ELSEVIER

International Journal of Pharmaceutics 169 (1998) 33–44

international journal of pharmaceutics

W/O/W multiple emulsions of insulin containing a protease inhibitor and an absorption enhancer: biological activity after oral administration to normal and diabetic rats

A. Silva-Cunha [d], M. Chéron [c], J.L. Grossiord [b], F. Puisieux [a], M. Seiller [a,*]

[a] *Laboratoire de Pharmacie Galénique et Biopharmacie, URA CNRS 1218, UFR des Sciences Pharmaceutiques, 5 rue Jean Baptiste Clément, 92296 Châtenay-Malabry, France*

[b] *Laboratoire de Physique Pharmaceutique, UFR des Sciences Pharmaceutiques, 5 rue Jean Baptiste Clément, 92296 Châtenay-Malabry, France*

[c] *Laboratoire de Physicochimie Biomoléculaire et Cellulaire (CNRS URA 2056), Université Pierre et Marie Curie, case 138, 4 place Jussieu 75252, Paris, France*

[d] *Faculdade de Farmacia da Universidade Federal de Minas Gerais, Avenida Olegario Maciel, 2360, 30180-112 Belo Horizonte/MG, Brazil*

Received 6 October 1997; received in revised form 24 February 1998; accepted 27 February 1998

Abstract

In this work, the biological effects of w/o/w multiple emulsions of medium-chain triglycerides containing sodium insulin alone or with a protease inhibitor, or with an absorption enhancer, or with a protease inhibitor and an absorption enhancer, were compared to a w/o/w multiple emulsion containing zinc insulin. The release mechanism of all multiple emulsions was the swelling–breakdown phenomenon after dilution of the emulsions under hypo-osmotic conditions. The biological effects after oral administration to normal and diabetics rats showed a larger decrease of glycemia with the multiple emulsions containing sodium insulin than with the multiple emulsion containing zinc insulin. However, there was no significant difference between the hypoglycemic effects induced by the emulsions containing sodium insulin. These results suggest that the aggregation state of insulin molecules might be the major factor responsible for increasing the extent of intestinal insulin absorption. Thus, the nature of the insulin plays a fundamental role and, at the concentration used in this work, the addition of sodium taurocholate was not able to modify its aggregation state in aqueous solution, as confirmed by circular dichroism studies.

Keywords: W/O/W multiple emulsion; Insulin; Protease inhibitor; Absorption enhancer; Oral route; Diabetic and normal rats; Glycemia

* Corresponding author.

0378-5173/98/$19.00
PII S0378-5173(98)00102-1

1. Introduction

The challenge of successfully developing pharmaceutical systems for oral administration of insulin is great. As well as the ease of this route, oral administration of insulin would be more appropriate in the treatment of diabetes because, after intestinal absorption, insulin is channelled directly to the liver following the route of physiological pancreatic insulin and playing a very positive role by reducing peripheral hyperinsulinemic effects (Gwinup et al., 1990). In fact, with peripherally infused insulin, ideal blood glucose control is achieved only with concomitant peripheral hyperinsulinemia. However, it seems that this abnormal physiological condition is a very important factor in the development of atherosclerosis (Nordestgard et al., 1997). Thus, the development of an oral dosage form providing adequate bioavailability of insulin, although it may not completely replace parenteral therapy, would certainly supplement it.

In order to meet this challenge we aimed to develop an oral dosage form whose insulin bioavailability would be sufficient to promote a hypoglycemic response at the same level as obtained with parenteral dosage forms. We have previously reported that oral administration of insulin entrapped in w/o/w multiple emulsions reduced the blood glucose level of diabetic rats (Silva-Cunha et al., 1997a). However, in this study low efficiency and large variations of glycemia were found after administration of these multiple emulsions.

There are two main hypothesis to explain the low efficiency of our insulin systems:

(1) the poor permeability of insulin through intestinal epithelium associated with the low potential for absorption enhancement of the multiple emulsions;

(2) the inactivation of insulin, after its release from the emulsions, by the peptidases present in the intestinal tissue. In fact, multiple emulsions could protect the hormone against luminal enzymatic degradation, but this protection would disappear after hormone release.

However, some attractive alternatives to improve transmucosal bioavailability of insulin exist: (i) by using insulin species with a weak tendency to form aggregates, (ii) by inhibiting enzymatic tissue metabolism, and (iii) by increasing membrane permeability (Kidron et al., 1982; Shao et al., 1993; Yamamoto et al., 1994). Furthermore, an advantage of this system would be that, when encapsulated in the multiple emulsions, the protease inhibitor or absorption promoter could be used in small amounts and would not affect the digestive process.

Thus, in order to increase insulin bioavailability from our emulsions, like others authors using different dosage forms (Hirai et al., 1981; Linde and Gunnaesson, 1985; Aungst and Rogers, 1988; Morishita et al., 1992), we have developed new multiple emulsions of medium-chain triglycerides (MCT) containing porcine sodium insulin, aprotinin (a protease inhibitor) and sodium taurocholate (an absorption enhancer). Sodium insulin was used with a view to minimizing its aggregation in aqueous solution and, due to reduction of the size of insulin aggregates, to increase its absorption in the gastrointestinal tract (Liu et al., 1991; Li et al., 1992). The protease inhibitor was used in an attempt to protect the hormone against degradation by proteolytic enzymes after its release from multiple emulsions and the absorption enhancer was used to increase the mucosal absorption of insulin by various mechanisms.

Consequently, the objective of the present work was to investigate the biological effects of these new multiple emulsions, in the hope of obtaining a large reduction in glycemia.

