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Preparation and characterization of a peptide containing w/o emulsion

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Abstract

Human insulin as a model peptide drug was incorporated into a water-in-oil emulsion prepared by high-pressure homogenization. The formulation of the emulsion was developed with regard to oral compatibility and enhancing properties of the excipients. A fine and stable dispersion of the aqueous phase was achieved by a valid complex of emulsifiers dissolved in a mixture of triglycerides. The resulting droplet size distribution was determined by microscopical means and photon correlation spectroscopy (PCS). The stability of insulin in the formulation was examined by ELISA and HPLC methods over a period of 3 months. Human insulin as a high molecular mass peptide (5800 Da) stayed immunologically active after preparation and the formulation showed high compatibility with the peptide in longer term stability by HPLC. The emulsion was able to protect insulin against gastric degradation in vitro without further encapsulation. Simultaneous incorporation of aprotinin as a suitable protease inhibitor was performed.

Keywords: Peptide; w/o emulsion; Insulin; Aprotinin; Oral administration; Stability

1. Introduction

In order to achieve improved bioavailability of peptide drugs after oral administration there are two main aspects to be considered in formulation development: enzymatic degradation in gastrointestinal fluids and low absorption of the drug at the mucosal site. High molecular weight and low lipophilicity of most peptide drugs are the two molecular properties that imply low penetration through biological membranes. Additionally, the digestive processes cause degradation of the active substance in the lumen, in the brush border and also in the cytosol of the enterocytes (Lee et al., 1991). There are several approaches in drug formulation to achieve both protection against degradation and enhanced absorption of peptide drugs at the intestinal wall, such as the addition of enhancers, mixed micellar solutions, liposomes (Choudhari et al., 1994), peptide-loaded nanocapsules (Damgé et al., 1990), lipid formulations, colon targeting (Saffran et al., 1986), mucoadhesive systems (Lehr, 1991) and chemical modification (Yamada et al., 1992).

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Lipoidal adjuvants like phosphatidylcholine and oleic acid have been discussed for modifying the drug transport of water-soluble macromolecules (Muranishi, 1985). In a mixture of surfactants in arachis oil, insulin was able to reduce blood glucose after oral administration (Touitou and Rubinstein, 1986). Water-in-oil formulations with incorporated insulin were used for absorption experiments resulting in a dose dependent reduction of blood glucose after intraduodenal application (Engel et al., 1968). Also, plasma insulin responses correlated to the amount of intrajejunally administered w/o/w emulsion (Shichiri et al., 1974). A w/o emulsion was found to improve the peroral bioavailability of the endekapeptide cyclosporin A as compared to a commercially available solution (Ritschel et al., 1990).

The aim of this study was to incorporate human insulin as a model peptide drug and aprotinin as a protease inhibitor into a water-in-oil emulsion that was to be developed with respect to low toxicity of the excipients and fine dispersion of the peptide solution in the oil phase. The activity and content of the peptide in the formulations were determined by chromatographic and immunological means. Microscopy, photon correlation spectroscopy and viscometry were used for the characterization of the physical properties. The protection of the incorporated peptide against gastric juice with proteolytic activity was evaluated in vitro.

2. Materials and methods

2.1. Chemicals

Semisynthetic human insulin (28 IU/mg) was kindly donated by Hoechst AG (Frankfurt a.M., Germany) and aprotinin (6950 KIU/mg) was a gift from Bayer AG (Leverkusen, Germany). Soybean phospholipid of high purity (80–85% phosphatidylcholine, Epikuron® 180) was supplied by Lucas Meyer GmbH (Hamburg, Germany) and Cremophor® w/o 7 (ethoxylated castor oil) was provided by BASF AG (Ludwigshafen, Germany). Porcine pepsin (0.7 FIP U/mg) was purchased from E. Merck (Darmstadt, Germany). Olive oil of pharmaceutical grade was purchased from H. Lamotte (Bremen, Germany) and C8–C12 medium chain triglycerides (Miglyol® 812) were supplied by Huels AG (Witten, Germany). All other chemicals were obtained from commercial sources and were of analytical reagent or chromatographical grade.

