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Biopharmaceutical characterisation of insulin and recombinant human growth hormone loaded lipid submicron particles produced by supercritical gas micro-atomisation

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ABSTRACT

Homogeneous dispersions of insulin and recombinant human growth hormone (rh-GH) in tristearin/phosphatidylcholine/PEG mixtures (1.3:1.3:0.25:0.15 w/w ratio) were processed by supercritical carbon dioxide gas micro-atomisation to produce protein-loaded lipid particles. The process yielded spherical particles, with a 197 ± 94 nm mean diameter, and the insulin and rh-GH recovery in the final product was 57 ± 8% and 48 ± 5%, respectively. In vitro, the proteins were slowly released for about 70–80 h according to a diffusive mechanism. In vivo, the insulin and glucose profiles in plasma obtained by subcutaneous administration of a dose of particles containing 2 μg insulin to diabetic mice overlapped that obtained with 2 μg of insulin in solution. Administration of a dose of particles containing 5 μg insulin resulted in faster and longer glycaemia reduction. Oral administration of 20 and 50 μg insulin equivalent particles produced a significant hypoglycaemic effect. The glucose levels decreased since 2 h after administration, reaching about 50% and 70% glucose reduction in 1–2 h with the lower and higher dose, respectively. As compared to subcutaneous administration, the relative pharmacological bioavailability obtained with 20 and 50 μg equivalent insulin particles was 7.7% and 6.7%, respectively. Daily subcutaneous administration of 40 μg of rh-GH-loaded particles to hypophysectomised rats induced similar body weight increase as 40 μg rh-GH in solution. The daily oral administration of 400 μg rh-GH equivalent particles elicited a slight body weight increase, which corresponded to a relative pharmacological bioavailability of 3.4% compared to subcutaneous administration.

1. Introduction

Biotech drugs, namely proteins and oligonucleotides, are thought to become the main source of therapeutics in the near future (Tsuji and Tsutani, 2008). Nevertheless, the therapeutic exploitation of such molecules will rely on the possibility to develop suitable formulations that can satisfactorily overcome the intrinsic limitations to their use, namely physicochemical instability and low bioavailability (Orive et al., 2003).

As peptide drugs are often indicated for chronic conditions, the requirement for long term daily injections has obvious drawbacks (Nestor, 2007). Although the oral route offers the advantages of self-administration with high patient acceptance and compliance, poor membrane permeability hampers oral, as well as other non-invasive administration routes (Morishita and Peppas, 2006). To enable effective transmucosal protein delivery, attention has been focused on formulations which improve transmembrane absorption and prevent from degradation (Jorgensen et al., 2006).

Micro- and nano-sized systems (e.g., liposomes, lipid and polymeric nanoparticles, micelles, etc.) have been found to provide advantages over traditional formulations for protein delivery. The entrapment of protein drugs into these systems provides for (1) a way to hide and protect the proteins from degradation during storage and delivery and (2) sustained release (Almeida and Souto, 2007; Bilati et al., 2005; Mundargi et al., 2008).

Among particulate formulations, solid lipid nanoparticles have been successfully explored for drug delivery because they combine the benefits of liquid lipid-based colloidal systems (e.g., emulsions and liposomes) and solid systems (Joshi and Müller, 2009; Kluge et al., 2009; Mehnert and Mader, 2001). These products possess excellent tissue biocompatibility, biodegradability, composition flexibility and small size, making them suitable for a variety of applications. Furthermore, these formulations have been found to enhance the drug bioavailability after oral or local administration. On the other hand, solid lipid particle manufacturing techniques are

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not easily adaptable to protein processing as they operate under harsh conditions; namely, high temperature, pressure, and shear stress, and in a few cases, involve the use of organic chemicals, which are detrimental to protein stability. Moreover, often these techniques are difficult to scale-up for industrial production.

In recent years, much effort has been made to provide solutions which meet these existing manufacturing needs. In particular, techniques based on supercritical fluids have been developed to process polymer and lipid materials and produce particulate pharmaceutical. These techniques can be properly adapted to protein processing as they can avoid denaturation and degradation phenomena and may be exploited to produce pharmaceutical grade protein delivery system formulations (Caliceti et al., 2004; Davies et al., 2008; Okamoto and Danjo, 2008; Reverchon et al., 2008; Reverchon et al., 2009; Tandya et al., 2007).

Recently, we described a novel supercritical fluid gas micro-atomisation process for preparation of protein-loaded lipid particles (Salmaso et al., 2009). This technique is an improved version of "Particle from Gas Saturated Solution" (PGSS) developed to process materials melting under supercritical conditions. In our previous study, we demonstrated that the introduction of a co-axial air injection device in the typical PGSS equipment can yield submicron particles. Furthermore, a preliminary study performed with insulin as the protein model showed that the use of selected excipient mixtures and the optimisation of operating conditions resulted in high product yield, protein loading and preservation of biological protein activity.

Since we demonstrated that the gas micro-atomisation process was suitable for the fabrication of submicron protein-loaded lipid particles, we further investigated the biopharmaceutical and in vivo performance of these particles. Aimed at evaluating the general applicability of the manufacturing process to produce therapeutically efficient drug delivery systems, the study was performed using insulin and recombinant human growth hormone (rh-GH), two proteins of relevant pharmaceutical interest with significantly different physicochemical properties. Protein-loaded lipid particles were produced under optimised operative conditions and their biopharmaceutical properties were evaluated. In vivo investigations were undertaken using appropriate animal models to evaluate the pharmacokinetic and pharmacodynamic performance of these formulations after either subcutaneous or oral administration.