2. Materials and methods

2.1. Materials

Porcine sodium insulin was kindly donated by Eli Lilly and Company (Indianapolis, IN); aprotinin (AP) (6602 kI.U./mg) was kindly donated by Bayer Pharma (France); porcine zinc insulin (27.8 I.U./mg HPLC) and sodium taurocholate were obtained from Sigma (Sigma, France). The following substances were used to prepare 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.

Male Wistar rats were used in this study. Diabetes was induced in rats by an intraperitoneal injection of streptozotocin (Sigma, France) dissolved in citrate buffer, pH 4.5. One daily administration of 40 mg/kg body weight was made on three consecutive days. Rats were considered diabetic when their fasting glycemia was >250 mg/dl and were fasted for least 16 h before experiments.

2.2. Methods

2.2.1. Preparation of emulsions

Four different emulsions, containing porcine sodium insulin (ME 1), sodium insulin + aprotinin (AP) (ME 2), sodium insulin + sodium taurocholate (TC) (ME 3), sodium insulin + TC + AP (ME 4), and a multiple emulsion containing zinc insulin (ME 5), as control, were prepared.

The multiple emulsions were obtained by a two-step process (Silva-Cunha et al., 1997a). The primary emulsion consisted of (w/w) 45% oily phase, 5% lipophilic emulsifier and 50% aqueous insulin solutions. The w/o/w multiple emulsion consisted of (w/w) 75% of primary emulsion and 25% of an aqueous solution containing 4% hydrophilic emulsifier. The two stages of emulsification were carried out at 15°C. The speed of agitation was 3000 rpm for 30 min in the first step and 900 rpm for 20 min in the second.

The preparation of the aqueous insulin solutions has been described previously (Silva-Cunha et al., 1997b), and their compositions are shown in Table 1.

2.2.2. Physico-chemical characterization

Multiple emulsions were characterized by granulometric, conductimetric, rheological and HPLC analysis, as described previously (Silva-Cunha et al., 1997b).

2.2.3. Circular dichroism studies

Circular dichroism (CD) spectra were recorded with a Jobin Yvon Mark IV dichrograph using a thermostated cuvette holder. $\Delta\epsilon$ (M^{-1} cm^{-1}) is

Table 1
Composition of the insulin solutions

Substances	Sol. 1	Sol. 2	Sol. 3	Sol. 4	Sol. 5
Zinc insulin (mg/ml)	—	—	—	—	2.0
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	1.8

Table 2
Initial body weight and glycemia of the rats in different experimental protocols

Protocol no.	Rat condition	Treatment procedure	Body weight (g)	Glycemia before experiments (mg/ml)	Condition of animals
1	Normal	Subcutaneous	217 ± 17	96.1 ± 3.4	Non-fasted
2	Normal	Subcutaneous	340 ± 18	93.6 ± 5.0	Non-fasted
3	Normal	Oral route	300 ± 21	52.8 ± 5.6	Fasted
4	Normal	Oral route	350 ± 20	53.4 ± 6.8	Fasted
5	Normal	Oral route	350 ± 25	51.3 ± 7.4	Fasted
6	Diabetic	Oral route	255 ± 27	351.8 ± 35.8	Fasted

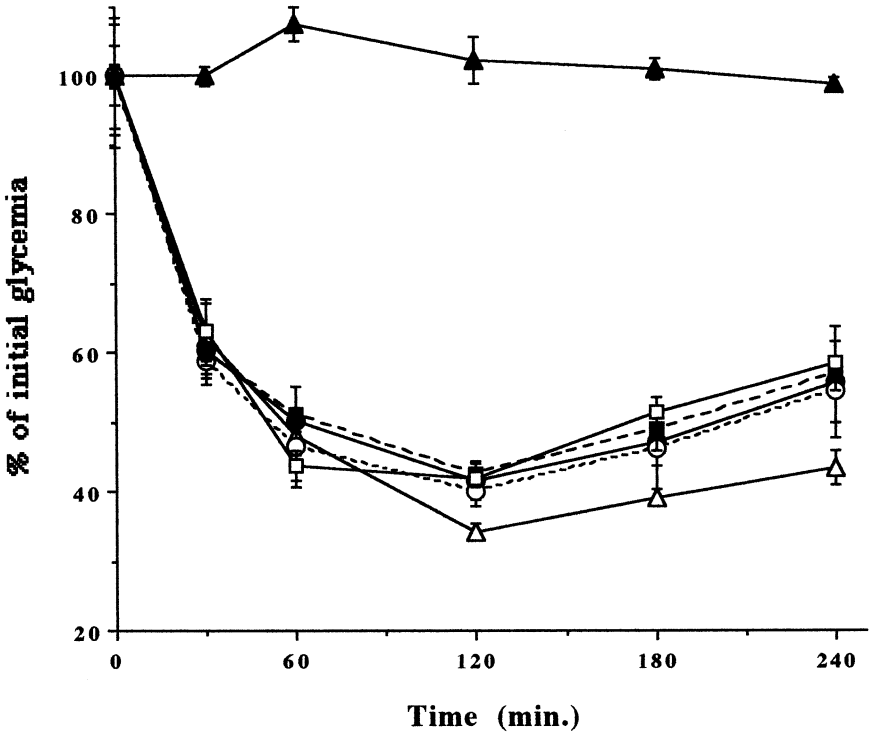

Fig. 1. Glycemia after administration of insulin solutions s.c. to non-fasted normal rats: (○) sol. 1; (●) sol. 2; (□) sol. 3; and (■) sol. 4. Controls: (△) zinc insulin solution and (▲) phosphate-buffered saline solution (PBS) (mean ± S.D., $n = 4$).

the differential molar dichroic absorption coefficient. Sodium insulin solutions (sol. 1–4) and zinc insulin solution (sol. 5) were scanned from 300 to 250 and from 250 to 200 nm at a scanning speed of 5 mm/min and at a temperature of 25°C.

