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W/O/W multiple emulsions of insulin containing a protease inhibitor and an absorption enhancer: preparation, characterization and determination of stability towards proteases in vitro

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Abstract

W/O/W multiple emulsions composed of medium-chain triglycerides containing insulin with aprotinin, a protease inhibitor, and/or sodium taurocholate, an absorption enhancer, were prepared by a two-step emulsification method at 15°C. Whatever the nature of the substance introduced in the internal aqueous phase, all the emulsions obtained had very similar properties. The yield of insulin encapsulation, measured by HPLC analysis, was approximately 95%. These systems were stable during at least 3 months of storage at 4–6°C. The main release mechanism was a swelling-breakdown phenomenon after dilution of the emulsions under hypo-osmotic conditions. These emulsions were able to protect insulin against enzymatic degradation in vitro. © 1997 Elsevier Science B.V.

Keywords: W/O/W multiple emulsion; Insulin; Protease inhibitor; Absorption enhancer; Proteolytic degradation; Stability

1. Introduction

Since the discovery of insulin, several attempts have been made to design a system which would allow its administration by the oral route. Indeed

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the development of a such system would represent real medical and scientific progress in the treatment of diabetes. In addition to patient comfort, oral administration of insulin would be the most convenient and the most physiological route, because in this way insulin would be directly channelled from the intestine to the liver and thus avoid peripheral hyperinsulinemic effects, which are now considered to be a very important factor in the development of arteriosclerosis (Gwinup et al., 1990; Kennedy, 1991). However, there are three main obstacles to the absorption of this hormone in the gastrointestinal tract: (i) its degradation by luminal and cellular peptidases; (ii) the self-association of insulin molecules in aqueous solution and (iii) its very hydrophilic character (Touitou and Rubinstein, 1986; Schilling and Mitra, 1990).

The main strategy employed to attempt to bypass these obstacles, especially degradation by proteolytic enzymes, and to improve enteral insulin bioavailability has been to use new pharmaceutical forms, such as nanocapsules, microspheres, liposomes, cyclodextrins (Damgé et al., 1988; Morishita et al., 1992; Choudhari et al., 1994; Shao et al., 1994) and multiple emulsions. The potential of the last of these systems as vehicles of drugs by oral administration has been demonstrated over the last few years. Several interesting studies can be found in the literature describing multiple emulsions destined for oral administration containing, for example, anticancer agents, anti-inflammatory drugs, antibiotics and vaccines (Omotosho et al., 1990; Roy and Gupta, 1993; Khopade et al., 1996; Hearn et al., 1996). As far as insulin is concerned, the first studies were carried out by Engel et al. (1968) and Shichiri et al. (1974, 1975, 1976) and more recently by Matsuzawa et al. (1995). However, in these studies low efficiency and large variations of glycemia and plasma insulin responses were found after administration of multiple emulsions.

In our preceding studies, with the aim of obtaining better results than these previous authors, we have developed W/O/W multiple emulsions consisting of triglycerides easily metabolized by the organism (soybean oil or medium-chain triglycerides — MCT) and a commercial insulin solution. We found that: (i) in vitro, the yield of encapsulation was superior to 95%, the multiple emulsions obtained were stable over a period of 3 months, the release mechanism was a swelling-breakdown phenomenon after dilution of the emulsions under hypo-osmotic conditions, and these systems offered a total protection of insulin when in contact with pepsin, α-chymotrypsin and trypsin solutions; (ii) in vivo, that insulin could be absorbed from multiple emulsions after oral administration to diabetic rats. However, the level of intestinal absorption remained low (Silva-Cunha et al., 1997a,b).

In order to increase the bioavailability of insulin from our emulsions, we are now developing multiple emulsions consisting of MCT, but containing porcine sodium insulin (Na-Ins), a protease inhibitor and an absorption enhancer. The choice of this type of insulin is based on the fact that this molecule, in aqueous solution, has an lower tendency to form larger species (hexamers) than zinc insulin (Shao et al., 1993). It would be expected that reducing the aggregation of the hormone in the internal aqueous phase of the emulsions would improve its absorption in the gastrointestinal tract. The protease inhibitor selected, aprotinin (AP), had been already used with very good results by Morishita et al. (1992). Similarly, the absorption enhancer, sodium taurocholate (TC), was selected because cholic acid and its analogues have been used to increase the bioavailability of insulin by different routes of administration with very satisfactory results (Ziv et al., 1987; Zhou and Po, 1991).