2. Materials and methods

2.1. Materials

Tristearin, Tween 80, 5 kDa poly(ethylene glycol) (PEG 5000) and dimethylsulfoxide were obtained from Fluka (Buchs, Switzerland). Streptozotocin, glucose Trinder kit, glucose standard solution and bovine insulin (5.7 kDa) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human growth hormone (rh-GH, 21 kDa) was a kind gift of Bio-Ker (Pula, Italy). Phosphatidylcholine (Epikuron 200) was donated by Degussa (Padova, Italy). Acetonitrile was from Merck (Darmstadt, Germany) and CO_2 was purchased by Air Liquid (Padova, Italy). The 100 kDa dialysis membranes (Float-A-Lyzer tubes) were from Spectrum Laboratories (Los Angeles, CA, USA). The C-18 reverse phase Jupiter column (250 mm × 4.6 mm, 300 Å) was furnished by Phenomenex (Torrance, CA, USA).

2.2. Animals

Two month old male Balb/c mice, weighing 22–25 g, were provided by the Department of Pharmaceutical Sciences of University of Padua. Four week old hypophysectomised female Sprague–Dawley rats, weighing 75–80 g, were obtained from Charles River (Como, Italy). Animal care and handling were performed in accordance with the provisions of the European Economic Community Council Directive 86/209 (recognised and adopted by the Italian Government with the approval decree D.M. No. 230/95-B) and the NIH publication No. 85-23, revised in 1985.

2.3. Particle production

The lipid mixture was prepared by dispersing 1.3 g phosphatidylcholine and 0.25 g of PEG 5000 into 1.3 g of melted tristearin at 120 °C. The mixture was stirred continuously for about 20 min, until complete homogenisation, and then the temperature was decreased to 65 °C. The melted lipid mass was supplemented with 1 ml of DMSO containing 150 mg of insulin or rh-GH. The mixture was continuously stirred while the temperature was left to slowly decrease to solidification. Two grams of the lipid/protein mixture was loaded into the melting chamber of the gas micro-atomisation supercritical apparatus described elsewhere (Salmaso et al., 2009), which was pressurised with CO_2 at 150 bar, heated to 40 °C, and maintained under stirring. All the equipment modules (connections, valves, nozzle, etc.) were thermostated at 50 °C. After 30 min, the valve connecting the melting chamber with the collecting vessel was opened and the peristalting pump forcing the melted mass spraying into the precipitation chamber was activated. Simultaneously, a 0.18 l/h air flux at 200 bar was co-axially injected. The CO_2 flowed continuously through the precipitation chamber at 1200 Nl/h (normal litres/h at 273 K and 1 atm). At the end of the run, the dry material was collected from the bottom of the precipitation vessel where a circular stainless microfilter set (100 mm diameter) was placed.

2.4. Differential scanning calorimetry analysis

Five milligrams of tristeratin, PEG and the lipid tristearin/PEG/phosphatidylcholine mixture in DMSO were analysed by a Q10P/PDSC differential scanning calorimetry (TA Instruments, New Castle, DE, USA). The analysis was performed by sample heating from 25 °C to 80 °C. The heating rate was 2 °C while the cooling rate was 1 °C up to the instrument end point.

2.5. Dimensional analysis

Determination of the particle size was performed by photon correlation spectroscopy using a Nicomp 380 single-angle photon correlation spectrometer (Particle Sizing Systems, Santa Barbara, CA) fitted with a 632.5 nm laser. Ten milligram particles were dispersed under vortexing in 100 ml distilled water, sonicated in an ice bath for 30 min, and analysed.

2.6. Optical analysis

Transmission electron microscopy (TEM) analyses were performed by using a TECNAI G2 cryo-TEM apparatus (FEI, Oregon, USA) by adsorption of the sonicated particle dispersion onto glow-discharged carbon-coated copper grids and processed by liquid nitrogen for analysis.

2.7. Dispersibility and stability analyses

Ten-milligram particle samples were dispersed under stirring in 20 ml of 0.02 M phosphate buffer/0.15 M NaCl, pH 7.4. The dispersions were sonicated in ice bath for 5, 10, 15, 20, 25 and 30 min. After each sonication run, the samples were spectrophotometrically analysed at 600 nm. For each analysis set, the optical density was normalised by the maximal optical density value (end point, 100%).

The particle dispersions obtained after 30 min of sonication were centrifuged at 3400 rpm for 0, 5, 10, 20, 30, 40 and 60 min and analysed at 600 nm after each centrifugation run. For each analysis set, the optical density was normalised by the value obtained before centrifugation (time 0 min, 100%).

2.8. Insulin and rh-GH loading

The insulin and rh-GH content in the lipid particles was assessed by extraction and chromatographic analysis. Particle samples (10 mg) were supplemented with 1 ml DMSO, melted at 55 °C, and then 1 ml of H_2O containing 0.05% TFA was added. After 5 min of vortexing, the mixtures were centrifuged at 12,000 rpm for 10 min. The insulin content in the supernatant was analysed by RP-HPLC chromatography using an analytical C-18 column eluted with H_2O/0.05% TFA (eluent A) and acetonitrile/0.05% TFA (eluent B) in a gradient mode (0–3 min 20% eluent B; 3–23 min from 20% to 75% eluent B). The UV detector was set at 220 nm. The protein amount was determined on the basis of a protein titration curve ($y = 33875x$; $R^2 = 0.9893$). The rh-GH content in the supernatant was analysed by RP-HPLC using an analytical C-18 column eluted with H_2O/0.05%TFA (eluent A) and acetonitrile/0.05%TFA (eluent B) in a gradient mode (0–3 min 45% eluent B, 3–23 min from 45% to 70% eluent B). The UV detector was set at 210 nm. The protein amount was determined on the basis of a protein titration curve ($y = 15995x$; $R^2 = 0.9965$).

2.9. Insulin and rh-GH release

Insulin and rh-GH loaded particle samples (10 mg) was suspended in 1 ml 0.02 M phosphate buffer/0.15 M NaCl (pH 7.4), vortexed for 15 s, and sonicated in an ice bath for 10 min. The suspensions were introduced in dialysis tubes with a 100 kDa molecular weight cut-off and dialysed against 10 ml of the same buffer at 37 °C. At scheduled time intervals, 10 ml of the dialysis receiving volume was withdrawn and replaced with fresh buffer. The protein content in the released buffer was evaluated by RP-HPLC according to the methods reported above. For comparison, 0.2 mg/ml protein solutions were dialysed according to the same protocol reported above.

