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Solid lipid micro-particles carrying insulin formed by solvent-in-water emulsion–diffusion technique

M. Trotta*, R. Cavalli, M.E. Carlotti, L. Battaglia, F. Debernardi

Dipartimento di Scienza e Tecnologia del Farmaco, via P. Giuria 9, 10125 Turin, Italy

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Abstract

The study aimed to produce solid lipid insulin-loaded micro-particles by the solvent-in-water emulsion–diffusion technique, using isobutyric acid as solvent phase, glyceryl monostearate or cetyl palmitate as lipid, soya lecithin and taurodeoxycholate as emulsifiers. Isobutyric acid, a partially water-miscible solvent with low toxicity, was used due to its high insulin-solubilization capacity. Solid lipid micro-particles of spherical shape were prepared by simple dilution of the emulsion with water. To increase the lipid load the process was conducted at 50 °C, and in order to reach sub-micron size, a high-shear homogeniser was used. Insulin encapsulation efficiency was about 80%. Analysis of microsphere content after processing showed that insulin did not undergo any chemical modification within the micro-particles. The in vitro release of insulin from the micro-particles was very low, and an initial burst effect of 20% of the dose was observed. After treatment of the solid lipid micro-particles with pepsin solution, an insulin loss of about 24% of the total englobed insulin was observed. The solid lipid micro-particles appear to have interesting possibilities as delivery systems for oral administration of insulin.

Keywords: Lipid micro-particles; Peptides; Insulin; Oral administration

1. Introduction

Daily subcutaneous injection of insulin is troublesome for patients with insulin-dependent diabetes. Among the many routes investigated for insulin administration, the oral route seems to be the most convenient for ease of long-term administration, and because this peptide undergoes a hepatic first-pass effect by which it contributes to the inhibition of the hepatic glucose output (Alleman et al., 1998). However, after oral administration of free insulin, less than 0.5% of the dose is absorbed and the peptide is degraded by luminal proteolytic enzymes.

Incorporating or encapsulating peptides in polymeric or non-polymeric particles should have the effect of protecting the drug against degradation by

* Corresponding author. Tel.: +39 011 6707 667; fax: +39 011 6707 687.
E-mail address: michele.trotta@unito.it (M. Trotta).

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the proteolytic enzymes present in the gastrointestinal tract.

Several approaches have been investigated to prepare drug-loaded micro-particles. Among them, the use of polyalkyl cyanoacrylate (Damgè et al., 1988; Michel et al., 1991) has been extensively reported. Particles are generally prepared by the interfacial polymerisation technique (Al-Khouri et al., 1986), but a lack of protection against proteolytic enzymes has been found when they are suspended in water. These observations indicate that, with the emulsion polymerisation technique, hydrophilic peptides tend to diffuse out to the surface of the particles formed, which impedes their protection.

Morishita et al. (1992, 1993) obtained better results by coating microspheres of methacrylic acid copolymer with Eudragit L100 to avoid dissolution of the drug, which would have been adsorbed rather than incorporated. The highest efficiencies against trypsinic and chymotrypsinic degradation were achieved with insulin-loaded particles containing trypsin inhibitor and chymostatin, respectively.

The use of alternative materials, such as lipids, as matrix materials for sustained-release formulations for peptides and proteins has rarely been reported (Domp, 1993; Amselm et al., 1992; Almeida et al., 1997). A clear advantage of SLN is the fact that the lipid matrix is made of physiological lipids which decreases the danger of acute and chronic toxicity.