The objectives of this work were: (i) the development of the formulations themselves and the determination of their physico-chemical properties, (ii) the study of the release mechanism of the encapsulated insulin, and (iii) the in vitro evaluation of the protection of the insulin by the multiple emulsion against pepsin, α-chymotrypsin, trypsin and against pancreatin, in the presence or absence of sodium taurocholate.

Table 1
Composition of the insulin solutions

Solutions	Solution 1	Solution 2	Solution 3	Solution 4
Sodium insulin (mg/ml)	2.0	2.0	2.0	2.0
Aprotinin (units/ml)	—	1000	—	1000
Sodium taurocholate (mM/ml)	—	—	10	10
Sodium chloride (mg/ml)	1.8	1.7	1.6	1.6

2. Materials and methods

2.1. Materials

Porcine sodium insulin was kindly donated by Eli Lilly and Company (Indianapolis, IN); aprotinin (AP) (6602 KIU/mg) was kindly donated by Bayer Pharma (France); pepsin (3100 units/mg), trypsin (11 000 units/mg), α-chymotrypsin (52 units/mg), pancreatin (USP equivalent) and sodium taurocholate were obtained from Sigma, France). The following substances were used to multiple emulsions: lipophilic emulsifier, Abil EM-90®, a silicone-based polymeric emulsifier (Goldschmidt, France); hydrophilic emulsifier, Tween 80®, polyoxyethylene sorbitan monooleate (ICI, Clamart, France) and medium-chain triglycerides (MCT) (Société Industrielle des Oléagineux, France) as the oily phase.

All other chemicals used in this study were analytical reagent or HPLC grade.

2.2. Methods

2.2.1. Preparation of emulsions

Four different emulsions containing porcine sodium insulin (Na-Ins) (ME 1), Na-Ins + aprotinin (AP) (ME 2), Na-Ins + sodium taurocholate (TC) (ME 3) and Na-Ins + TC + AP (ME 4) were obtained.

The multiple emulsions were prepared by a two-step process (Silva-Cunha et al., 1997a). The primary emulsion consisted of (w/w) 45% oily phase, 5% Abil EM-90® and 50% of an aqueous solution of insulin. The W/O/W multiple emulsion consisted of (w/w) 75% of primary emulsion and 25% of an aqueous solution containing 4% Tween 80®. The two stages of emulsification were carried out at 15°C using an Rayneri® agitator. The speed of agitation was 3000 rpm for 30 min in the first step and 900 rpm for 20 min in the second.

The aqueous insulin solutions were prepared as follows: weighed amounts of Na-Ins were dissolved in a few drops of 0.1 N HCl in order to facilitate dissolution. Phosphate-buffered saline (PBS) at pH 7.4 was then added and, after addition of AP and TC where appropriate, PBS was added to obtain a final insulin concentration of 2.0 mg/ml. In order to obtain approximately equal osmotic pressure in these insulin solutions, different amounts of NaCl were then added. The osmolar concentration of the solutions was measured with a Roebling® automatic Osmometer (Bioblock Scientific, France). NaCl was also used as an aqueous phase marker.

The composition and properties of the different insulin solutions are shown in Table 1 and Table 2.

2.2.2. Granulometric analysis

2.2.2.1. Microscopic analyses. After preparation of the emulsions, microscopic observations were made with an optical microscope (Laboval 4, Bioblock, France) at 1000 × magnification after diluting in the appropriate external phase. This analysis allowed us to verify the multiple character of the emulsion by measuring the size of globules and by observing the percentages of simple and multiple globules.

2.2.2.2. Measurement by particle counting. For the determination of the mean diameter of multiple globules, the W/O/W multiple emulsions were dispersed in Isoton® electrolyte solution and the particle size was measured with a Coulter counter

Table 2
Properties of the insulin solutions

Solutions	Solution 1	Solution 2	Solution 3	Solution 4
pH	7.4	7.4	7.4	7.4
Conductivity[a] (mS)	5.4	5.3	5.8	5.8
Osmolar concentration[a] (Osm/kg)	0.28	0.29	0.32	0.33

[a]Values are means ($n = 3$, S.D. $< 0.5\%$).

(Coulter® Multisizer II, Coulter Coultronics, France).