2.10. In vivo studies: insulin loaded submicron particles

Balb/c mice were intraperitoneally treated with 65 mg/kg of streptozotocin (40 mg/ml in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4); the mice were boosted one week after the first treatment. After 5, 8 and 11 days from the second streptozotocin treatment, 30 μl of blood were collected by retrobulbar puncture. The blood samples were centrifuged for 2 min at 3500 rpm and 10 μl of plasma were added to 40 μl of 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4. The glucose concentration in the diluted plasma was estimated using the glucose Trinder Kit and a titration curve obtained with a standard glucose solution. One hundred mice with glucose levels in the range of 400–500 mg/dl were divided into 10 groups of 10 animals each. The animals in Group 1 (reference) were subcutaneously treated with 200 μl of 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4. The animals in Groups 2–4 were subcutaneously treated with 200 μl of phosphate buffer containing 0.1 mg of protein free particles (Group 2); 2 μg of insulin (Group 3); or 2 μg insulin equivalent particles (Group 4). The animals in Group 5 (reference) were orally treated with 200 μl of 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4. The animals in Groups 6–10 were orally treated with 200 μl of phosphate buffer containing 1 mg of protein free particles (Group 6); 20 μg of insulin (Group 7); 50 μg of insulin (Group 8); 20 μg of insulin equivalent particles (Group 9); or 50 μg of insulin equivalent particles (Group 10). At scheduled times, 50 μl of blood was collected by retrobulbar puncture and centrifuged. Plasma samples (10 μl) were diluted and the glucose concentrations were estimated as reported above.

Plasma samples (10 μl) were used for insulin analysis as determined by ELISA using a human insulin-specific ELISA kit (Linco Research Inc., St. Charles, MO).

2.11. In vivo studies: rh-GH loaded submicron particles

Forty-nine hypophysectomised rats were divided into seven groups of seven animals each. The animals of Group 1 were not treated (control). The animals of Groups 2–4 were treated by daily subcutaneous administration of 200 μl of 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4, containing 40 μg of rh-GH, 40 μg of rh-GH equivalent particles and 1.5 mg protein free particles, respectively. The animals of Groups 5–7 were treated by daily oral administration of 500 μl of phosphate buffer containing 400 μg of rh-GH, 40 μg of rh-GH equivalent particles, and 16 mg protein free particles, respectively. The body weight increase was assessed daily.

2.12. Experimental data elaboration

The insulin concentrations and glucose values at the experimental time points were analysed by the computer program Kinetica to calculate the insulin bioavailability ($AUC_{0\rightarrow t}$) and the pharmacological bioavailability ($AAC_{0\rightarrow t}$). AAC was calculated as the area above the glycaemic curve considering the initial glucose level as the baseline point (starting and end point, 100%). The relative pharmacokinetic bioavailability of insulin after subcutaneous (PKRBsc) or oral (PKRBos) administration of the protein-loaded particles was calculated as follows:

$$\text{PKRBsc} = \left[\frac{(\text{AUC}_{\text{particle sc}}/\text{dose})}{(\text{AUC}_{\text{solution sc}}/\text{dose})}\right] \times 100$$

$$\text{PKRBos} = \left[\frac{(\text{AUC}_{\text{particle os}}/\text{dose})}{(\text{AUC}_{\text{solution sc}}/\text{dose})}\right] \times 100$$

The relative pharmacological bioavailability of insulin after subcutaneous (PDRBsc) or oral (PDRBos) administration of the protein-loaded particles was calculated as follows:

$$\text{PDRBos} = \left[\frac{(\text{AAC}_{\text{particle os}}/\text{dose})}{(\text{AAC}_{\text{solution sc}}/\text{dose})}\right] \times 100$$

$$\text{PDRBsc} = \left[\frac{(\text{AAC}_{\text{particle sc}}/\text{dose})}{(\text{AAC}_{\text{solution sc}}/\text{dose})}\right] \times 100$$

The relative pharmacological bioavailability of rh-GH was calculated as follows:

$$\text{PDRBsc} = \left[\frac{(\text{Body weight increase}(\%)_{\text{particles sc}}/\text{dose})}{(\text{Body weight increase}\,(\%)_{\text{solution sc}}/\text{dose})}\right] \times 100$$

$$\text{PDRBos} = \left[\frac{(\text{Body weight increase}\,(\%)_{\text{particles os}}/\text{dose})}{(\text{Body weight increase}(\%)_{\text{solution sc}}/\text{dose})}\right] \times 100$$

3. Results

3.1. Particle preparation and characterisation

The combination of tristerain, phosphatidylcholine, 5 kDa PEG and DMSO yielded homogeneous low melting temperature mixtures. The DSC thermograms of tristerain, PEG and the tristearin/PEG/phosphatidylcholine/DMSO mixture are reported in Fig. 1.