2.2. Equipment

A Micron Lab 40 high-pressure homogenizer (APV Gaulin GmbH, Lübeck, Germany) was used to prepare w/o emulsions. Transmission electron microscopy (TEM) was performed on an EM 300 (Philips, Kassel, Germany). Samples were frozen by a jet-freeze device with liquid propane (JFD 030, Baltec, Walluf, Germany) and freeze-fractured using a BAF 400 (Balzers, Wiesbaden, Germany). The photon correlation spectroscopy (PCS) unit consisted of a Malvern PCS RR 102 (Malvern Instruments, Great Malvern, UK) and a digital correlator (ALV 3000, ALV, Langen, Germany). A rotational viscometer (Rotovisco RV 20, Haake, Karlsruhe, Germany) was used to determine the absolute viscosity. Photometric measurements were performed in a diode array spectrophotometer (model 8452 A, Hewlett Packard GmbH, Bad Homburg, Germany). HPLC analyses were carried out using a high-pressure pump (model 300, Gynkotek GmbH, Germering, Germany), a variable-wavelength UV detector (Kontron Instruments GmbH, Eching, Germany), an ODS Hypersil 5 μm column (250z × 5 mm i.d., Shandon Southern Prod. Ltd, Cheshire, UK) and a precolumn (25 × 5 mm i.d., Macherey-Nagel, Düren, Germany). Enzyme-linked immunoassay (ELISA) was performed on an autoanalyser (ES 300, Boehringer Mannheim GmbH, Mannheim, Germany) with reagents from the same supplier.

2.3. Methods

2.3.1. Composition and preparation of the w/o emulsions

Taking the IEP at pH 5.4 to be the point of lowest solubility of human insulin (h-insulin) in an aqueous medium, a suitable buffer system for

Table 1
Solubility of h-insulin in two buffer systems of various pH values after 24 h of gentle shaking at room temperature

	pH (acetate buffer, $I = 0.05$)					pH (phosphate buffer, $I = 0.05$)				
	4.0	4.1	4.2	4.4	5.0	6.7	6.9	7.1	7.3	7.4
h-insulin solubility (mg/ml)	9.6	8.1	4.8	4.3	0.7	0.5	1.5	2.9	7.5	12.0

the aqueous phase is required to adjust the pH to a value above or below this point, in order to incorporate a sufficient amount of dissolved drug into the emulsion. On the other hand, the degradation of insulin which is found to increase significantly at pH values below 5 and above 8 must be taken into account (Brange and Langkjær, 1992). The solubility of the freeze-dried h-insulin powder was determined over the pH ranges of 4.0–5.0 in an acetate buffer system (ionic strength $I = 0.05$) and 6.7–7.4 in a phosphate buffer system (PBS) ($I = 0.05$) by photometric measurement at 276 nm after 24 h of gentle shaking at room temperature (Table 1). PBS of pH 7.4 was used in the preparation of w/o emulsions to combine high solubility and low degradation of h-insulin in the inner phase.

The composition of the w/o emulsion with a content of 30 IU/g of h-insulin is listed in Table 2. To prepare the aqueous phase insulin powder was dispersed in a part of PBS pH 7.4 ($I = 0.05$). The suspension was acidified to pH 3 with 1 N HCl until the peptide was completely dissolved. Subsequently, the pH was adjusted to 7.4 by the addition of an equivalent amount of 1 N NaOH solution and the remaining PBS was added to the solution. It was then filtered (0.2 μm) to reduce microbial contamination.