2.2.3. Determination of the yield

The encapsulation yield of the W/O/W emulsions containing insulin was determined by two methods: a conductimetric test and HPLC analysis.

2.2.3.1. Conductimetric test. The conductimetric test allowed the measurement of the weight fraction $\beta(t)$ of the electrolyte released into the external aqueous phase at a given time t, $\beta(t) = M(t)/M_o$, where M_o is the initial amount incorporated and $M(t)$ is the amount present in the external phase at a given time t (Florence and Whitehill, 1982).

Conductivity was measured with a Conductivity Meter CDM3 (Copenhagen).

2.2.3.2. HPLC analysis. Analytical determination of insulin was performed by the method described by Schilling and Mitra, 1991. The concentration of insulin was determined at room temperature by reverse-phase HPLC with a C_{18} column (Nova-Pack, Waters) 4 μm, 4.6 × 250 mm. An auto-injector (WISP Model 712, Waters Millipore, Saint Quentin en Yvelines, France), a UV detector (model 484, Waters Millipore, Saint Quentin en Yvelines, France) adjusted to 220 nm and an integrator (model 745, Waters Millipore, Saint Quentin en Yvelines, France) were used.

Samples were prepared for HPLC as follows: the W/O/W emulsion was diluted 10-fold with an aqueous solution of NaCl at the same osmolar concentration as that of the internal aqueous phase. Considering that the oil/water partition coefficient of insulin is almost zero, the diffusion of insulin across the oil membrane after dilution would be negligible. After centrifugation (3500 × g, 15 min) insulin was extracted from the simple W/O emulsion in the top layer using the mobile phase for HPLC as the extraction solution, in 10-fold excess with respect to the emulsion. After extraction, the aqueous phase was separated by centrifugation (3500 × g, 15 min) and filtered with a Millipore filter (0.45 μm) before HPLC analysis.

2.2.4. Release kinetics

Release kinetics were evaluated by three methods: conductivity measurements, HPLC analysis and rheological analyses.

The amount of electrolyte released was evaluated by conductivity measurements after dilution (10-fold) of the multiple emulsions in hypo (water) and iso-osmotic conditions (glucose solutions).

Insulin release was evaluated by HPLC analysis after dilution (10-fold) of the multiple emulsions in hypo (water) and iso-osmotic conditions (NaCl solution). HPLC analysis was carried out after sample preparation as described above.

The rheological analyses were performed at 20 ± 1°C using a controlled-stress rheometer CSL100 (Carri-Med, Rheo, Palaiseau, France) with cone-plate geometry. The sample, diluted (10-fold) in hypo (water) and iso-osmotic conditions (glucose solutions), was subjected to a constant shear rate (100 s^{-1}) and the evolution of the viscosity coefficient (h) was recorded (Grossiord et al., 1993; Jager-Lezer et al., 1997).

2.2.5. Long-term stability

The stability of the preparations was examined at regular intervals over a period of 3 months of storage at 4–6°C by HPLC analysis and conductimetric tests.

Table 3
Properties of the multiple emulsions containing insulin

Properties		ME 1	ME 2	ME 3	ME 4
Microscopic aspect (μm)	Aqueous globules	<3	<3	<2	<2
	Oily globules	3–16	4–18	7–20	7–25
Oily globule size	Oily globules (μm)	7.3 ± 5.9	7.3 ± 4.2	11.8 ± 6.5	12.0 ± 7.3
Conductivity	μS	6.1	5.2	8.2	12.5
Yield	HPLC ($n = 3$)	96.3 ± 1.9	94.2 ± 1.1	92.2 ± 0.9	87.5 ± 2.4
(%)	Conductivity[a]	97.6	97.8	96.3	94.8

[a]Values are means ($n = 3$, S.D. < 0.5%).

2.2.6. In-vitro protection from digestive enzymes

The stability of encapsulated insulin in the presence of digestive enzymes was determined by the amount of undegraded insulin remaining after incubation of W/O/W multiple emulsions of insulin in the presence of pepsin, trypsin or α-chymotrypsin. The enzyme solutions were prepared as follows: pepsin, 10 units/ml in 0.05 M glycine buffer, pH 1.4; trypsin, 7500 units/ml in 0.05 M phosphate buffer, pH 7.9; and α-chymotrypsin, 52 units/ml in 0.05 M phosphate buffer, pH 7.9 (Michel et al., 1991). Two-hundred milligrams of multiple emulsion were diluted to 1.0 ml with a NaCl solution at the same osmolar concentration as that of the internal aqueous phase and mixed with an equal volume of the enzyme solution. Incubations were carried out at 37°C for 30 min. After incubation, the total amount of insulin was determined by HPLC analysis after sample preparation as described above. Multiple emulsions without insulin were mixed with the insulin solutions and used as controls under the same experimental conditions.