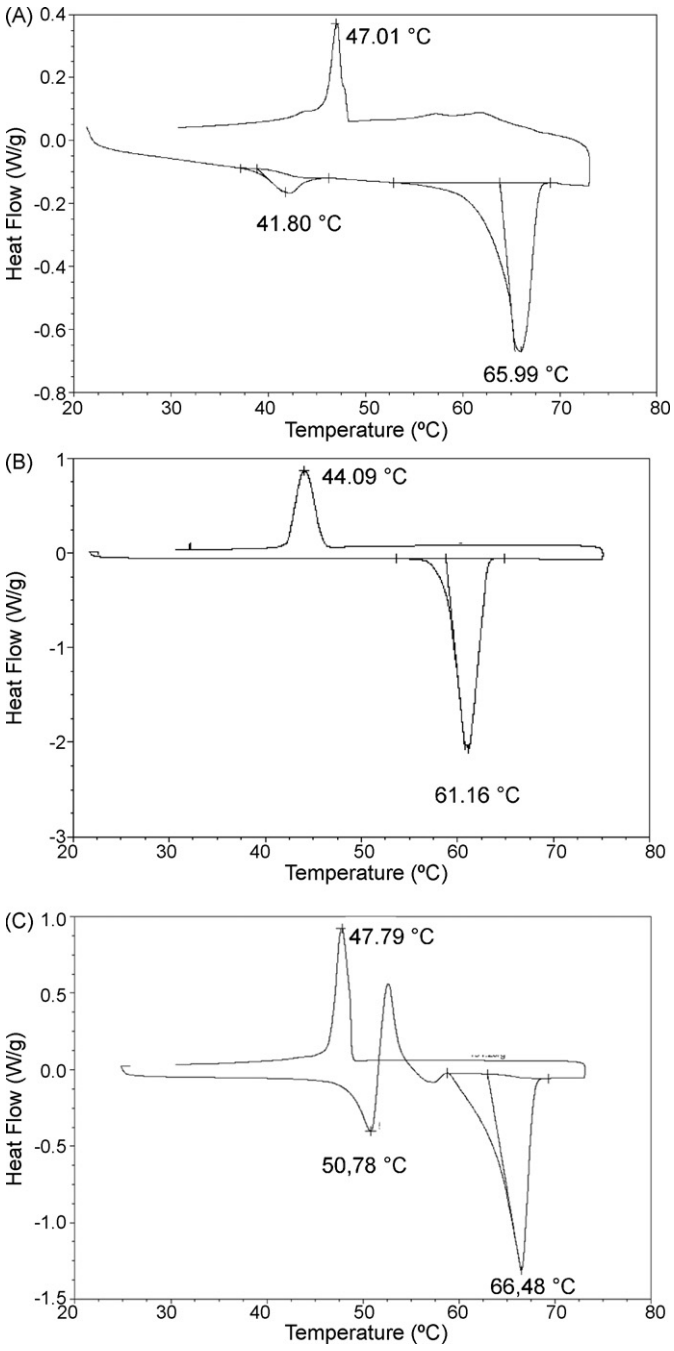

Fig. 1. Differential scanning calorimetry thermograms of: (A). tristearin/PEG/phosphatidylcholine/DMSO (1.0:0.2:1.0:1.0 w/w/w/w ratio); (B). 5 kDaPEG; (C). tristearin.

The thermogram of the lipid mixture reported in Fig. 1A shows the disappearance of the endotherm peak at 61.16 °C of PEG (Fig. 1B) and the thermal modification of tristerain (Fig. 1C). The endothermal peak corresponding to the α form at 50.78 °C disappeared as well as the exothermal peak at 52.7 °C while the presence of the β form is confirmed. However, when these measurements are performed under CO_2 pressure, the area of this peak is reduced. Therefore, the combined effect of composition and pressure are able to homogeneously disperse the mixture before atomisation.

The protein/lipid dispersions were processed by gas micro-atomisation. The supercritical equipment, described elsewhere (Salmaso et al., 2009), was equipped with a peristaltic pump to facilitate the spraying process. The lipid mass placed into the melting chamber of the supercritical apparatus was melted under the supercritical CO_2 conditions (150 bar, 40 °C) and sprayed through a micrometric nozzle into the precipitation vessel by a co-axial air assisted device. Under optimised operative conditions, 67.8 ± 4.7% product yield (final material/processed product, w/w%) was obtained.

The product obtained by supercritical gas micro-atomisation was a white fluffy powder easily dispersible in aqueous solution. Dispersibility studies carried out by light scattering analysis at 600 nm showed that small aggregates could be completely disaggregated by 20 min sonication. Light scattering showed that prolonged sonication (up to 60 min) did not change the particle size. The colloidal dispersions were fairly stable against prolonged centrifugation. After 60 min centrifugation at 3400 rpm, only 15% of the dispersed material precipitated.

Fig. 2A shows the particle size distribution obtained by dynamic light scattering analysis of tristearin/PEG/phosphatidylcholine (1.0:0.2:1.0 w/w/w ratio) formulation. The particle size was 197 ± 94 nm (0.22 ± 0.02 PDI) on the basis of volume-weighted Gaussian distribution analysis. The cumulative frequency curve shows that 10% volume-weighted particles had size lower than 75 nm, 50% particles had size lower than 120 nm and 90% of particles had size lower than 280 nm. The low chi-squared and fit error values (0.86 and 4.67, respectively) confirmed the unimodal size distribution as well as the good dispersion stability throughout the 30 min analysis. The transmission electron microscopy (TEM) confirmed the results obtained by light scattering. Fig. 2B shows that spherical shape particles were produced. The smooth spherical shape of the sonicated particles seems to confirm that sonication does not provoke significant particle fracturation.

3.2. Protein loading and in vitro release

The insulin and rh-GH loading were evaluated by protein extraction and HPLC analysis. The extraction and analytical protocol was previously validated by the addition of known amounts of insulin or rh-GH in DMSO to melted phosphatidylcholine/PEG/tristearin mixtures. Over 85% of the added insulin or rh-GH was extracted.

The insulin and rh-GH loading efficiency were 57 ± 8% and 48 ± 5% (processed protein/recovered protein, w/w), respectively, corresponding to a 2.9 ± 0.4% and 2.4 ± 0.25% protein loading (loaded protein/particle, w/w).

The in vitro release studies were performed by incubating the protein-loaded particles under physiological conditions (phosphate buffer at pH 7.2 and 37 °C). Sink conditions were maintained by replacing the releasing buffer at the sampling times. For comparison, the dialysis profiles of insulin and rh-GH in solution have been reported. The release profiles depicted in Figs. 3 and 4 show the insulin and rh-GH release profiles. The dialysis of the protein in solution was found to take place in few hours, while the dialysis of the proteins encapsulated into the particles was found to occur in about 4 days, as a consequence of the slow protein released from the lipid formulation.