A suspension of phospholipid Epikuron® 180 in the mixture of triglycerides was stirred for 24 h at room temperature to obtain a clear solution of 10% (w/w) phospholipid as recommended for enhancing the emulsifying ability of lecithins (Von Kleinsorgen, 1980). Cremophor® w/o 7 (clear phase without sediment) was added to this lipid solution. The mixture was heated to 70° C in order to obtain a homogeneous oil phase and cooled to room temperature immediately to prevent phospholipid degradation. The aqueous phase was poured into the lipid mixture and a preliminary emulsion was formed by high-speed stirring (6000 rpm, 30 s). Finally, the w/o emulsion was homogenized five times at a pressure of 500 bar at room temperature in the Micron Lab 40. The formulations were warmed by homogenization to 36–38° C.

Since there was a reduction in solubility by the precipitation of dissolved h-insulin and aprotinin in the applied aqueous medium, simultaneous incorporation of aprotinin was achieved by the preparation of a separate aprotinin emulsion following the same procedure as described above. Instead of h-insulin the protease inhibitor was dissolved in the aqueous phase of pH 7.4 without acidification and neutralization. The homogenized aprotinin emulsion and the insulin emulsion were mixed by stirring to yield a w/o emulsion containing peptide drug and protease inhibitor (Table 3).

Table 2
Composition of a w/o emulsion containing 30 IU/g of h-insulin

Epikuron® 180	6.38 g
Olive oil	28.71 g
Miglyol® 812	28.71 g
Cremophor® w/o 7	13.20 g
h-insulin	118 mg
NaCl + H_2O (HCl + NaOH)	q.s.
PBS pH 7.4 ($I = 0.05$)	ad 110.0 g

2.3.2. Insulin extraction

An extraction fluid composed of 50 ml of PBS pH 7.4 ($I = 0.05$) and 50 ml of methanol was

Table 3
Composition of a w/o emulsion containing 30 IU/g of h-insulin and 2500 KIU/g of aprotinin

h-insulin emulsion (50 IU/g)	60.0 g
aprotinin emulsion (6250 KIU/g)	40.0 g

applied to extract h-insulin for analytical determinations. 150 mg of w/o emulsion and 1.5 ml of extraction fluid were mixed intensively (vortexing for 30 s) and were centrifuged subsequently (12 000 rpm, 10 min). An aliquot of the separated aqueous phase was used for HPLC analysis. The recovery of h-insulin was 97 ± 3.5% (n = 8).

2.3.3. Analytical methods

Analytical determination of h-insulin by high-pressure liquid chromatography (HPLC) was performed at room temperature by a modified version of a method described by Szepesi and Gazdag (1981). The conditions employed were: column, ODS-Hypersil 5 μm, 250 × 5 mm i.d.; detection, at 215 nm; eluent, methanol-acetonitrile-aqueous solution of monobasic sodium phosphate (0.01 M) containing 0.1 M sodium sulfate (volume ratio 135:28:90). The eluent was acidified with concentrated phosphoric acid to pH 2.5. The flow rate used was 1.8 ml/min.

Immunological activity of h-insulin was analysed by enzyme-linked immunoassay (ELISA). Samples were diluted for analysis in a solution of purified bovine serum albumin (3.5% w/v) in isotonic phosphate buffer pH 7.4.

2.3.4. Particle size determination and viscometry

The particle size of the dispersed aqueous phase was examined by light microscopy and transmission electron microscopy (TEM). For TEM the formulations were freeze-fractured (Folger and Müller-Goymann, 1994). To characterize the particle size distribution, photon correlation spectroscopy (PCS) was performed (632,8 nm laser, 90° measurement). The w/o emulsions were diluted in n-hexane (35% w/v) to reduce viscosity and samples were subsequently analysed at 25° C incubation temperature. The mean radius of the intensity distribution of each sample was calculated based on 10 measurements. The absolute vicosities and rheological properties of the w/o systems were determined on a rotational viscometer at 25° C.