2.2.4. Biological evaluation of insulin solutions

In all experiments, the doses of insulin administered by subcutaneous injection or by the oral route were 0.11 and 5.4 mg of insulin/kg of body weight, respectively.

Blood samples for measurement of glycemia were collected from the tail vein, under ether anesthesia. Glycemia was measured by an enzymatic method (Glucose-Test kit, Sigma, France).

Body weights and initial glycemia of the experimental groups are detailed in Table 2.

Blood samples were collected 5 min before administration and 30 (only in the subcutaneous injection experiments), 60, 120, 180 and 240 min after administration.

2.2.4.1. Subcutaneous injection of insulin solutions to normal rats (Protocol 1). A sample of each insulin solution (sol. 1–5) was diluted to a concentration of 0.22 mg/ml with phosphate-buffered saline solution (PBS). The diluted samples were injected by subcutaneously in non-fasted normal rats.

2.2.4.2. Subcutaneous injection of the aqueous phase of diluted multiple emulsions to normal rats (Protocol 2). Two samples of each emulsion containing sodium insulin were diluted to a concentration of 0.22 mg of insulin/ml, the first under hypo-osmotic conditions (water) and the second under iso-osmotic conditions (NaCl solution). The diluted samples were stirred for 60 min at 12°C, centrifuged (3500 × g, 15 min) and the centrifugal supernatants were filtered on a Millipore filter (0.45 nm). The filtrates thus obtained were administered (s.c.) to non-fasted normal rats.

2.2.4.3. Single oral administration to normal rats (Protocol 3). In an initial study, insulin solutions at the same dose as in the multiple emulsions of insulin were administered to normal rats. In a second study (Protocol 4), multiple emulsions containing insulin and a multiple emulsion without insulin, but containing AP and TC (MEwi), as a control, were administered by gavage into the stomach of normal fasted rats. The blood sample collection and glycemia measurement were as previously described.

Finally (Protocol 5) we performed a second experiment with normal rats based in the studies carried out by Damgé et al. (1988). In this experiment, normal rats were divided into two groups and received, after an overnight fast, multiple emulsions containing insulin (ME 1–5) or PBS solution. Ninety minutes later both groups received an oral administration of glucose (2.5 g/kg body weight). Glycemia was measured in blood samples 5 min before and 60 and 120 min after administration of glucose.

2.2.4.4. Single oral administration to diabetic rats (Protocol 6). Multiple emulsions containing insulin and a multiple emulsion without insulin (MEwi), as a control, were administered by gavage into the stomach of diabetic fasted rats. The blood sample collection and glycemia measurement were as previously described.

2.2.5. Statistical analysis

Tests for significant differences between means were made by analysis of variance (ANOVA). Reference to a significant difference in the subsequent text refers to a level of $p < 0.05$.

3. Results

Five different w/o/w multiple emulsions (ME 1–5) containing sodium and zinc insulin at an approximate concentration of 0.75 mg/g emulsion were obtained. The main physico-chemical properties of these emulsions have been previously

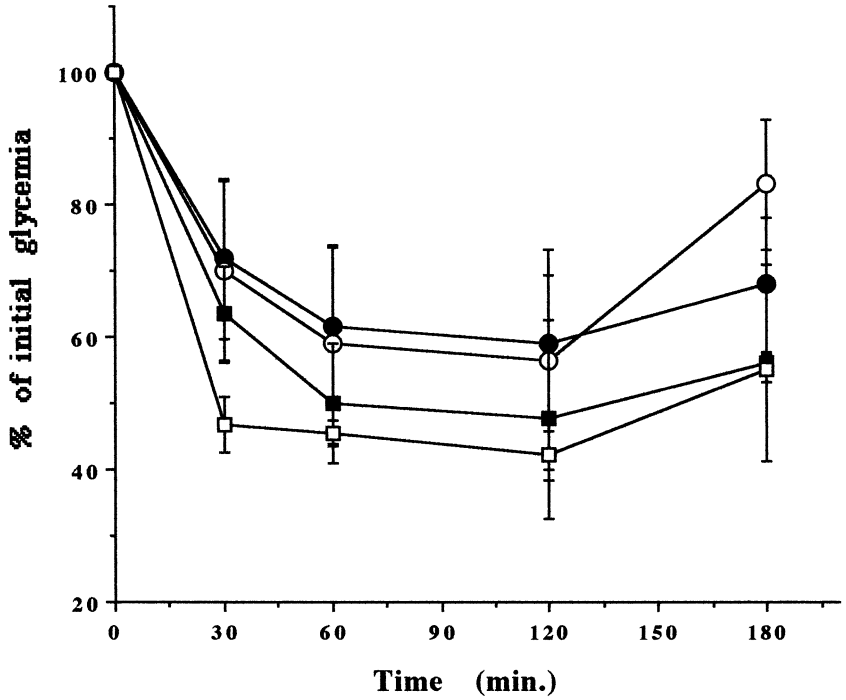

Fig. 2. Glycemia after administration of the aqueous phase of the multiple emulsions diluted under hypo-osmotic conditions s.c. to non-fasted normal rats: (○) ME1; (●) ME 2; (□) ME 3; and (■) ME 4 (mean ± S.D., $n = 4$).