For the pancreatin degradation study, a preliminary determination of the resistance of insulin in solution in the presence of different concentrations of pancreatin was carried out as follows: 0.5 ml of the insulin solutions (1.0 mg of protein) were incubated at 37°C for 30 min with an equal volume of pancreatin solution, at different concentrations in PBS (pH 7.4). After determination of the optimal ratio of insulin and pancreatin concentrations catalysing rapid insulin degradation, the resistance of the insulin within emulsions was determined in presence or absence of sodium taurocholate (TC at 10 and 20 mM).

The osmolar concentrations of the buffer solutions used in these enzymatic degradation studies were 1010.00 and 0.36 Osm/kg for glycine and phosphate buffer (PBS), respectively.

3. Results

3.1. Characterization of emulsions

Four different W/O/W multiple emulsions (ME 1–4) containing Na-insulin at an approximate concentration of 0.75 mg/g emulsion were obtained. It is important to note (Table 3) that whatever the nature of the solutes in the internal aqueous phase, the emulsions had very similar properties: low conductivity and a good yield of insulin entrapment. However, it was noted that the inclusion of TC (ME 3 and ME 4) slightly decreased the size of the aqueous globules while increasing the size of the oily globules. Thus, the mean diameter of the aqueous globules could be estimated as a maximum of 2 μm for the emulsions containing TC and 3 μm for emulsions without TC. On the other hand, the mean diameter of the multiple globules was about 7 and 12 μm for the emulsions without and with TC (ME 1 and ME 2), respectively.

A second consequence of the addition of TC was as light reduction in the percentage entrapment of insulin. As indicated (Table 3), approximately 95% of the added insulin, measured by HPLC analysis, was incorporated into the preparations without TC and approximately 90% into emulsions containing TC. However, the NaCl entrapment efficiency, measured by the conductivity

Table 4
Long-term stability of the multiple emulsions

Emulsion	Yield (%)	1 day	30 days	60 days	90 days
ME 1	Conductivity[a]	97.6	97.6	97.3	95.6
	HPLC ($n=3$)	96.3 ± 1.9	96.3 ± 2.3	—	95.1 ± 3.1
ME 2	Conductivity[a]	97.8	97.7	97.4	96.0
	HPLC ($n=3$)	94.2 ± 1.1	94.1 ± 1.0	—	93.1 ± 1.8
ME 3	Conductivity[a]	96.3	96.0	94.5	91.8
	HPLC ($n=3$)	92.2 ± 0.9	90.6 ± 1.5	—	87.4 ± 2.0
ME 4	Conductivity[a]	94.8	92.8	90.3	88.8
	HPLC ($n=3$)	87.5 ± 2.4	86.7 ± 2.6	—	83.1 ± 3.3

[a]Values are means ($n=3$, S.D. $<0.5\%$).

assay, showed that there was no significant difference between the preparations.

The stability of all the multiple emulsions, on storage at 4–6°C for a period of 3 months, was satisfactory (Table 4). However, the presence of TC in internal aqueous phase slightly decreased the stability of these systems during this storage period.

3.2. Release

Release kinetics studies yielded identical results to these obtained in our previous work (Silva-Cunha et al., 1997a). As far as NaCl release was concerned, there was no significant release from multiple emulsions diluted under iso-osmotic conditions (Fig. 1). However, under hypo-osmotic conditions, NaCl was released (Fig. 2).

The release of Na-insulin from the W/O/W emulsions diluted under hypo and iso-osmotic conditions was in accord with the NaCl release studies. Thus, under iso-osmotic conditions, no release was observed (data not shown), whereas under hypo-osmotic conditions Na-insulin was released from the multiple emulsions (Fig. 3).

In both studies, the release of the encapsulated substances was slightly increased from multiple emulsions containing sodium taurocholate. Moreover, we observed a good correlation between NaCl and insulin release studies.