The linear release against time$^{0.43}$ plot indicates that the proteins were released according to a diffusion mechanism (Ritger and Peppas, 1987). The equation calculated from the linear plots of insulin and rh-GH release was $y = 14.62x - 5.20$ ($R^2 = 0.978$) and $y = 17.37x - 22.28$ ($R^2 = 0.979$), respectively.

3.3. Pharmacokinetic and pharmacodynamic studies with insulin loaded lipid particles

Pharmacokinetic and pharmacodynamic studies were carried out by subcutaneous and oral administration of insulin solutions or insulin-loaded particles to diabetic mice. Diabetes was induced in Balb/C mice by streptozotocin treatment mice according to a

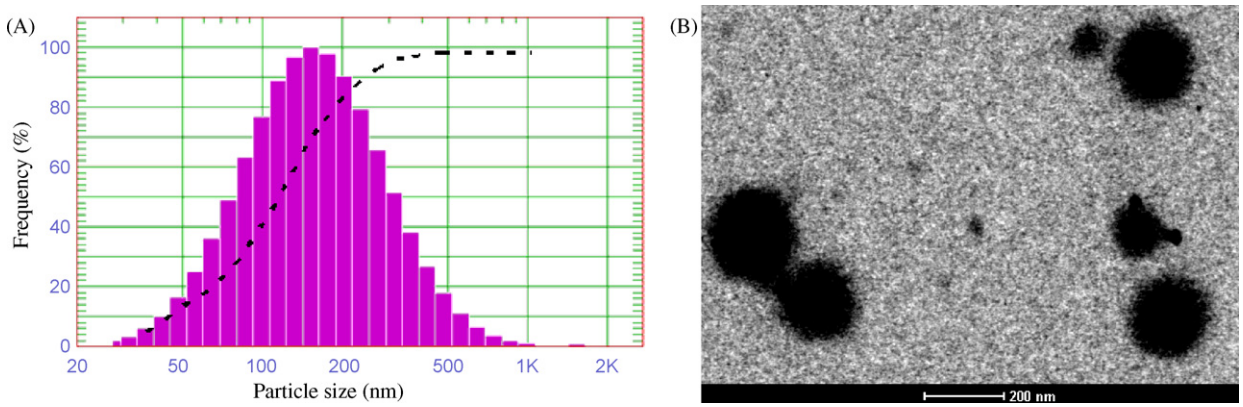

Fig. 2. (A). Volume-weighted size distribution profile of tristearin/PEG/phosphatidylcholine (1.0:0.2:1.0 w/w/w ratio) particles obtained by the gas micro-atomisation supercritical process: relative frequency distribution (histogram) and cumulative frequency (- - -); (B). TEM image of lipid particles.

protocol reported elsewhereThe citation (Marschütz et al., 2000). As controls, animal groups were subcutaneously and orally treated with buffer and protein free particles.

Fig. 5A and B shows the insulin and glycaemic time course profiles in plasma after subcutaneous administration of insulin in solutions or in lipid particles to diabetic mice. The administration of 2 μg insulin equivalent particles and 2 μg insulin in solution produced overlapping insulin and glucose behaviours in plasma. The maximum reduction in glucose concentration was observed 1 h after administration, and the pharmacological effect, which paralleled the insulin concentration profile, lasted for about 3 h. Table 1 reports the insulin bioavailability (AUC) and the pharmacological bioavailability (AAC) calculated by analysis of the experimental data. The relative pharmacokinetic bioavailability (PKRBsc) and the relative pharmacological bioavailability (PDRBsc) obtained with 2 μg insulin equivalent loaded particles were 96% and 118%, respectively.

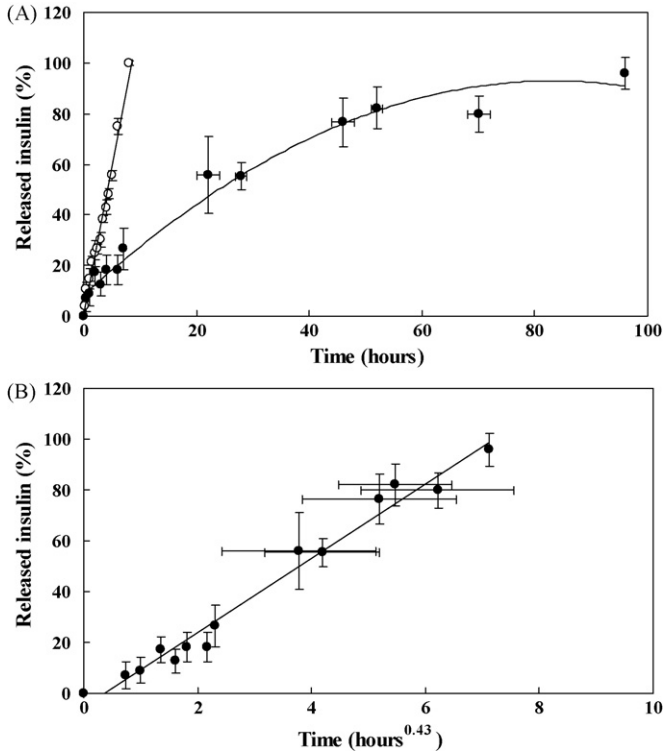

Fig. 3. In vitro dialysis profiles of insulin in solution (○) or in lipid particles (●) incubated at 37 °C. The experiments were replicated five times.