Different methods for the preparation of insulin-loaded lipid micro-particles have been proposed. With the classical solvent evaporation method (Reithmeier et al., 2001), the drug is dispersed in an organic solution of the matrix material, followed by emulsification in an external aqueous phase to form a solid oil–water emulsion. Choosing water-immiscible solvents (e.g. methylene chloride) resulted in high encapsulation efficiencies, if the more hydrophilic ethyl acetate and ethyl methyl ketone had been used, water could have diffused from the external aqueous phase into droplets, causing dissolution of the insulin crystals and their subsequent diffusion into the external aqueous phase, thus reducing encapsulation efficiency. Regardless of the organic solvent used, microscopic inspection revealed insulin crystals located on the surface of particles. This phenomenon could be explained by expulsion of the incorporated insulin crystals from the lipid crystal lattice during the solidification of the micro-particles (Westesen, 1996).

When insulin was incorporated as a solution to form a W–O–W emulsion (Reithmeier et al., 2001), the encapsulation efficiency was also closely dependent on the organic solvent, ranging from 50% with methylene chloride to 2.6% with ethyl acetate, and the encapsulation efficiency was much lower compared to the solid oil–water technique, suggesting that dissolved insulin was lost to the outer aqueous phase to a higher degree as compared to a peptide in a solid state.

With the melt dispersion technique (Mehnert, 2001), a lipid melt is used instead of a solution of the lipid matrix materials in an organic solvent. Homogenisation is carried out at a temperature above the melting point of the lipid and can, therefore, be regarded as the homogenisation of an emulsion. Solid particles are expected to be formed by the subsequent cooling of the emulsion to room temperature or below. A large number of drugs, including very hydrophilic molecules, have been postulated to be incorporated in lipid matrixes, but few data exist on the localisation site and the physical state of the drug molecule; in most cases, burst release is also observed.

Whatever the preparation method, due to their hydrophobic nature lipid matrixes may be more appropriate to incorporate lipophilic drugs, which can easily be dissolved in melted lipids. On the contrary, hydrophilic proteins are expected to be poorly micro-encapsulated in lipid particles, tending to partition in the water phase during the process.

In previous work (Trotta et al., 2003), solid lipid nanoparticles were prepared by the emulsion–diffusion technique using butyl lactate or benzyl alcohol as oil phase. The process is based on the water miscibility of these solvents. Upon transferring a transient oil-in-water emulsion into water, lipophilic material dissolved in the organic solvent solidifies instantly due to diffusion of the organic solvent from the droplets to the continuous phase. Using optimised formulations, glyceryl monostearate nanospheres below 200 nm were obtained.

The present study aimed to encapsulate insulin in lipid micro-particles by the emulsion–diffusion technique using isobutyric acid as partially water-miscible solvent, and to evaluate its stability and in vitro release behaviour.

2. Experimental

2.1. Materials

Bovine insulin (M_w 5807), isobutyric acid, taurodeoxycholic acid (TDC), trifluoroacetic acid (TFA), lysozyme and pepsin were from Sigma Chemical Co. (Milano, Italy). Cetylpalmitate (CP) and glyceryl monostrearate (GMS) were from Goldschidt (Essen, Germany). Soya lecithin (Epikuron 200) was from Lucas Meyer (Hamburg, Germany). A-21 desamido insulin was prepared by storing the bovine insulin in 0.01 M HCl at 50 °C for 48 h (Brange, 1987). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MO). All other chemicals were of analytical grade and used without further purification.

2.2. Methods

2.2.1. GMS and CP apparent solubility

A series of samples were prepared by adding increasing amount of GMS or CP to 1 ml of isobutyric acid or 1 ml of water-saturated isobutyric acid. The samples were sealed and stirred at 25 ± 1 °C or 50 ± 1 °C for 12 h. The lipid solubility of the sample was considered to be at highest GMS or CP content, which appeared transparent on visual observation.

2.2.2. Insulin apparent solubility

The solubility of insulin at 25 ± 1 °C and 50 ± 1 °C in isobutyric acid or in water-saturated isobutyric acid was evaluated by determining the minimum amount of solvent required to solubilize a known amount of drug at the chosen temperature.