The rheological profiles (Fig. 4) were similar for all multiple emulsions, showing, for samples diluted under hypo-osmotic conditions, a very rapid increase in the apparent viscosity followed by a similarly rapid decrease. However, a slight increase in the swelling peak was observed for the emulsions containing sodium taurocholate. Under iso-osmotic conditions, no change in apparent viscosity was observed for any of the emulsions.

3.3. In-vitro protection against digestive enzymes

The protection of the Na-insulin against pepsin, trypsin and α-chymotrypsin afforded by encapsulation within the multiple emulsions was remark-

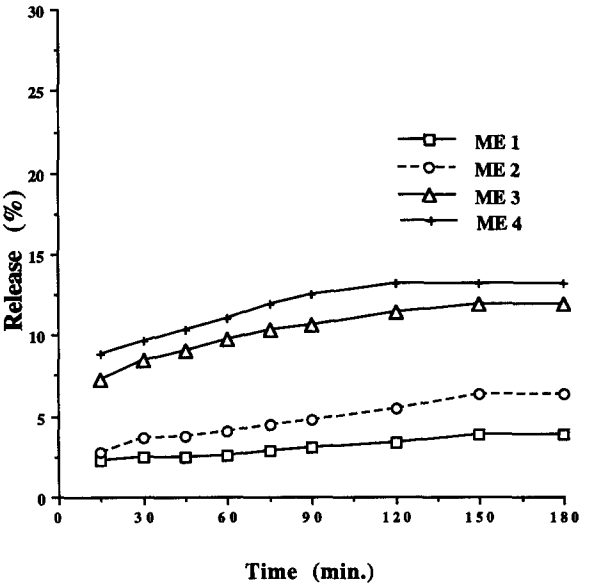

Fig. 1. Release rate of NaCl from multiple emulsions diluted under iso-osmotic conditions. Values are means ($n=3$, S.D. $<0.3\%$).

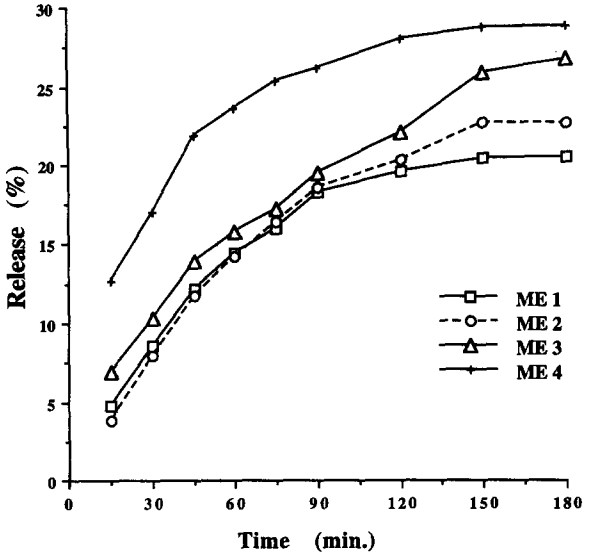

Fig. 2. Release rate of NaCl from multiple emulsions diluted under hypo-osmotic conditions. Values are means ($n = 3$, S.D. $< 0.3\%$).

able: almost all the entrapped insulin was recovered at the end of incubation period (Fig. 6), while the free insulin in control preparations was degraded (Fig. 5).

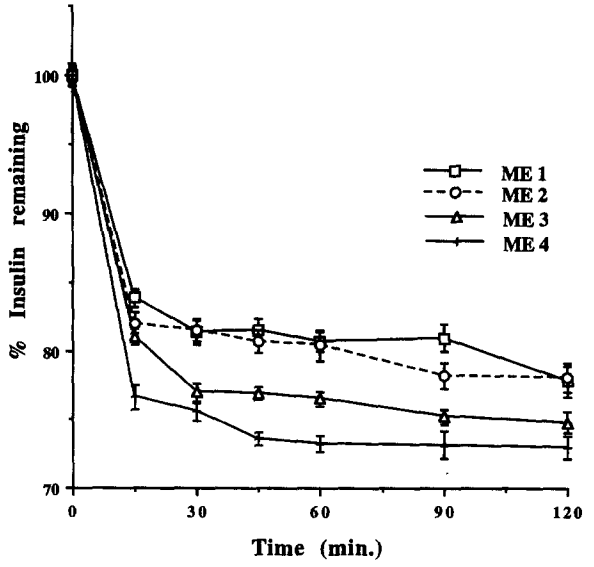

Fig. 3. Percentage of intact insulin as a function of time for the multiple emulsions diluted under hypo-osmotic conditions. Values are means ($n = 3$) $\pm$ S.D.