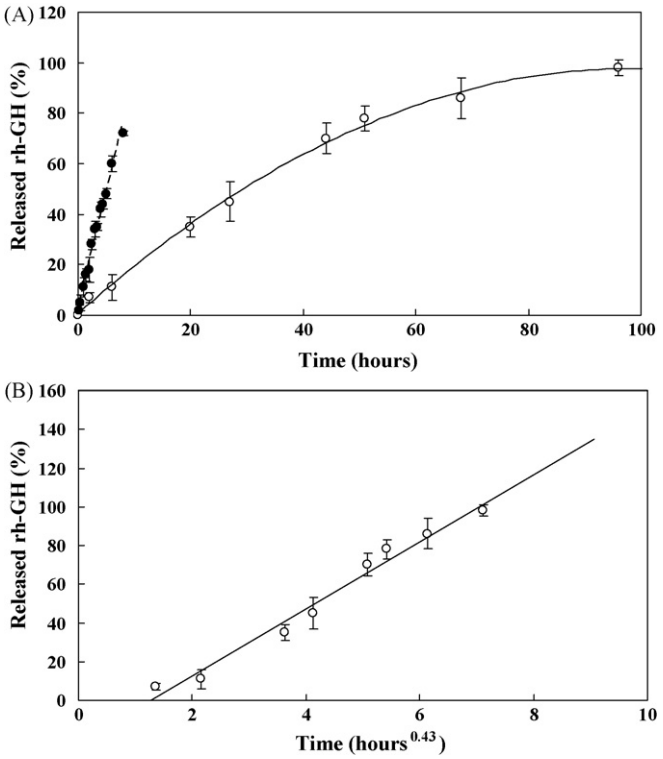

Fig. 4. In vitro dialysis profiles of rh-GH in solution (●) or in lipid particles (○) incubated at 37 °C. The experiments were replicated five times.

Table 1
Pharmacokinetic and pharmacodynamic parameters calculated by subcutaneous and oral administration of insulin in solution and insulin formulated in lipid particles: AUC (area under the curve) values were calculated from the insulin pharmacokinetic profiles described in Figs. 4 and 5Figs. 4A and 5A; AAC (area above the curve) values were calculated from the insulin pharmacodynamic profiles described in Figs. 4B and 5B.

	AUC (Insulin μg ml^{-1} h)	AAC [(Glucose ml^{-1})% h]
Subcutaneous administration		
2 μg insulin in solution	9.3	132
2 μg insulin equivalent insulin loaded particles	9.0	157
Oral administration		
20 μg insulin equivalent insulin loaded particles	10.2	102
50 μg insulin equivalent insulin loaded particles	15.2	221

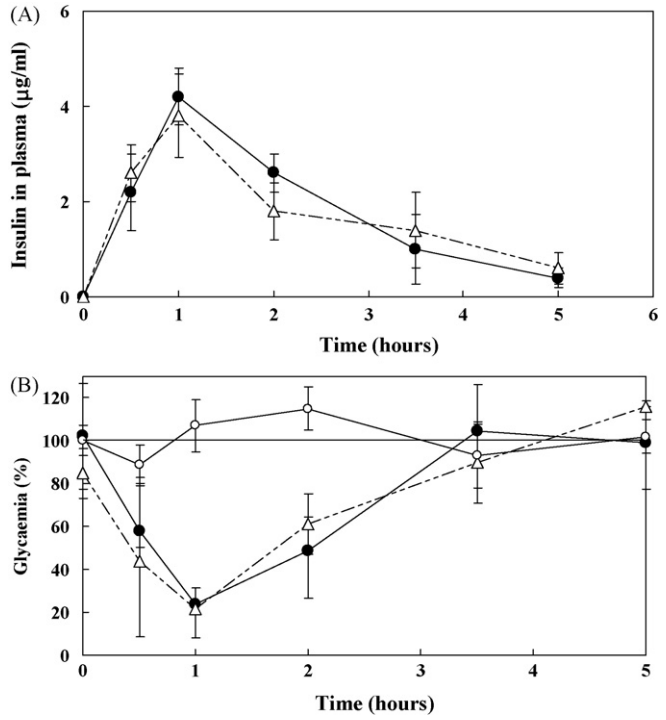

Fig. 5. Pharmacokinetic (A) and pharmacodynamic (B) profiles by subcutaneous administration of 2 μg insulin in solution (●), 2 μg insulin in lipid particles (△) and protein free particles (○) to diabetic mice.

Fig. 6A and B shows the insulin and glucose concentrations in plasma after oral administration of 20 and 50 μg insulin equivalents in lipid particles or equal protein amounts in solution. The administration of 20 and 50 μg insulin in solution did not elicit detectable insulin concentrations in plasma. Accordingly, no hypoglycaemic effect was observed. On the contrary, the administration of 20 and 50 μg insulin equivalent particles produced detectable protein concentrations in plasma and about 50% and 70% reduction in the glucose levels, respectively. The time course profiles described in Fig. 6B show that the lipid particles display a 2 h onset time, and the maximal hypoglycaemic effect was observed 3 h after administration. The pharmacological activity lasted for 3 and 8 h in the case of 20 and 50 μg insulin loaded particles, respectively. The PKRBos of 20 and 50 μg insulin equivalent particles were 11% and 6.5%, respectively, while the PDRBos were 7.7% and 6.7%, respectively.

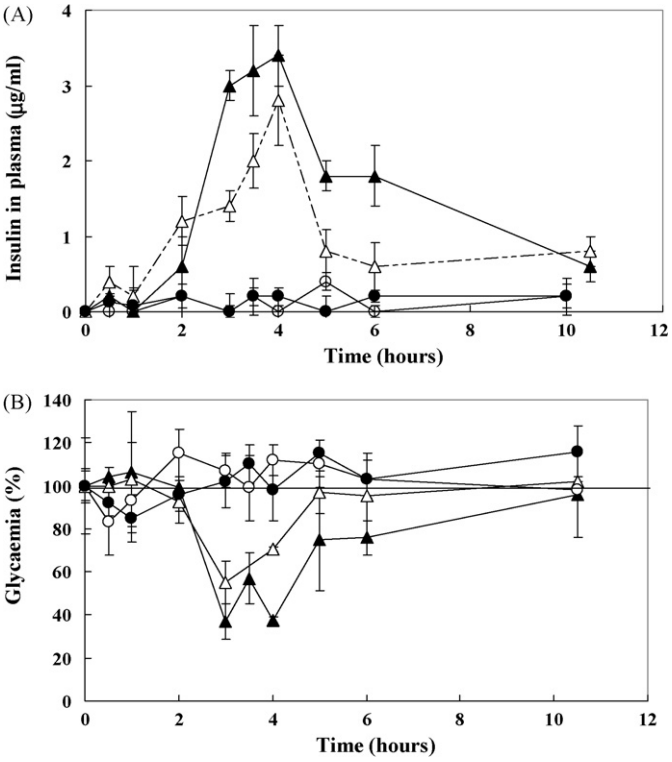

Fig. 6. Pharmacokinetic (A) and pharmacodynamic (B) profiles by oral administration of 20 μg insulin in solution (○), 50 μg insulin in solution (●), 20 μg insulin in lipid particles (△) and 50 μg insulin in lipid particles (▲) to diabetic mice.