2.3.5. Protection against gastric juice

To assess the protective effect against gastric degradation, 500 mg of insulin phosphate buffer solutions or w/o emulsions (30 IU/g) were incubated (37° C) and shaken with 1 ml of simulated gastric juice (pH 1.2) for 60 min in a water bath shaker. The gastric fluid was prepared by acidifying an isotonic solution of sodium chloride with hydrochloric acid. Enzymatic degradation of 0.5 and 5 FIP U/ml, respectively, was achieved by the addition of pepsin. The enzyme was dissolved in the gastric juice immediately before starting the experiment to prevent loss of activity. To terminate enzymatic action and to ensure insulin stability for analysis after incubation, the aqueous insulin solutions were adjusted to pH 4 with acetate buffer and methanol was added to reach a concentration of 50% (v/v) which prevented further enzymatic degradation. The w/o emulsions were separated from the gastric juice by centrifugation (3000 rpm, 2 min) and an aliquot of 150 mg was mixed with 1.5 ml of a mixture of a phosphate buffer system (pH 7.4, I = 0.05) and methanol for insulin extraction (see above) and for termination of digestive processes. All samples were analysed by HPLC.

Table 4
Photon correlation spectroscopy (PCS) of a placebo and an insulin (30 IU/g) w/o emulsion

Emulsion/homogenization	PCS radius (nm) (±SD)	Polydispersity value (±SD)
Placebo/1 × 500 bar	32.7 (±2.9)	0.51 (±0.04)
Placebo/3 × 500 bar	24.1 (±0.4)	0.31 (±0.07)
Placebo/5 × 500 bar	24.3 (±1.1)	0.30 (±0.06)
Insulin/1 × 500 bar	43.3 (±2.1)	0.58 (±0.04)
Insulin/3 × 500 bar	25.3 (±0.7)	0.36 (±0.05)
Insulin/5 × 500 bar	25.6 (±1.1)	0.29 (±0.08)

3. Results

3.1. Physical properties

In the process of homogenization the opaque emulsions cleared up significantly after three cycles of 500 bar, becoming an opalescent, isotropic system. The Tyndall effect indicated a colloidal dispersion of the peptide solution in the protective oil phase. In contrast to an unsterile preparation of the w/o formulation there was no visible phase separation or enhanced turbidity for at least 1 month of storage in the refrigerator (4–8° C) and at room temperature. Microbiological examination after 4 months (4–8° C) of a batch with phase separation yielded a colony of microorganisms (*Byssochlamys nivea*, *Ascomycetales*). Within 1 week after preparation the characterization of the entire particle size distribution using PCS resulted in mean diameters of the intensity distribution of about 50 nm (Table 4). Transmission electron microscopy (TEM) photographs of placebo and insulin emulsion demonstrated diameters of the aqueous droplets in the range of 30–400 nm (Fig. 1). No multiple layers of surfactants were detected at the visible interfaces. Considering the limit of about 800 nm, light microscopy was used to examine the largest particles in the formulations. A few droplets of up to 3 μm diameter were detected in samples of placebo and insulin w/o emulsion (30 IU/g) after five cycles of 500 bar homogenization.

Identical results were obtained for both placebo and insulin emulsions (Fig. 2) by means of rotational viscometry. Almost linear plots of shearing stress vs rate of shear, passing through the origin, indicated Newtonian bodies. Up and down consistency curves were identical. An absolute viscosity of 420 ± 20 mPa s was represented by the slope throughout the entire measurements. The viscosity of the oil phase (92 ± 2 mPa s) was increased 4-fold after incorporation of the aque-