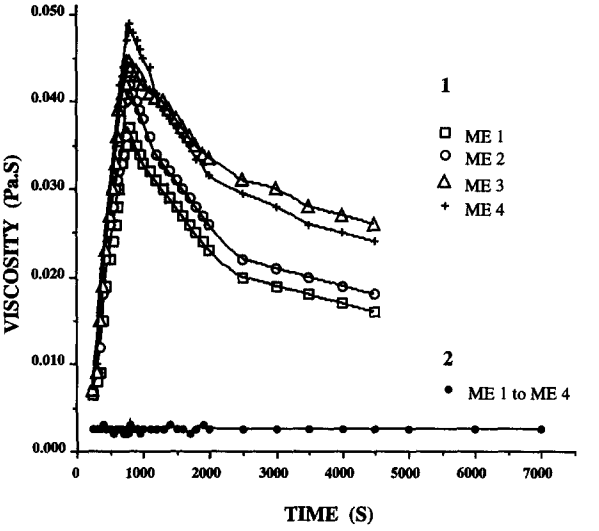

Fig. 4. Rheological behaviour of the multiple emulsions diluted under hypo- (1) and iso- (2) osmotic conditions.

In the preliminary determination of the sensitivity of the insulin solutions to pancreatin, almost 100% of the insulin was degraded by a concentra-

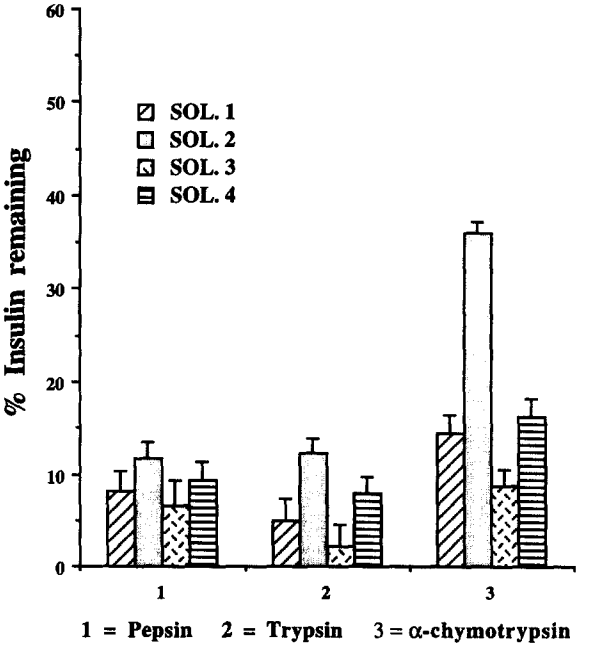

Fig. 5. Percentage of intact insulin in solutions incubated with pepsin, trypsin and α-chymotrypsin. Values are means ($n = 3$) $\pm$ S.D.

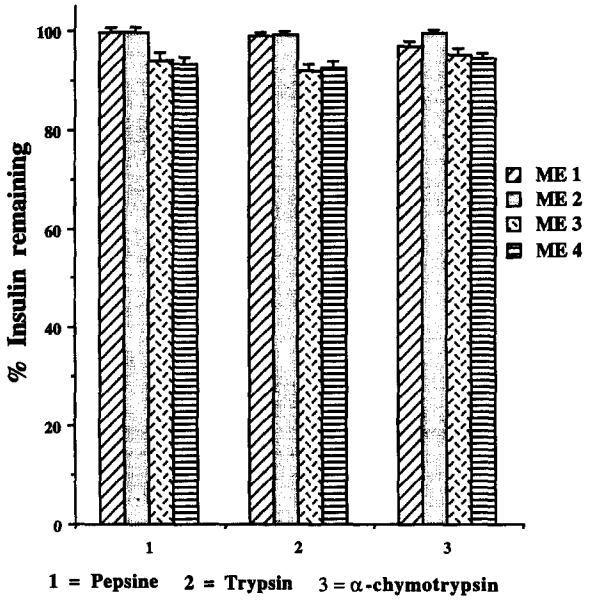

Fig. 6. Percentage of intact insulin in multiple emulsions incubated with pepsin, trypsin and α-chymotrypsin. Values are means ($n = 3$) ± S.D.