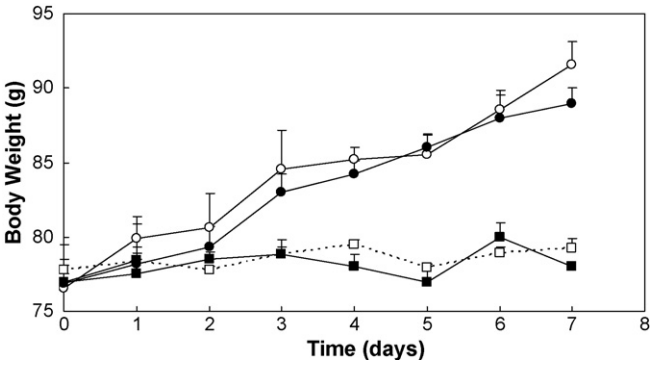

Fig. 7. Pharmacodynamic profiles by subcutaneous daily administration of 40 μg rh-GH in solution (○), 40 μg rh-GH in lipid particles (●), 0.1 mg protein free particles (■) and buffer (□).

3.4. Pharmacodynamic studies of rh-GH loaded lipid particles

The pharmacodynamic effect of rh-GH loaded particles was evaluated by subcutaneous and oral administration of the formulation to hypophysectomised female rats (Bangham, 1989; Wilkinson et al., 2007).

Fig. 7 shows that daily subcutaneous administration of 40 μg of rh-GH in lipid particles induced similar body weight increases to the hormone in solution. At the end point, the body weight of animals treated with rh-GH in solution and in lipid particles increased 19.7% and 15.7%, respectively. Therefore, the PDRBsc of the lipid formulation was 80%. On the contrary, the protein free particles did not have any pharmacological effect.

The body weight increase profiles obtained after daily oral administration of 400 μg of rh-GH in solution or in lipid particles depicted in Fig. 8 indicate that neither rh-GH in solution nor protein free lipid particles elicited a pharmacological effect, while the protein-loaded lipid particles induced a weight increase of 6.7%

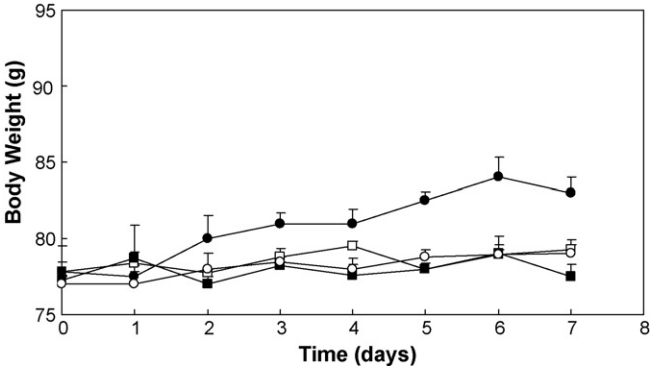

Fig. 8. Pharmacodynamic profiles by oral daily administration of 400 μg rh-GH in solution (○), 400 μg rh-GH in lipid particles (●), 1 mg protein free particles (■) and buffer (□).

after seven days of treatment. The PDRBos for the subcutaneous administration of rh-GH in solution was 3.4%.

4. Discussion

Tristearin, phosphatidylcholine, and PEG were selected for preparation of lipid particles because these pharmaceutical excipients, approved by the main regulatory agencies either for oral or parenteral formulations, possess the appropriate physicochemical features for supercritical processing and can yield delivery systems with suitable biopharmaceutical properties (Chen, 2008; Hauss, 2007; Schulze and Winter, 2009; Spilimbergo et al., 2006). Preliminary DSC studies showed that the lipid mixtures did not present the typical endothermal peaks corresponding to PEG and the thermogram profile of tristerain was profoundly changed with disappearance of the endothermal peak corresponding to the α form and dramatic decrease of the enthalpy of the β form (Oh et al., 2006). Therefore, the combination of different materials, the presence of DMSO and the pre-melting treatment produce mixtures with melting points of about 40–45 °C. The melting temperature further decreased by about 10–15 °C under supercritical CO_2, allowing it both to operate under mild conditions suitable for labile drugs and to avoid troubleshooting during supercritical processing.

The optimization of the equipment and operative conditions, namely melting temperature, CO_2 pressure, melting time, spraying rate, and tangential air flow pressure, yielded submicron droplets that rapidly solidified in the precipitation chamber. In contrast with previous studies, which showed that the gas micro-atomisation process produces particles with bimodally distributed size (Salmaso et al., 2009), the insulin and rh-GH formulations prepared in the present investigation showed unimodal distribution profiles. The bimodal size distribution was ascribed to partial agglomeration of sprayed lipid droplets in the precipitation chamber, which may be favoured by inhomogeneous melting and viscosity of the processed mixtures. Additionally, a discontinuous spraying due to a pressure gradient could also produce inhomogeneous particles. In order to overcome these problems the temperature in the melting chamber was slightly decreased, thus increasing the CO_2 dissolution in lipids (Sampaio de Sousa et al., 2006). Also, the supercritical apparatus was equipped with a peristaltic pump, which was activated to assist the mixture spraying into the precipitation chamber. As a consequence, a more homogeneous melting spraying was achieved and the multi-modal size precipitation was avoided.