2.2.3. Insulin apparent partition coefficient

The apparent partition coefficient of insulin was determined by dissolving a weighed amount of insulin in isobutyric acid-saturated with water. Two millilitres of this solution was added to 2 ml of water saturated with isobutyric acid. This mixture was shaken for 5 min and then stored for 12 h at 50 ± 1 °C. The insulin concentration in water phase was determined by RP-HPLC and the apparent partition coefficient was calculated.

2.3. Insulin stability

Preliminary experiments on insulin stability in the isobutyric acid-saturated water, in the presence and in the absence of emulsifiers, were performed in order to assess the optimal experimental conditions. A 0.01% wt/V insulin solution in isobutyric acid-saturated water in the absence and in the presence of 0.5% lecithin or 0.5% TDC was prepared. The samples were placed in a thermostatic water bath (Tecno Galenica, Milano, Italy) and kept at 50 ± 1 °C. At scheduled times, an aliquot was withdrawn, filtered and analysed by RP-HPLC and SE-HPLC.

2.4. Solid lipid micro-particles preparation

Isobutyric acid and water were mutually saturated in order to ensure initial thermodynamic equilibrium of both liquids. Typically, weighted amount of lipid and insulin were dissolved in water-saturated isobutyric acid and this organic solution was emulsified at 50 ± 1 °C with isobutyric acid-saturated water containing lecithin and TDC. The composition (% w/w) of the O–W emulsion was: 10.0% water-saturated isobutyric acid, 87.9% isobutyric acid-saturated water, 1.0% lipid, 0.5% lecithin, 0.5% TDC, 0.1% insulin. The emulsion was emulsified using an Ultra Turrax (IKA, Staufen, Germany) at 15000 rpm for 1 min. The lipid particles were precipitated by quickly adding water (10.0 ml) to the initial emulsion (5.0 ml) to extract the solvent into the continuous phase. Finally, the particles were isolated by centrifugation at 22000 × g for 10 min (Beckman Coulter, California, USA) and washed twice with 0.01 M HCl and three times with water.

2.4.1. Particle characterization

The particle size and Z-potential of the lipid dispersions were determined by the laser light scattering technique (Brookheven, New York, USA). Measurements were obtained at an angle of 90°. The dispersions were diluted with water for size determination or with 0.005 M KNO_3, in order to achieve the prescribed conductibility for Z-potential determination.

The morphology of the particles was determined by TEM (CM 10 Philips, The Netherlands). An aliquot of the washed lipid suspension (2 ml) was incubated with a solution of osmium tetroxide at 2% (15 μl) for 75 min and then a uniform deposit was produced, spraying the

suspension on to the microscope grid by means of an aerosol-sampling device.

2.4.2. Differential scanning calorimetry (DSC)

DSC was performed with a Perkin-Elmer differential calorimeter (Norwalk, CT, USA). GMS bulk material (1–1.2 mg) and a suspension of GMS microparticles obtained by ultracentrifugation (10–12 mg) was placed in conventional aluminium pans and a scan speed of 5 °C/min was employed.

2.4.3. Insulin encapsulation efficiency and insulin stability in micro-particles

The amount of insulin loaded into the microparticles was determined after dissolution of a weighed amount of dry micro-particles (5 mg) in 1.0 ml of methanol and insulin was extracted in 2 ml of hydrochloric acid of concentration 0.01 M. The suspension was centrifuged at 22000 × g for 10 min and the solution was analysed for insulin content by reverse-phase (RP-HPLC) and size exclusion (SE-HPLC) chromatography in order to detect the different degradation products of insulin.

No insulin transformation products, such as A-21 desamido insulin and covalent dimer insulin, were found by the above procedure using native insulin. The results are expressed as encapsulation efficiency, i.e. the ratio between the actual and theoretical insulin-loading percent.