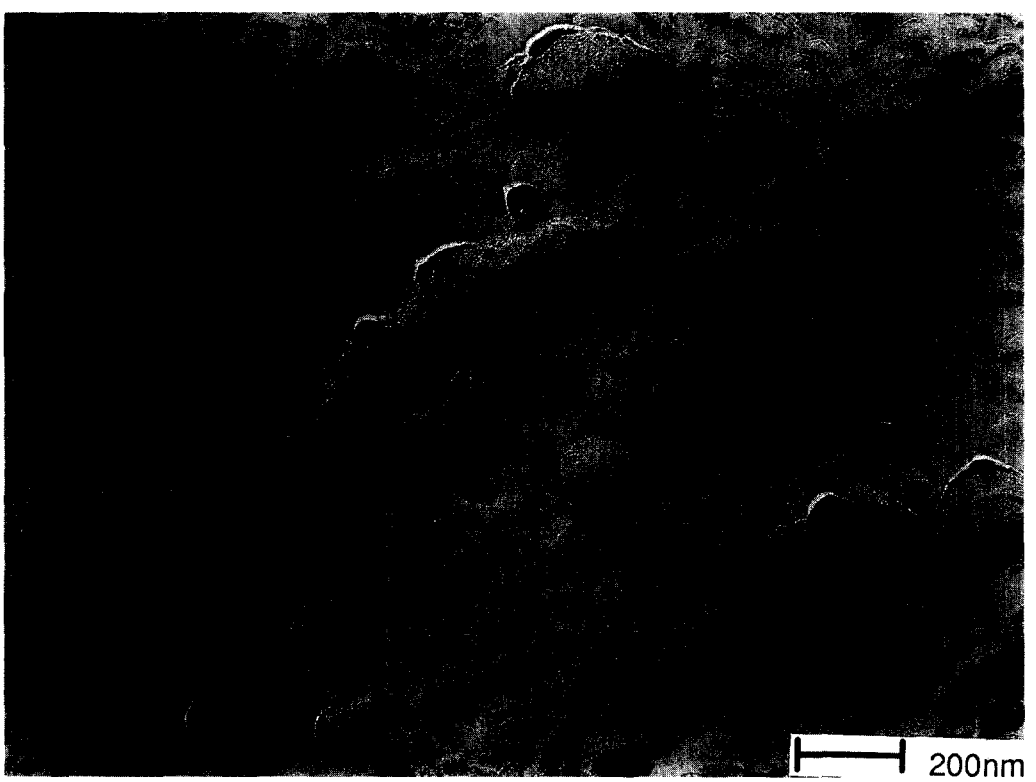

Fig. 1. TEM photograph of a freeze-fractured insulin (30 IU/g) w/o emulsion.

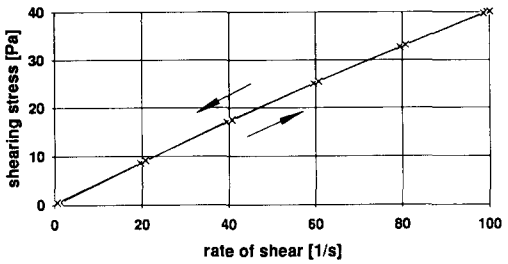

Fig. 2. Up and down flow curve of the w/o emulsions at 25° C in a rotational viscometer.

ous phase which amounted to 30% (w/w) in the formulations.

3.2. Stability of the peptide drug in the formulations

Insulin (30 IU/g) was stable in the w/o emulsion for the examined period of 7 days by immunological analysis (ELISA) (Fig. 3). The structure of the peptide was able to withstand the preparation procedure. The analytical method caused a variation in the results by high sample dilution and immunological vacillations. HPLC determinations over a period of 3 months resulted in a slow decrease to 90% for storage at 4–8° C and to 75% for storage at room temperature (Fig. 4). The distribution of the drug in the emulsions proved to be homogeneous. Because of nonsterile preparation without preservatives, microbiological effects might be involved in the detected degradation. Additional incorporation of aprotinin (2500 KIU/g) into a separately dispersed aqueous phase did not have any significant influence on the stability of insulin for at least 30 days of storage according to HPLC.

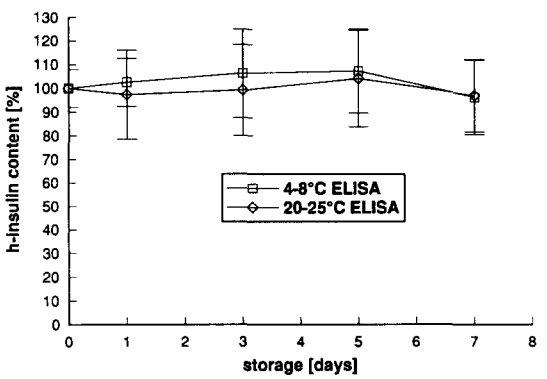

Fig. 3. Short term h-insulin stability in the w/o emulsion (30 IU/g) by ELISA ($n = 3$, mean ± SD).