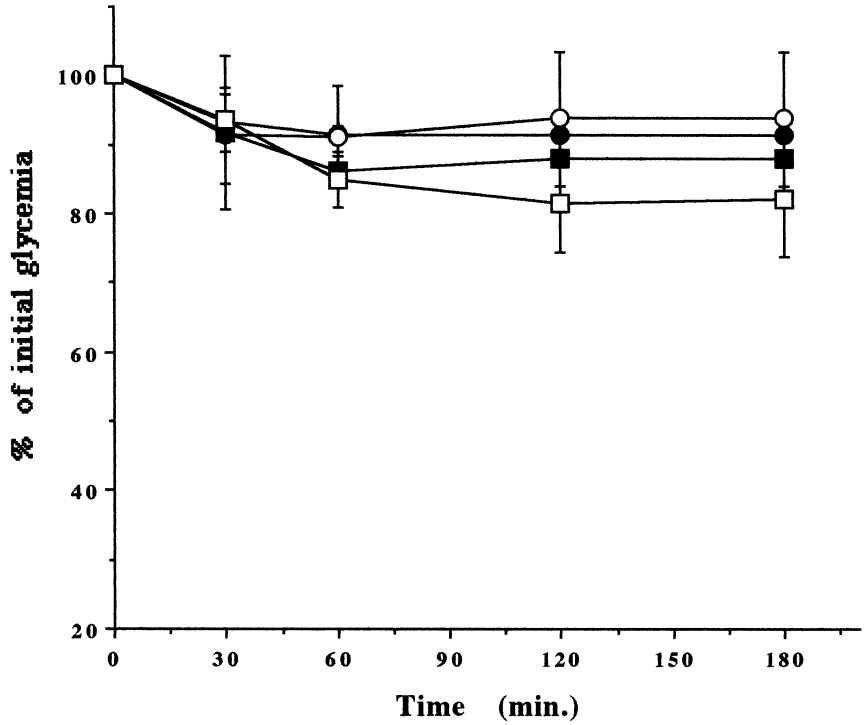

Fig. 3. Glycemia after administration of the aqueous phase of the multiple emulsions diluted under iso-osmotic conditions s.c. to non-fasted normal rats: (○) ME1; (●) ME 2; (□) ME 3; and (■) ME 4 (mean ± S.D., $n = 4$).

discussed (Silva-Cunha et al., 1997b): in brief, it should be noted that the emulsions, although containing different encapsulated ingredients, showed similar properties.

In the present study, the percentage of reduction of initial glycemia was used as the parameter to evaluate insulin absorption.

As expected, after subcutaneous administration (Fig. 1) of insulin solutions to normal rats a rapid and marked decrease of glycemia was obtained. These results indicated an equivalent biological activity of all insulin solutions.

In accord with the results of an in vitro study of insulin release, a decrease of glycemia was observed after subcutaneous administration to normal rats of the aqueous phase obtained from multiple emulsions diluted under hypo-osmotic conditions (Fig. 2). In contrast, only a slight hypoglycemic effect was observed after subcutaneous administration of the aqueous phase obtained from these emulsions diluted under iso-osmotic conditions (Fig. 3). Although no significant difference was observed between the different aqueous phase administered, a more pronounced tendency for glycemia reduction was found with aqueous phases obtained from multiple emulsions containing TC (ME 3 and 4).

As expected, as a single oral dose in normal rats, no hypoglycemic effect was observed after oral administration of the insulin solutions (Fig. 4) or with the multiple emulsion without insulin (MEwi) (Fig. 5). In contrast, with multiple emulsions containing insulin a slight hypoglycemic effect was observed from 120 min after administration. However, no significant difference between these emulsions was noted (Fig. 5).

It was interesting to note that the increase in blood glucose, induced by an oral administration of glucose solution, was initially identical for all groups of rats which received multiple emulsions. However, 120 min after glucose administration, the initial glycemia was almost restored in the groups of rats which received multiple emulsions containing sodium insulin, while in the groups of

rats which received multiple emulsions containing zinc insulin no reduction of glycemia was noted (Fig. 6). The rise in glycemia level was approximately four times more pronounced in the group of rats which received phosphate-buffered saline (PBS) before glucose administration than in the rats that received multiple emulsions. The formation of a barrier by the emulsion above the mucosal membrane in the gastrointestinal tract could reduce glucose absorption and would explain the differences encountered between the different groups of rats.

Finally, from 120 min after oral administration (Fig. 7) of the emulsions containing sodium insulin (ME 1–4) to diabetic rats a significant decrease of glycemia was observed, while in the case of the multiple emulsion containing zinc insulin (ME 5) and the multiple emulsion without insulin (MEwi) a slight or no hypoglycemic effect were observed, respectively. However, there was no significant difference in the hypoglycemic effects induced by emulsions containing sodium insulin. The reduction of initial glycemia obtained with these was about 35%.

Fig. 8a,b shows CD spectra of insulin solutions (sol. 1–5) at a concentration of 2.0 mg/ml. In agreement with other work (Liu et al., 1991; Li et al., 1992), a significant difference between the CD spectra of zinc (sol. 5) and sodium insulin solutions (sol. 1–4) was observed. This difference in molar dichroic absorption may be attributed to different conformations in aqueous solution of the two species of insulin: dimers predominate with sodium insulin and hexamers with zinc insulin. No significant difference between the sodium insulin solutions (sol. 1–4) was found, suggesting that there was no dissociation of insulin dimers into monomers with the addition of sodium taurocholate, at a concentration of 10 mM.