To determine the protective ability of the microparticles for insulin under conditions simulating the human gastric environment, free insulin and microparticles-incorporated insulin (5 mg) were placed in a vessel containing 5 ml of simulated gastric fluid, prepared by dissolving 2.0 g of sodium chloride and 3.2 g of pepsin in 7.0 ml of 1 M HCl, and water to 1000 ml. During the experiment, temperature and stirring speed were maintained at 37 ± 1 °C and 100 rpm. After 1 h, the micro-particles were collected by centrifugation, transferred to 1 ml of methanol, and insulin was extracted with 2 ml of hydrochloric acid 0.01 M and the suspension was centrifuged. The solution and the sample of free insulin were analysed for insulin content by RP-HPLC and SE-HPLC.

2.4.4. In vitro release studies

Release experiments were performed by suspending a weighed amount of dried micro-particles (10 mg) in 20 ml, pH 2.2 phosphate-citrate buffer solution containing 0.02% (w/w) polysorbate 80 as dispersing agent. During the experiment (24 h), the temperature and stirring speed were maintained at 37 ± 1 °C and 100 rpm. At scheduled times, 0.5 ml of the sample was removed and centrifuged for 5 min at 22,000 × *g*. Each sample was analysed by RP-HPLC and the amount of insulin was calculated by means of a calibration curve.

2.5. Insulin analysis

RP-HPLC and SE-HPLC were used to determine the amount of insulin in the different samples. The chromatograph was equipped with a Shimadzu HPLC system (Shimadzu, Milano, Italy), set at 220 nm. For the RP-HPLC mode a Kromasil C18 column (TeKnokroma, Barcelona, Spain) was employed. The mobile phase was a mixture of 0.1% TFA–acetonitrile (75:25, v/v) and was delivered at 1 ml/min. The retention time was 11 min and 12.5 min for insulin and A-21 desamido insulin, respectively. For the SE-HPLC, a TSK gel G2000SWXL (Tosoh Bioscience, Stuttgart, Germany) was employed. The mobile phase was an aqueous solution of potassium phosphate monobasic 50 mM, adjusted to pH 6.8 with potassium hydroxide. Flow rate was 1 ml/min and retention time was 10.2 min; lysozyme (M_W 14.3 KDa), retention time 8.5 min, was used as reference to detect the presence of insulin dimers.

2.6. Data report

Each set of experiments described so far was repeated at least three times. Results were reported as mean ± standard deviation (S.D.) in text, and statistical analysis was performed using a two-tailed Student's *t*-test.

3. Results and discussion

The solvent-in-water emulsion–diffusion technique was chosen to encapsulate insulin; insulin was chosen as an unstable, water-soluble model protein for which the physical stability issues have been studied in detail (Brange, 1994). The primary requirement to obtain solid lipid micro-particles with high insulin entrapment efficiency is to use a solvent with high solubilization ca-

Table 1
Insulin and lipid apparent solubility at different temperatures

	Isobutyric acid		Water-saturated isobutyric acid	
	50 °C	25 °C	25 °C	50 °C
Insulin	Insoluble	Insoluble	54 mg/ml	178 mg/ml
GMS	< 50 mg/ml	2200 mg/ml	< 50 mg/ml	420 mg/ml
CP	< 50 mg/ml	5200 mg/ml	< 50 mg/ml	380 mg/ml

pacity, both for insulin and for the lipid. Moreover, this solvent must easily be removed (extraction or evaporation) from the emulsion. Toxicologic problems arising from solvent residues should also be considered.

After exploratory experiments, isobutyric acid as solvent, and cetylpalmitate and glyceryl monostearate as lipid, were chosen to investigate the possibility of preparing insulin-loaded lipid micro-particles.

Table 1 reports the solubility of insulin and of the lipids in isobutyric acid and in water-saturated isobutyric acid. Drug and lipid solubilities were only found to be acceptable for preparing the primary emulsion at about 50 °C and, in particular, a very high solubility was noted for insulin in the water-saturated solvent.