tion of 2.0 mg/ml of pancreatin (Fig. 7) with solutions 1, 3 and 4 and 80% of degradation was obtained with solution 2. However, after incubation of the multiple emulsions with 2.0 mg/ml of pancreatin (ratio of insulin to pancreatin 1:2) practically no insulin degradation was observed

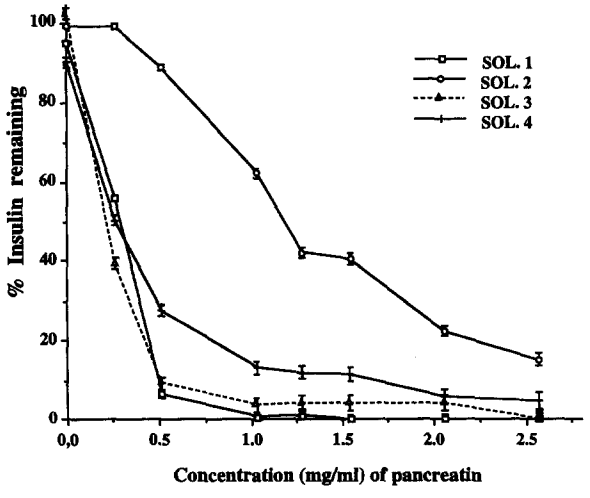

Fig. 7. Percentage of intact insulin in solutions incubated with pancreatin. Values are means ($n = 3$) ± S.D.

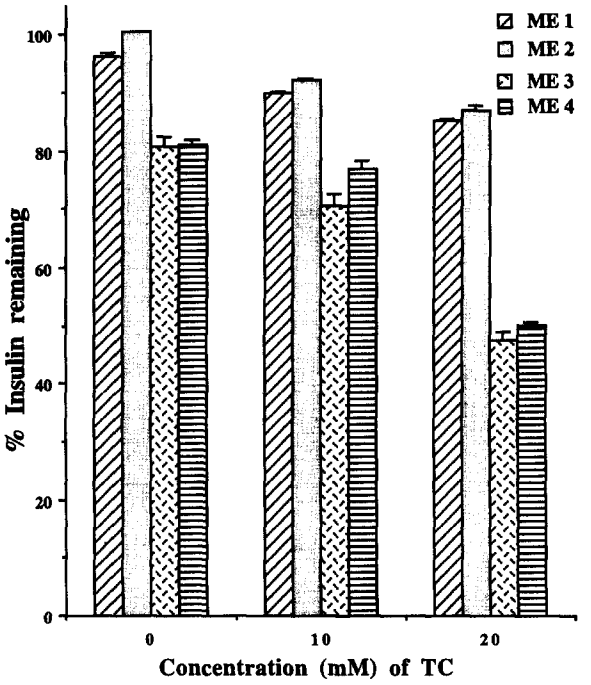

Fig. 8. Percentage of intact insulin in multiple emulsions incubated with pancreatin in presence or absence of sodium taurocholate (TC at 10 and 20 mM). (Ratio between insulin and pancreatin = 1:2) Values are means ($n = 3$) ± S.D.

for ME 1 and ME 2 and only 20% degradation with ME 3 and ME 4 (Fig. 8). The addition of sodium taurocholate (TC) at 10 and 20 mM to the incubation medium provoked an increase in the rate of hormone degradation, which was more pronounced for emulsions containing this bile salt within their internal aqueous phase.

So, the presence of aprotinin (solution 2) decreased the rate of hormone degradation in the free insulin control, but the presence of sodium taurocholate seemed to counteract the protective effect of the protease inhibitor (Fig. 5 and Fig. 7).

4. Discussion

Degradation of insulin is a major factor influencing its absorption by the oral route. We recently reported that W/O/W multiple emulsions, composed of soybean oil or medium-chain triglycerides (MCT), were able to protect insulin against

degradation by pepsin, trypsin and α-chymotrypsin in vitro. However, the hypoglycaemic effects obtained with these systems in diabetics rats, although significant, were weak. Therefore, to optimize the efficacy of these emulsions it was important to increase the extent of insulin absorption.