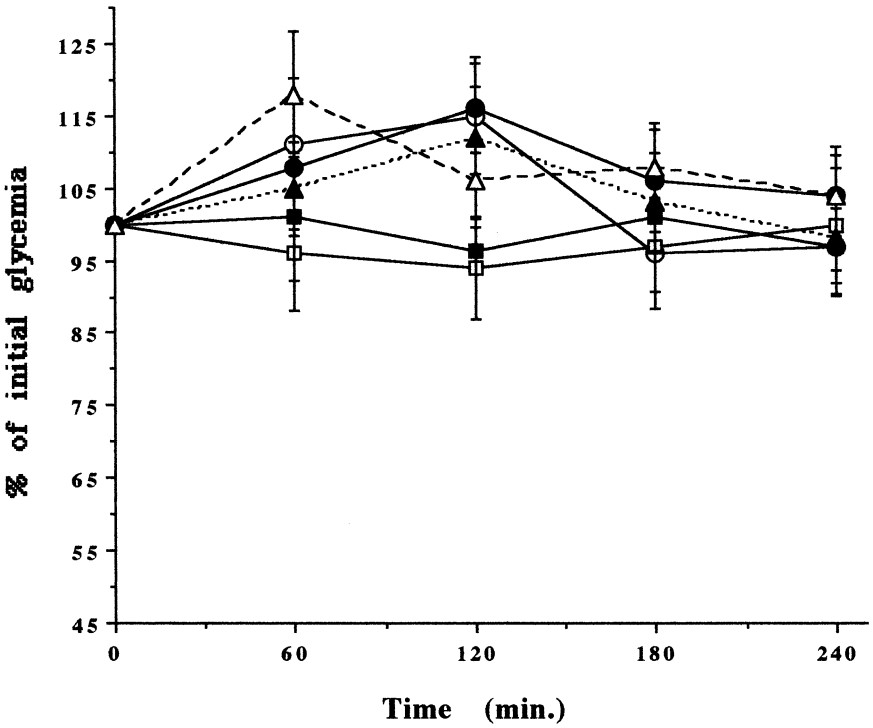

Fig. 4. Glycemia after oral administration of insulin solutions to fasted normal rats: (○) sol. 1; (●) sol. 2; (□) sol. 3; and (■) sol. 4. Controls: (△) zinc insulin solution and (▲) phosphate-buffered saline solution (PBS) (mean ± S.D., $n = 6$).

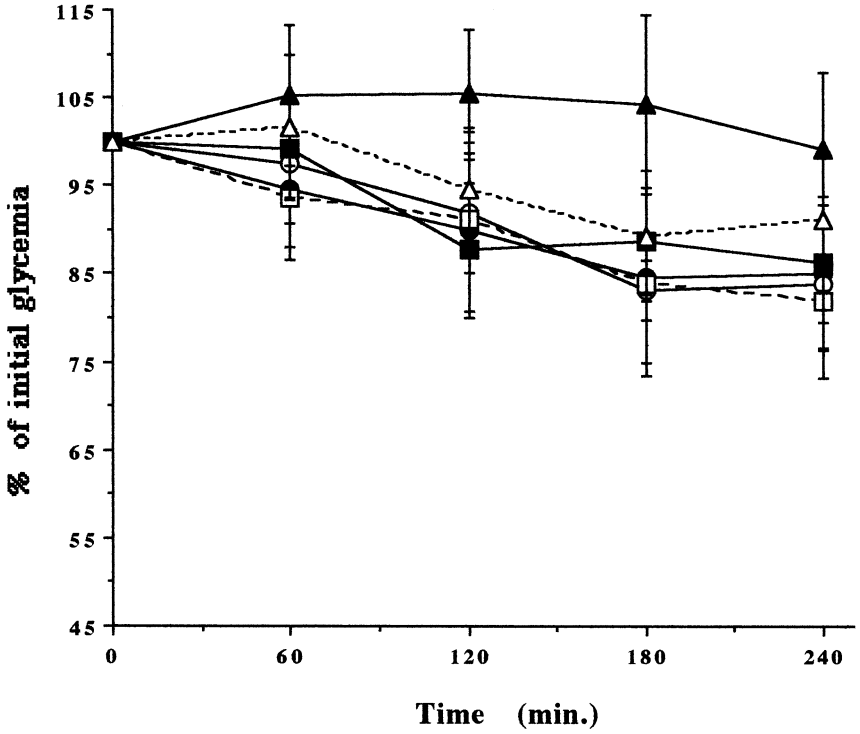

Fig. 5. Glycemia after oral administration of multiple emulsions to fasted normal rats: (○) ME1; (●) ME 2; (□) ME 3; and (■) ME 4. Controls: (▲) MEwi and (△) ME 5 (mean ± S.D., $n = 8$).

4. Discussion

In this study we examined w/o/w multiple emulsions, containing sodium insulin alone or with aprotinin (AP), or with sodium taurocholate (TC), or with both AP and TC, as an oral dosage form for insulin administration which could overcome the barriers against insulin absorption.

Several reports have shown that the ileum is the preferential site of insulin absorption in the gastrointestinal tract (Ziv et al., 1987; Bendayan et al., 1990; Morishita et al., 1993), but these results also suggested that at the ileum, even when the luminal enzymes are absent, insulin is still subject to enzymatic proteolytic degradation by the enzymes adsorbed at the surface of the mucosal layer. Thus, absorption of insulin, injected into the ileum loop after removal of the luminal enzymes, was strongly enhanced when insulin was coadministered with aprotinin. Inhibition of insulin degradation at the site of injection has been proposed as one possible means of enhancing its bioavailability. However, in our study, the difference between hypoglycemic effect obtained after administration of the multiple emulsion containing sodium insulin alone (ME 1) and that containing sodium insulin and aprotinin (ME 2 and 4) was not significant.

