassi, C.A., Schiavon, O., 1985. Surface modification of protein: activation of monomethoxypolyethylene-glycols by phenylchloroformates and modification of ribonuclease and superoxide dismutase. Appl. Biochem. Biotechnol. 11, 141–152.

Veronese, F.M., Monfardini, C., Caliceti, P., Schiavon, O., Scrawen, M.D., Beer, D., 1996. Improvment of pharmacokinetic, immunological and stability properties of asparaginase by conjugation to linear and branched monomethoxypoly(ethylene glycol). J. Control. Release 40, 199–209.

Wissenschaftliche Tabellen Geigy, eighth ed., Basel, 1977.

Yeh, P.Y., Bentley, M.D., Clark, I.L., Kim, G., Smith, P.L., 2000. Intestinal transport of PEGylated small drug molecule, In: Proceedings of the 27th International Symposium on Contr. Rel. Bioact. Mater., Paris, France, pp. 59–60.