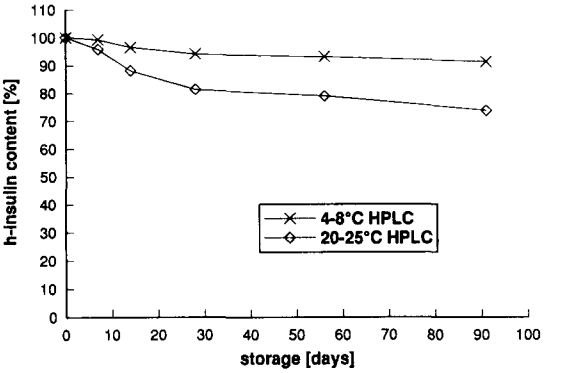

Fig. 4. Longer term h-insulin stability in the w/o emulsion (30 IU/g) by HPLC ($n = 3$, mean values, SD 0.5–1.0%).

3.3. Protection of h-insulin against gastric degradation

Simulation of gastric conditions for 1 h resulted in the effective protection of the incorporated peptide drug against enzymatic degradation by pepsin (5 FIP U/ml). Insulin in aqueous solution was destroyed rapidly within 15 min by an even lower protease activity (0.5 FIP U/ml) under the same conditions (Fig. 5a,b). Obviously, the access of enzymes to the dispersed aqueous phase was prevented by the w/o formulation, implying a phase stability of the emulsion which was subjected to body temperature and mechanical stress.

4. Discussion

In this study high-pressure homogenization proved to be a valid method for preparing peptide-containing w/o emulsions. The compounds were selected with regard to good digestibility and compatibility for oral administration. Olive oil, medium chain triglycerides and lecithin consist of naturally occurring substances that are closely connected with daily nutrition. Cremophor® w/o 7 at least is reported to be of low

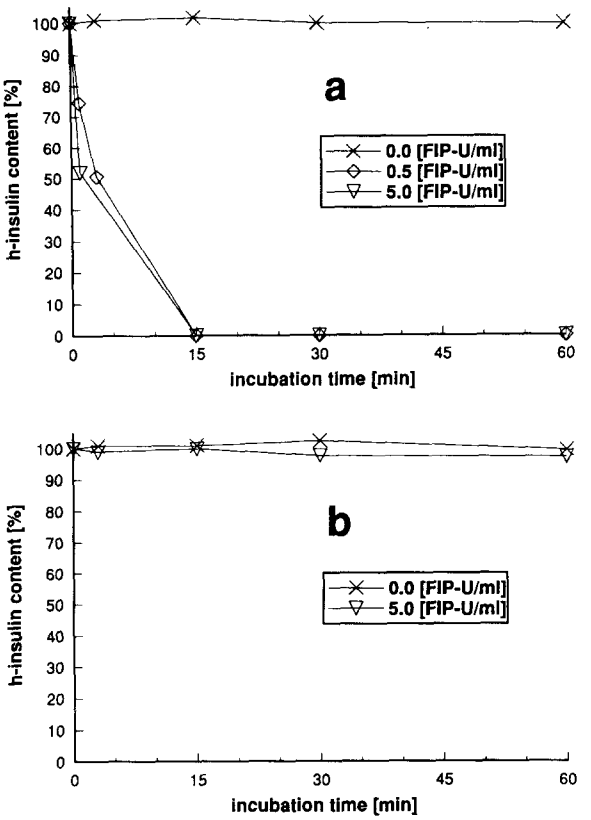

Fig. 5. (a,b) Simulated gastric degradation of h-insulin under influence of pepsin activity expressed in FIP U/ml: (a) in solution; (b) in w/o emulsion ($n = 2$, deviation $\pm$ 2–5%). Unit definition: 1 unit will hydrolyze hemoglobin to produce color equivalent to 1 μmol of tyrosine per min at pH 1.6 at 25° C (color by Folin reagent).