Bile salts have used to enhance insulin absorption by various routes of administration (Zhou and Po, 1991; Yamamoto et al., 1992). There are three major hypothesis to explain the mechanism of mucosal enhancement of protein absorption by bile salts: (i) the reduction of insulin degradation by the enzymes adsorbed within the mucosal layer (like aminopeptidase), (ii) the interaction of bile salts with cell membranes to form a reverse micelle which acts as a channel to increase membrane permeation, and (iii) the dissociation of molecular aggregates through micellar solubilization (Li et al., 1992; Yamamoto et al., 1994). In our study, the last hypothesis seems improbable

because no significant difference between the CD spectra analysis of the sodium insulin solutions was found. Thus, this result could indicate that addition of sodium taurocholate, at a concentration of 10 mM, to sodium insulin solution did not cause any significant change in the aggregation equilibrium of insulin molecules.

Furthermore, in our experiments, addition of sodium taurocholate to sodium insulin formulations (ME 3 and 4) seemed ineffective in enhancing intestinal absorption of insulin from multiple emulsions. In fact, no significant difference between hypoglycemic effects was observed after oral administration of the multiple emulsion containing sodium insulin alone (ME 1) or containing sodium insulin and sodium taurocholate (ME 3 and 4).

Moreover, the results obtained after subcutaneous administration of the sodium insulin solutions to normal rats indicated that the bioavailability of insulin was not changed by the addition of aprotinin and sodium taurocholate.

The hypoglycemic effects, obtained after subcutaneous administration to normal rats of the aqueous phase obtained from multiple emulsions diluted under different conditions, confirmed the results obtained in our in vitro investigations (Silva-Cunha et al., 1997b). Thus, in the present study, the main mechanism of solute release was a swelling–breakdown phenomenon occurring under hypo-osmotic conditions of dilution, and also confirmed, although no significant difference in hypoglycemia between the emulsions was noted, a tendency already observed in in vitro studies: the release of the encapsulated insulin was slightly increased from multiple emulsions containing sodium taurocholate.

The slight hypoglycemic effects obtained after oral administration of multiple emulsions containing insulin to normal rats when compared with effects with diabetic animals treated similarly could be explained by an autoregulation phenomenon in fasted normal rats because of their hypoglycemic condition (Damgé et al., 1988;

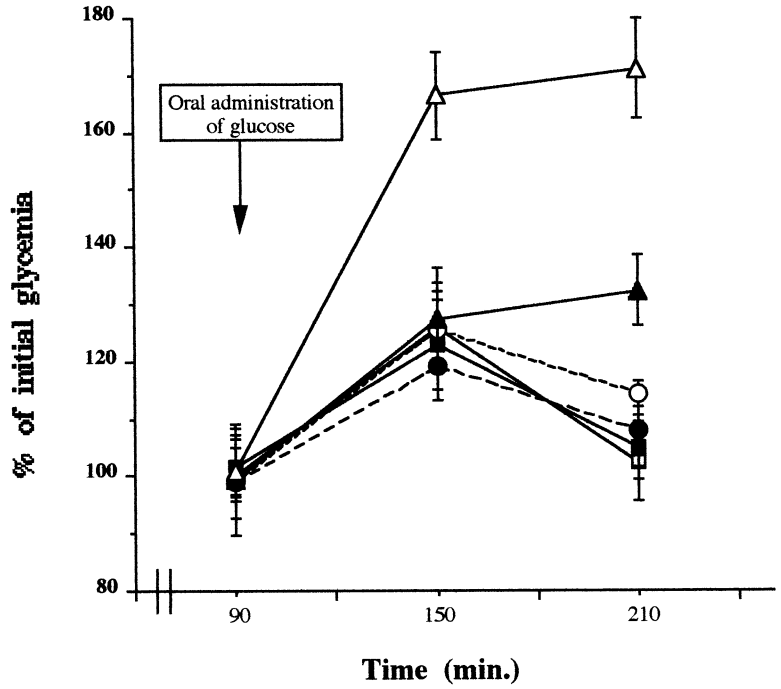

Fig. 6. Effect of oral administration of the multiple emulsions to fasted normal rats after administration of oral glucose given 90 min later: (○) ME1; (●) ME 2; (□) ME 3; and (■) ME 4. Controls: (△) phosphate-buffered saline solution (PBS) and (▲) ME 5 (mean ± S.D., $n = 8$).