Despite their lipid nature, the lipid particles displayed good dispersibility and stability in buffer, two important requisites for their pharmaceutical application. These properties were achieved by using small PEG amount in the formulation which could avoid burst protein release. In fact, previous studies showed that PEG-free tristearin/phosphatidylcholine particles undergo aggregation, while dispersible and fairly stable colloidal formulations were obtained by including high PEG amounts in the lipid formulations (Salmaso et al., 2009). Nevertheless, high PEG concentrations were found to produce burst protein release, which could compromise the therapeutic performance of the drug delivery system.

The general applicability of the gas micro-atomisation process for fabrication of protein delivery systems was investigating using insulin and rh-GH, two therapeutic proteins that possess different physicochemical properties, namely molecular weight, conformation, stability, hydrophobic/hydrophilic properties etc. Due to its high stability, insulin has been the molecule of choice in a number of investigations aimed at developing controlled protein delivery systems. Also, insulin is not inactivated by supercritical CO_2 processing, and micro- and nano-particulate formulations have been developed either for oral, pulmonary or parental delivery (Amidi et al., 2008; Khafagy et al., 2007; Jeandidier and Boivin, 1999). On the contrary, rh-GH is a fragile macromolecule, which easily undergoes chemical and physical inactivation during manipulation and storage. Furthermore, its large size and susceptibility to enzyme degradation represent major limits to transmucosal absorption (Cleland et al., 1997; Yoshiura et al., 2008; Lee, 2002; Leone-Bay et al., 1996). Nevertheless, there is unmet need either for long term or non-invasive rh-GH delivery, which may provide for a more convenient use.

The insulin and rh-GH content in the particles, determined either by extraction or at the end point of the release studies, was lower than what was theoretically expected. Actually, the incomplete protein recovery represents one of the main problems in the preparation of lipid or polymer micro- and nano-particles. Low drug loading efficiency may be due to a variety of reasons, namely (1) protein denaturation, aggregation or degradation during the manufacturing process, (2) interaction with equipment surfaces or formulation materials, (3) wasting etc (Wang, 2005). In the case of the gas micro-atomisation process the incomplete protein recovery may be ascribed to the partial precipitation in the melting chamber or to interactions with the lipid components of the formulation. Furthermore, although validation studies demonstrated that over 85% of the protein added to lipid mixtures could be recovered by solvent extraction, the supercritical process could promote strong protein/lipid interactions, which may prevent the protein recovery.

Protein/lipid interactions have been observed to take place during the in vitro release studies. Similarly to what has been reported by other authors, when the release study was performed by sampling instead of entire receiving volume withdrawal and replacement, the released protein concentration initially increased and then decreased, disappearing over time (García-Fuentes et al., 2003). The incubation of bare particles with insulin and rh-GH solutions even exhibited a decrease in protein concentration over time, confirming that insulin and rh-GH can interact with the excipients released in the medium. Possibly, the released proteins are taken up by phospholipid vesicles formed during the incubation in buffer, which are retained in the donor dialysis compartment, preventing the protein dialysis to the receiving compartment. However, when drug release was performed by complete withdrawal of the dialysis buffer, protein release was found to take place according to a typical diffusive mechanism for few days. Importantly, no burst effect was observed and the release rate was similar to that obtained with PEG-free formulations.

In vivo studies have shown that the gas micro-atomisation process does not impair the pharmacological activity of the loaded proteins, which can be easily inactivated by high temperature, shear stress and high pressure (Wang, 2005). Indeed, the subcutaneous administration of protein-loaded lipid particles yielded nearly 100% insulin relative pharmacokinetic and pharmacodynamic bioavailability, and 80% rh-GH pharmacodynamic bioavailability.

The similar pharmacokinetic and pharmacodynamic behaviours obtained by subcutaneous injection of insulin solution or lipid particles demonstrate that the protein is rapidly released from the formulation, even though complete in vitro protein release was achieved in few days. Similarly, the subcutaneous administration of rh-GH in solution and in lipid particles produced similar body weight increase profiles, confirming that, in vivo, the lipid formulations are rapidly disrupted providing for rapid protein release.

The lipid particle formulation was found to enhance the oral bioavailability of both insulin and rh-GH, producing effective therapeutic profiles. The 7% pharmacological bioavailability of insulin-loaded particles as compared to the subcutaneously administered insulin solution is in good agreement with the data reported in the literature showing that stearic acid/lecithin/poloxamer nanoparticles can yield relative pharmacological bioavailability ranging between 5 and 7%, with a maximal pharmacological activity was observed after 1 h from administration (Zhang et al., 2006). The delayed onset observed with the submicron particles obtained by

gas micro-atomisation compared to the smaller particles described in the literature is attributable to their different compositions and size. In fact, nanoparticles ranging between 30 and 100 nm in size have been found to display maximal interaction with the gut mucosa, facilitating absorption. The lipid formulation was also found to improve absorption of orally administered rh-GH, despite the poor physicochemical and absorption properties of this hormone. The relative pharmacological bioavailability was in good agreement with the data obtained by oral administration of rh-GH formulated in solid-in-oil suspension (Yoshiura et al., 2008). Accordingly, these results show that the lipid formulation is fairly stable in the gastrointestinal tract, thus protecting the protein from degradation by digestive protease in the stomach.

5. Conclusions

Supercritical techniques represent an emerging versatile opportunity for customised fabrication of formulations with tailored physicochemical and biopharmaceutical features. Nevertheless, many operative obstacles must be overcome to set up reliable and scalable processes to yield products with the required pharmaceutical properties.

The implementation of the supercritical PGSS process by introducing a peristaltic pump and co-axial air-flow assisted device allowed for setting up a new flexible gas micro-atomisation process that produces submicron lipid particles under mild conditions. Furthermore, the design of operative conditions and formulation composition can be adapted to process fragile molecules, namely proteins, to produce formulations with enhanced biopharmaceutical properties that may be exploited for non-invasive delivery of poorly absorbable drugs.

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