Thus, the aim of the present work was to obtain multiple emulsions composed of MCT with a protease inhibitor (AP) and an absorption enhancer (TC) included within the aqueous internal phase of the emulsions, in an attempt to enhance intestinal absorption of insulin. These substances have been reported to be effective in promoting insulin absorption through the intestinal wall.

In order to prevent aggregation of insulin in aqueous solution, porcine sodium insulin was used in these multiple emulsion formulations. Moreover, these smaller species are better able to diffuse across the gastrointestinal mucosal barrier (Liu et al., 1991; Li et al., 1992).

The remarkable qualities of these emulsions, prepared at low temperature, are related to the properties of the lipophilic emulsifier used: this polymeric emulsifier, due to its structure, provides a strong, thick and well-defined film around the water-in-oil droplets (Grossiord et al., 1993; Jager-Lezer et al., 1997). The fact that the osmolar concentration of the internal aqueous phase of the four emulsions was practically identical explains why these systems, despite different internal aqueous phase compositions, had very similar physico-chemical properties.

The conductivity and rheological studies confirmed that a swelling-breakdown phenomenon was the main mechanism of release from these emulsions, as for the emulsions described in earlier work (Silva-Cunha et al., 1997a). Thus, under hypo-osmotic conditions, an osmotic flux of water from external phase to the internal phase of the emulsions occurs and induces rupture of the oil membrane and release of the solute. On the rheological profiles, this influx of water under hypo-osmotic conditions corresponds with a very rapid increase in the apparent viscosity followed by a similarly rapid decrease. The similar rheological and release profiles obtained with the different emulsions shows that sodium taurocholate and aprotinin do not interfere with this mechanism. Moreover, the slight difference of the swelling rate between the different preparations may be explained by their granulometric distribution: the larger aqueous globules obtained in the case of ME 3 and ME 4 could provide an excess of lipophilic emulsifier which could migrate to the oil/water interface, thus increasing the swelling capacity of the oily membrane of the emulsions (Jager-Lezer et al., 1997).

When incorporated into W/O/W multiple emulsions, insulin was significantly more resistant to protease degradation than as the free polypeptide. This result can be explained by an effective protection afforded by the oily membrane which prevents contact between enzymes and the entrapped insulin. Moreover, the higher osmolarity (approximately among 1- to 3-fold greater) of the incubation medium compared with the internal aqueous phase prevented the rupture of the droplets of the W/O primary emulsion.

Since the small intestinal lumen contains a high concentration of bile salts (approximately 10 mM) and insulin degradation by enzymes might be more rapid under these conditions (Liu et al., 1991; Li et al., 1992; Matsuzawa et al., 1995), it was important to know whether the multiple emulsions could protect insulin against pancreatin in presence of sodium taurocholate (TC), a model bile salt.

The results showed that, although still significant, the protection offered by encapsulation was reduced by inclusion of sodium taurocholate in the incubation medium. This can probably be explained by the way TC molecules interact and disrupt of the oil membrane, facilitating insulin and triglyceride degradation by the enzymes. Nevertheless, the rate of degradation was more pronounced for emulsions containing TC within their internal aqueous phase when there was a high concentration of TC in the incubation medium.

It was clear that the presence of aprotinin in solution 2 enhanced the resistance of insulin to enzymatic degradation, but this increased protection offered by aprotinin decreased when sodium taurocholate was combined with aprotinin in solution 4. It is significant to note that in the

Na-insulin solution used the concentration of hexamer species is low and dimers and monomers predominate. It has been reported (Liu et al., 1991; Li et al., 1992; Shao et al., 1993) that smaller molecular species, although more easily absorbed by the intestine, are more susceptible to proteolytic attack than hexameric species.

Furthermore, it was also reported by Li et al. (1992) that the concentration of insulin monomers is increased in the presence of bile salts by solubilization in mixed micelles. In their work, it was noted that 10 mM of sodium glycocholate was needed to completely dissociate the dimers of sodium insulin into monomers. To elucidate the mechanism of bile salt-mediated insulin dissociation further circular dichroism studies are planned.

In conclusion, this study showed that W/O/W multiple emulsions containing insulin, a protease inhibitor and an absorption enhancer with very similar properties can be obtained at low temperature. These emulsions are stable and their release mechanism is a swelling breakdown phenomenon. Moreover, these systems are able to protect insulin against proteolytic degradation in vitro. The biological effects of these multiple emulsions are currently under investigation in diabetic and normal animals.

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