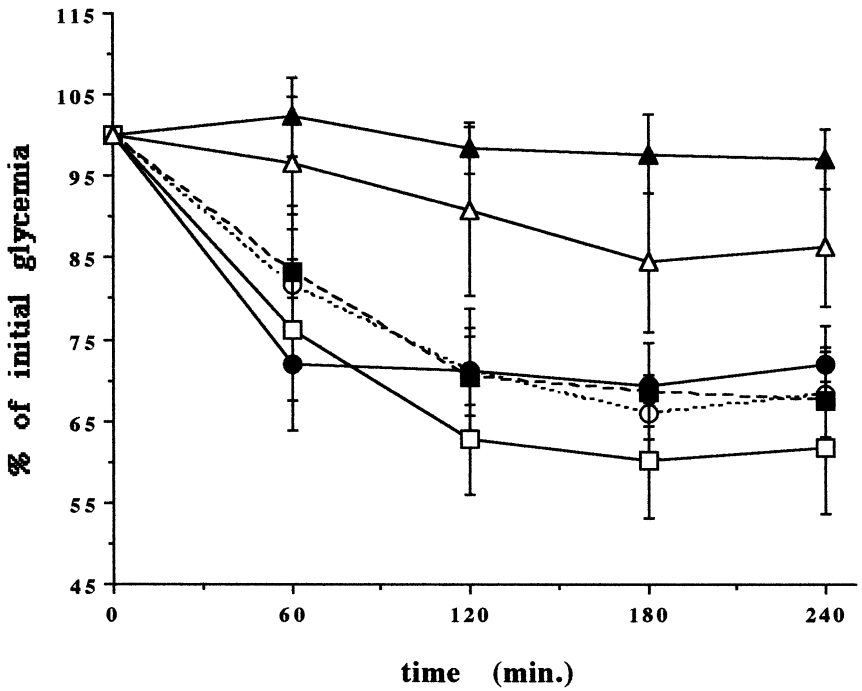

Fig. 7. Glycemia after oral administration of multiple emulsions to fasted diabetic rats: (○) ME1; (●) ME 2; (□) ME 3; and (■) ME 4 Controls: (▲) MEwi and (▽) ME 5 (mean ± S.D., $n = 8$).

Morishita et al., 1992). In fact, administration of glucose solution 90 min after emulsion administration seemed to eliminate this compensatory mechanism and a considerable hypoglycemic effect of the emulsions containing sodium insulin was observed.

After oral administration of the multiple emulsions to diabetics rats, a significant hypoglycemic effect was observed about 120 min after oral administration. One can speculate that the reason for this delay may be the progressive arrival of the emulsion droplets at the ideal site of insulin absorption in the gastrointestinal tract.

The results obtained after single oral administration to normal and diabetic rats showed that multiple emulsions containing sodium insulin alone (ME 1) was able to decrease blood glucose to the same level as multiple emulsions containing promoter adjuvants (ME 2–4). This observation seemed to suggest that the concentration of these adjuvants in the multiple emulsions was insufficient to increase insulin absorption. However, it was clearly evident that there was a significant difference between the hypoglycemic effects obtained with multiple emulsions containing sodium insulin and the multiple emulsion containing zinc insulin. It was also evident, after CD determinations, that there is a real difference in the association state of sodium insulin solution and zinc insulin solution. Thus, we could postulate, from this CD spectroscopy, that in all the sodium insulin solutions the largest aggregate was dimers.

Thus one can suggest that, with our multiple emulsions, the problem of low intestinal insulin bioavailability was partly resolved by reduction of insulin aggregation in aqueous media. As expected, the smaller molecular species (dimers) were more easily absorbed by the intestinal membrane.

In conclusion, the present work indicates that multiple emulsions containing sodium insulin (dimers) are able to reduce glycemia of diabetic rats. However, further studies, using a higher concentration of AP and TC, or using other adjuvants and/or using an insulin solution with the molecules completely dissociated (monomers), seem to be necessary to increase intestinal insulin absorption from these multiple emulsions.