To confirm the possibility of using this solvent to prepare the transient insulin-loaded emulsion, the apparent partition coefficient between isobutyric acid and water was determined. The value found (73 ± 6) indicates that most of the peptide was in the internal phase of the emulsion.

Moreover, the solubility in water of isobutyric acid (w/w) was about 16%: this made this solvent of interest in the preparation of micro-particles by the solvent-diffusion technique.

Insulin in solution is susceptible to chemical and physical degradation; deamidation at A-21 has been shown to be the main degradation product of insulin at low pH values; insulin can also form covalent aqueous soluble dimers.

The influence of isobutyric acid on the stability of insulin was next verified. Note that the term 'stability', as used here with regard to insulin, is defined as the ability of this peptide not to undergo transformation into A-21 desamido-insulin, assessed by RP-HPLC, and water-soluble aggregates assessed by SE-HPLC. HPLC analyses of samples from isobutyric acid-saturated water solutions of insulin were performed over time, and the results are reported in Fig. 1. As can be seen, no chemical degradation of insulin was found to occur. This

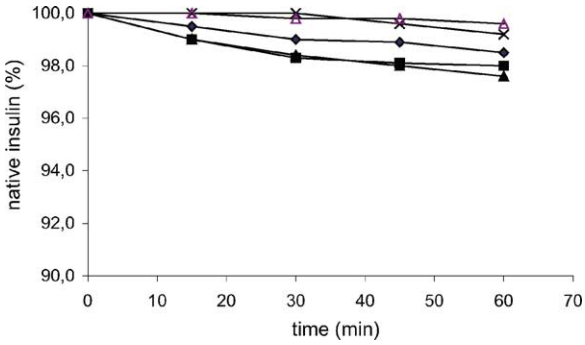

Fig. 1. Insulin stability in isobutyric acid-saturated water (△) by RP-HPLC in the presence of lecithin (×) by RP-HPLC; (♦) by SE-HPLC or TDC (■) by RP-HPLC; (▲) by SE-HPLC.

implies that, for a short period of time, the operative conditions did not significantly affect the stability of the peptide.

In the preparation of micro-particles from emulsions by the solvent-diffusion technique, the formulation of the emulsified systems to be used is of fundamental importance (Sah, 2000). Many attempts were therefore necessary to obtain O–W emulsions that were relatively stable at the working temperature. The emulsion composition and process parameters used were thus the result of this formulative study aimed at optimising the production of lipid micro-particles.

Fig. 2 shows a typical photomicrograph of the emulsion containing GMS. As can be seen, the majority of droplets were between 1 and 3 μm.

The subsequent dilution under a standard stirring rate of 5.0 ml of emulsion with an additional 10.0 ml of distilled water converted the micro-droplets into solid particles. The amount of water used was calculated such that it was sufficient to extract all the isobutyric acid from the oil phase of the emulsion.

Supercooled melts are not unusual in solid lipid nanoparticles systems (Bunjes et al., 1998); the term describes a phenomenon wherein lipid crystallization may not occur although the sample is stored at a temperature below the melting point of the lipid. As the advantage for SLN drug-carrier systems is essentially based on the solid state of the particles, solidification of the particles after dilution of the emulsions must be verified.

The status of the lipid particles was investigated using differential scanning calorimetry (DSC). Fig. 3