acute toxicity after oral administration in rats (LD 50 > 5 g/kg body weight) (Fiedler, 1989). The development of the formulation yielded an emulsion with a highly dispersed aqueous phase. Most droplets were smaller than 200 nm according to the TEM results. Fine dispersion of the internal phase caused the emulsion to become opalescent after being homogenized three times at 500 bar. The system was physically stable for at least 1 month of storage. Obviously, the physical stability of the formulations is primarily limited by microbial contamination. In general, a water-in-oil emulsion is less sensitive to microbiological growth than an aqueous solution or o/w emulsion. A long-term stability test with these formulations nevertheless would require a sterile preparation or the addition of preservatives, respectively, since contamination was detected after prolonged storage. In stability tests the formulation showed good compatibility with the peptide drug: immunological activity of h-insulin was found to be stable after preparation by means of ELISA, and 90% of the incorporated h-insulin was still analysed with HPLC after 90 days of storage at 4–8° C. A loss of the biological activity of insulin in an emulsion between 14 and 28 days, investigated by intraperitoneal injection, was reported by Patel et al. (1991).

To achieve a sufficient dose of the dissolved peptide in the internal phase, a phosphate buffer system as a suitable solvent for the model peptide was used. Both the peptide drug and the protease inhibitor were dissolved in the aqueous phase and were protected against the gastric juice after application by the oil phase. This was demonstrated in vitro using simulated gastric juice. Further encapsulation by enteric coated gelatin capsules, for instance, is feasible. It is likely that the release of both substances will occur simultaneously during lipolytic digestion by pancreatic agents in the small intestine. The precipitation of released insulin passing the IEP in the intestinal fluid (pH 6–8) is prevented by the neutral pH of the aqueous phase. Oleic acid and medium chain fatty acids are expected to be formed by lipolysis representing mucosal enhancing substances (Murakami et al., 1988; Muranushi et al., 1993). Furthermore, lysophosphatidylcholine (LPC) as a metabolite of the embedded lecithin in the formulation is able to increase the permeability of intestinal mucosa to macromolecules (Tagesson et al., 1985). Lecithin itself is known as a main compound of biological membranes and of the chylomicrons that are formed in the enterocytes to transport lipids via the lymph to the blood circulation. The close association of the release of the peptide drug and simultaneous fat digestion induced by the w/o emulsion may promote lymphatic uptake of the peptide drug. Thus, rapid metabolic degradation of the drug in the liver (first pass effect) would be bypassed (Lee et al., 1991). Oral application of lipid vesicles composed of vegetable oil, lecithin and glycerol resulted in

the enhanced lymphatic absorption of cyclosporin A (Yanagawa et al., 1989). However, this peptide drug is considerably more lipophilic than insulin. The inhibition of the intestinal proteases by aprotinin is to protect the peptide drug against enzymatic degradation in order to enhance the availability at the luminal absorption site of the small intestine. Aprotinin has been described to be suitable for this purpose (Kidron et al., 1989) and is already used in drug therapy (Trasylol®). These intestinal processes are currently under investigation in experiments with w/o formulations exposed to everted gut sacs under simulated digestion.

5. Conclusions

The model peptide drug h-insulin could be incorporated into a finely dispersed w/o emulsion. Additional incorporation of a sufficient dose of aprotinin as a protease inhibitor is possible. These formulations meet the requirements of compatibility and promising enhancing properties for studying the oral administration of peptide drugs. In stability tests the emulsions proved to be suitable for the incorporation of a peptide drug. In vitro degradation by simulated gastric juice was avoided by the protective external phase. Further studies are in progress to investigate the mucosal enhancing abilities of these systems.

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