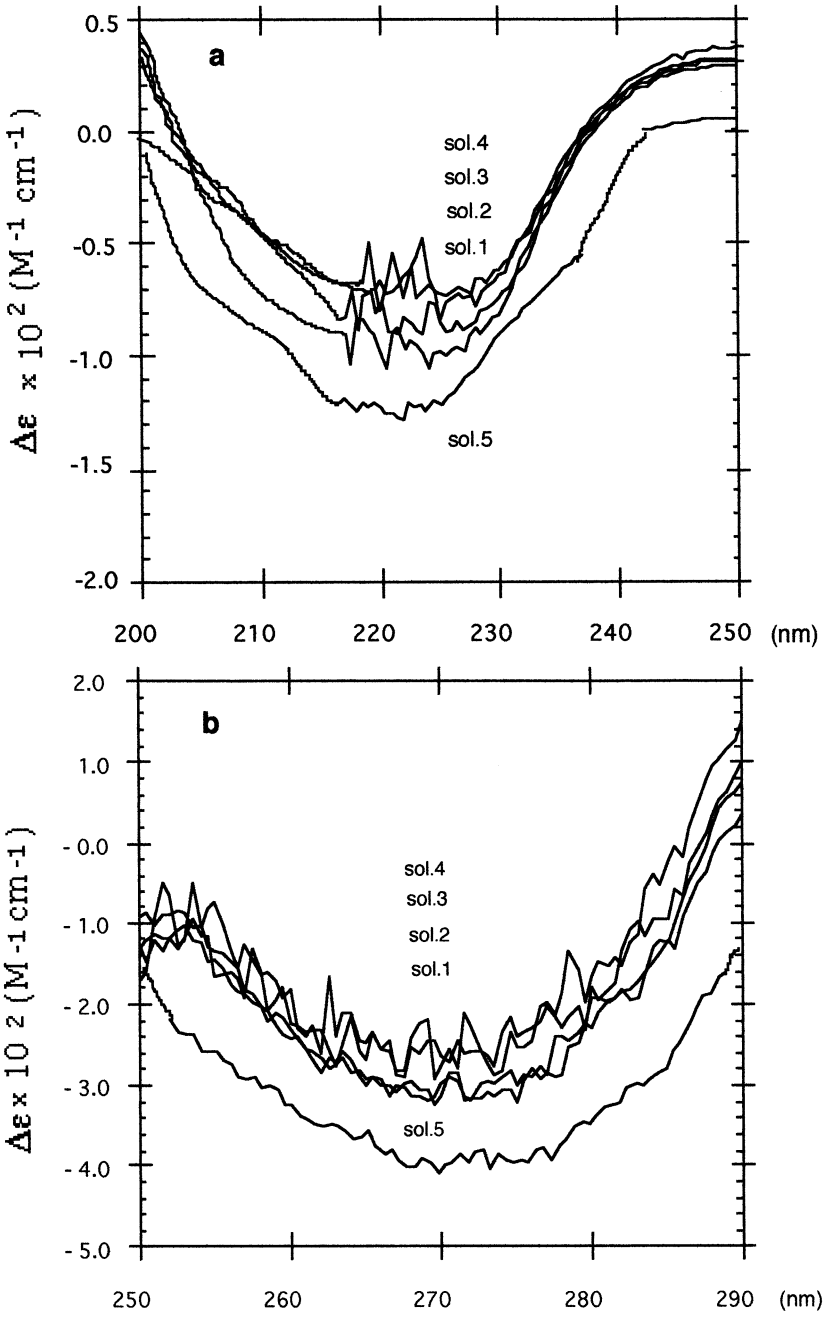

Fig. 8. (a,b) CD spectra of sodium (sol. 1–4) and zinc (sol. 5) insulin solutions.

Acknowledgements

We thank the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico-CNPq, Brazil, for supporting A.S.C. with a scholarship.

References

Aungst, B.J., Rogers, N.J., 1988. Site dependence of absorption-promoting actions of laureth-9, Na salicylate, Na_2EDTA, and aprotinin on rectal, nasal, and buccal insulin delivery. Pharm. Res. 5, 305–308.

Bendayan, M., Ziv, E., Ben-Sasson, R., Bar-On, H., Kidron, M., 1990. Morpho-cytochemical and biochemical evidence for insulin absorption by the rat ileal epithelium. Diabetologia 33, 197–204.

Damgé, C., Michel, C., Aprahamian, M., Couvreur, P., 1988. New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. Diabetes 37, 246–251.

Gwinup, G., Elias, A.N., Vaziri, N.D., 1990. A case for oral insulin therapy in the prevention of diabetic micro- and macro-angiopathy. Int. J. Artif. Organs 13, 393–395.

Hirai, S., Yashiki, T., Mima, H., 1981. Mechanisms for enhancement of the nasal absorption of insulin by surfactants. Int. J. Pharm. 9, 173–184.

Kidron, M., Bar-On, H., Berry, E.M., Ziv, E., 1982. The absorption of insulin from various regions of the rat intestine. Life Sci. 31, 2837–2841.

Li, Y., Shao, Z., Mitra, A.K., 1992. Dissociation of insulin oligomers by bile salt micelles and its effect on α-chymotrypsin-mediated proteolytic degradation. Pharm. Res. 9, 864–869.

Linde, B., Gunnaesson, R., 1985. Influence of aprotinin on insulin absorption and subcutaneous blood flow in type 1 (insulin-dependent) diabetes. Diabetologia 28, 645–648.

Liu, F., Kildsig, D.O., Mitra, A.K., 1991. Insulin aggregation in aqueous media and its effect on alpha-chymotrypsin-mediated proteolytic degradation. Pharm. Res. 8, 925–929.

Morishita, I., Morishita, M., Takayama, K., Machida, Y., Nagai, T., 1992. Hypoglycemic effect of novel oral microspheres of insulin with protease inhibitor in normal and diabetic rats. Int. J. Pharm. 78, 9–16.

Morishita, I., Morishita, M., Takayama, K., Machida, Y., Nagai, T., 1993. Side-dependent effect of aprotinin, sodium caprate, Na_2EDTA and sodium glycocholate on intestinal absorption of insulin Biol. Pharm. Bull. 16, 68–72.

Nordestgard, B.G., Agerholm-Larsen, B., Stender, S., 1997. Effect of exogenous hyperinsulinaemia on atherogenesis in cholesterol-fed rabbits. Diabetologia 40, 512–520.

Shao, Z., Li, Y., Krishnamoorthy, R., Chermak, T., Mitra, A.K., 1993. Differential effects of anionic, cationic, nonionic, and physiologic emulsifiers on the dissociation, α-chymotryptic degradation, and enteral absorption of insulin hexamers. Pharm. Res. 10, 243–251.

Silva-Cunha, A., Grossiord, J.L., Puisieux, F., Seiller, M., 1997a. Insulin in W/O/W multiple emulsions: biological activity after administration in normal and diabetic rats. J. Microencapsulation 14, 321–333.

Silva-Cunha, A., Grossiord, J.L., Puisieux, F., Seiller, M., 1997b. 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. Int. J. Pharm. 158 (1), 79–89.

Yamamoto, A., Hayakawa, E., Kato, Y., Nishiura, A., Lee, V.H.L., 1992. A mechanistic study on enhancement of rectal permeability to insulin in the albino rabbit. J. Pharmacol. Exp. Ther. 263, 25–31.

Yamamoto, A., Taniguchi, T., Rikyuu, K., Tsuji, T., Fujita, T., Murakami, M., Muranishi, S., 1994. Effects of various protease inhibitors on the intestinal absorption and degradation of insulin in rats. Pharm. Res. 11, 1496–1500.

Zhou, X.H., Po, A.L.W., 1991. Effects of cholic acid and other enhancers on the bioavailability of insulin from a subcutaneous site. Int. J. Pharm. 69, 29–41.

Ziv, E., Lior, O., Kidron, M., 1987. Absorption of protein via the intestinal wall. A quantitative model. Biochem. Pharmacol. 36, 1035–1039.