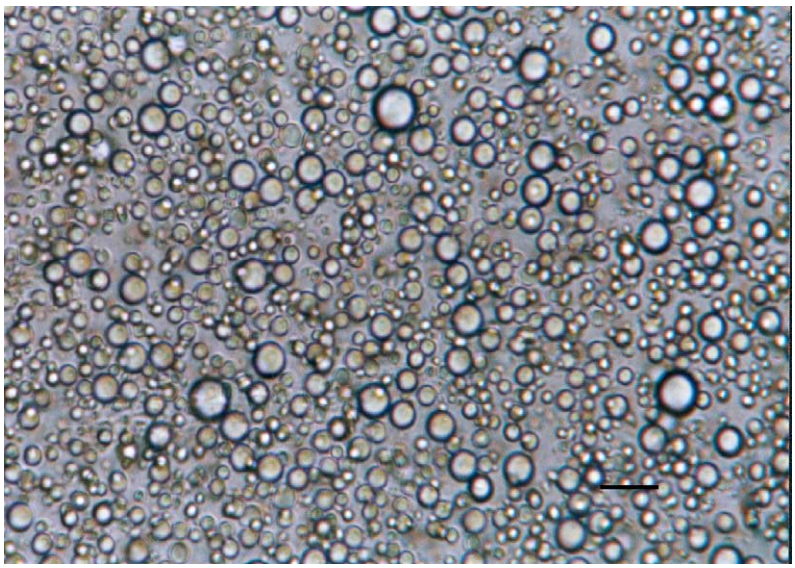

Fig. 2. Photomicrograph at 50 ± 2 °C of the emulsion containing GMS (bar = 5 μm).

shows the DSC thermograms of GMS bulk material, and of GMS particle suspension. The peak location of GMS particles is slightly shifted towards lower temperatures compared to that of the bulk material, probably because of the presence of residual solvent and surfactants into the GMS suspension. From the DSC results for the systems considered, it may be assumed that crystallization of the GMS particles began at room temperature after dilution of the emulsions.

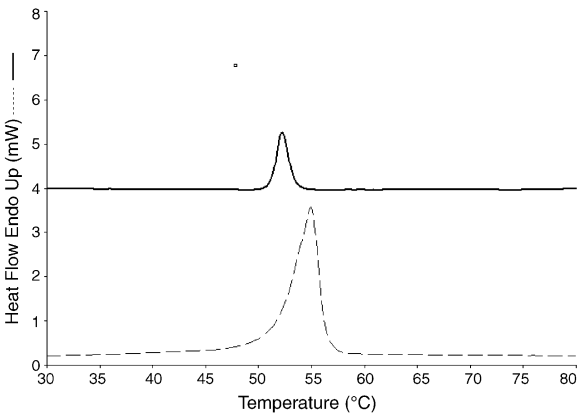

Fig. 3. Differential scanning calorimetry of GMS bulk material (– – –) and insulin-loaded GMS suspension (—).

Fig. 4 shows the transmission electron micrographs of the GMS particles. The TEM micrographs show that the solvent dilution process leads to the formation of spherical micro-particles with a smooth surface.

Table 2 reports the physicochemical properties, insulin-encapsulation efficiency and insulin protection towards pepsinogenic degradation of the loaded lipid micro-particles. As shown, these particles were found to be in the sub-2 μm size range, irrespective of lipid composition. Likewise, the type of lipid did not affect the encapsulation efficiency values achieved for insulin, which ranged from 78 to 84%. The micro-particles were negatively charged at their surface, as reflected in the *Z*-potential values, this charge not being significantly affected by the lipid composition of the micro-particles.

It is well known that insulin is easily destroyed and inactivated by digestive enzymes such as pepsin, trypsin and chymotrypsin, and blood glucose levels are not reduced following oral administration of free insulin alone. Thus, in order to enhance insulin's bioavailability, preparations, which can circumvent these enzymatic barriers, are required.

To assess the protective effect of lipid micro-particles against enzymatic degradation, the lipid preparations were incubated in the presence of pepsin.

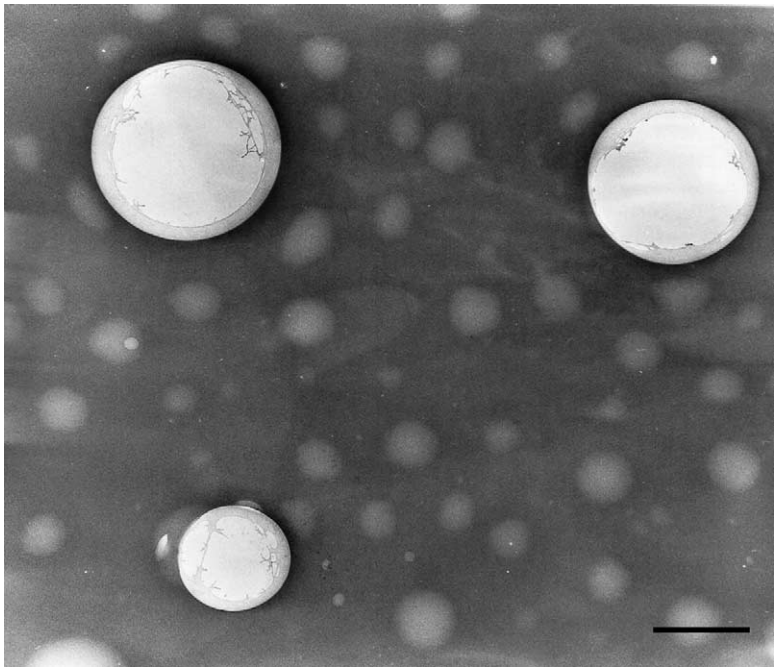

Fig. 4. Transmission electron microscopy (TEM) of insulin-loaded GMS particles (bar = 1 μm).

Insulin was found to be completely degraded during incubation in pepsin solution. On the other hand, with the lipid preparations, insulin loss was about 24% when they were exposed to pepsin solution. The protective efficiencies under the chosen experimental conditions were similar, for both GMS and CP particles.

The in vitro insulin-loaded particle-insulin release showed an initial bust of about 20% of the incorporated peptide and a continuous slow release thereafter (Fig. 5). A high percentage of the incorporated insulin was not released during the time period monitored. The initial burst value was quite similar to that observed in the stability studies in simulated gastric fluids, indicating that this percentage located on the surface of the particles was not removed by washing procedure.

These preliminary studies indicate that insulin can indeed be loaded within lipid micro-particles, however,

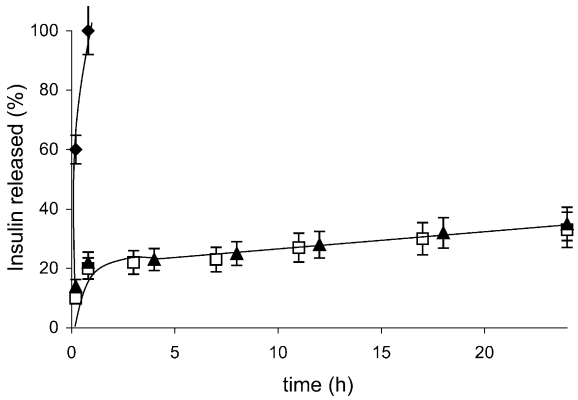

Fig. 5. In vitro release of insulin from GMS (□) and CP (▲) particles and insulin powder (♦).

Table 2
Physicochemical properties of insulin-loaded GMS and CP particles

Lipid	Mean particle size (nm)	Z-potenzial (mV)	Encapsulation efficiency (%)	Insulin loss (%) following incubation in pepsin solution
GMS	1335 ± 310	−12 ± 2	78 ± 5	24 ± 3
CP	1526 ± 388	−10 ± 2	84 ± 4	22 ± 4

more studies need to be performed in order to define the appropriate conditions for the in vitro release studies and to further optimize the release behaviour of lipid micro-particles.

4. Conclusions

The present study has shown that it is possible to produce insulin-loaded micro-particles with high encapsulation efficiency, with the emulsification-diffusion process, using isobutyric acid. A relatively high lipid load was obtained by increasing the process temperature. These micro-particles partially protected insulin from proteolytic degradation under the experimental conditions. The dosage forms reported here might be considered to be an interesting tool for the development of alternatives to parental administration.

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