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Hypoglycemic effect of novel oral microspheres of insulin with protease inhibitor in normal and diabetic rats

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Summary

The effectiveness of an oral administration of insulin microspheres (IMS) containing a protease inhibitor was evaluated in normal and diabetic rats. The dosage form is based on the incorporation of insulin with protease inhibitor into polyacrylic polymer (Eudragit L100). These preparations were administered orally with a 20 U/kg insulin dose by force-feeding to rats. Insulin absorption was evaluated by its hypoglycemic effect. IMS without protease inhibitor and with trypsin inhibitor (TI) or chymostatin (CS) produced no marked hypoglycemic response in both groups of rats. A significant continuous hypoglycemic effect was obtained after oral administration of IMS containing aprotinin (AP) or Bowman-Birk inhibitor (BBI) in both normal and diabetic rats when compared with controls. To calculate the relative efficacy, log dose/effect curves for i.v. insulin were obtained in both groups of rats. The efficacy of oral administration, relative to i.v., was assessed by measuring the cumulative percentage of change in serum glucose levels during the experimental period. The efficacy order of four protease inhibitors incorporated into IMS was AP $\geq$ BBI > CS=TI. The results suggest that the use of protease inhibitors as pharmaceutical adjuvants for IMS has the advantage of enhancing the efficacy of insulin.

Introduction

Routes of insulin administration favoring initial delivery to the liver, a target organ, are said to be more efficacious than others (Ritschel and Ritschel, 1984). Also, the importance of insulin delivery via the hepatic portal vein has been indicated for normalizing both blood glucose and insulin levels in the postprandial state (Goriya et al., 1980; Ritschel and Ritschel, 1984). An oral insulin administration could have an advantage by achieving portal insulin delivery in a convenient way. The development of an oral dosage form providing adequate bioavailability of insulin would revolutionize the treatment of diabetes.

The problems encountered with oral dosage forms of insulin are rapid enzymatic degradation in the gastrointestinal tract and poor membrane

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permeability. In order to develop an oral insulin dosage form, it is necessary that these enzymes be inhibited. Recent studies have shown that some protease inhibitors amplify the biological effect of insulin injected directly into the lumen of the intestine (Kidron et al., 1982; Fujii et al., 1985; Ziv et al., 1987). These studies suggested that it was feasible to absorb insulin from the intestine in the presence of the protease inhibitor.

As a new pharmaceutical approach to designing an oral dosage form of insulin, Eudragit L100 insulin microspheres (IMS) containing a protease inhibitor have been developed (Morishita et al., 1992). In a previous in vitro investigation, we found that the enteric polymer coating protected insulin from pepsinic degradation, and IMS containing a protease inhibitor could protect insulin against trypsinic and/or α-chymotrypsinic degradation. The purpose of this study was to estimate the biological efficacy of IMS containing a protease inhibitor after oral administration in normal and diabetic rats. In this study, serum glucose level was determined as a measure of insulin efficacy. The biological efficacy of insulin following oral administration of IMS was evaluated by comparison with the i.v. route.

Materials and Methods

Materials

Crystalline bovine insulin (Zn-insulin, 24.4 U/mg), aprotinin, soybean trypsin inhibitor, Bowman-Birk inhibitor and streptozotocin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Eudragit L100 was a gift from Higuchi Co., Ltd (Tokyo, Japan). Gelatin and a glucose B-Test kit (glucose oxidase method) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Chymostatin was obtained from the Peptide Institute Inc. (Osaka, Japan). All other agents were of analytical grade.

Preparations

IMS examined in this study were prepared according to the method reported by Morishita et al. (1991). Briefly, the procedure was as follows: weighed amounts of insulin with or without protease inhibitor were dissolved in 300 μl of 0.1 N HCl and then ethanol and Eudragit L100 were added with stirring at 1200 rpm. The resultant solution was poured into liquid paraffin with stirring at 1200 rpm for emulsification. By adding a gelatin solution (0.5% w/w) to the emulsion, Eudragit L100 was solidified and the microspheres were obtained. These microspheres were further coated with Eudragit L100. Thus, five types of microspheres containing insulin (2% w/w) alone and with each inhibitor (1% w/w) were prepared. Microspheres composed of only Eudragit L100 were also prepared for control experiments. Microspheres were sized by sieving and the fraction ranging from 180 to 500 μm was used for the study. All batches were prepared at least three times.

For intravenous dosing, solutions were prepared by dissolving an appropriate amount of crystalline bovine insulin in 0.1 M phosphate buffer, pH 7.4.

Animal experimental procedures

Animal experimental design and the summarized data observed in normal and diabetic rats are detailed in Table 1. Male Wistar rats were used in this study. Diabetes was induced in rats by an intraperitoneal injection of streptozotocin (once daily injection of 40 mg/kg body weight for 3 consecutive days) dissolved in citrate buffer at pH 4.5 (Liu et al., 1988). They were considered as diabetics when fasted glycemia was > 250 mg/dl at 2 weeks after streptozotocin treatment.

Both normal and diabetic rats were fasted at least 16 h before experiments, and were allowed water ad libitum. IMS containing protease inhibitor (20 U total insulin/kg body weight) were administered orally by force-feeding with 1 ml of water via a rubber tube under non-anesthesia. Rats were restrained in a supine position during administration and at each blood sampling. Approx. 5 min before insulin dosing, a 0.2 ml aliquot of blood sample was taken from the jugular vein. Subsequent blood samples were taken every 2 h for a total time period of 10 h. In order to calculate the efficacy of the oral route of insulin administration relative to i.v., insulin solution was administered intravenously via the jugular vein.

TABLE 1

Animal experimental design and summarized data observed in normal and diabetic rats

Preparation	No. of rats		Body weight (g)		Initial serum glucose level (mg/dl)		Insulin dose (U/kg)	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
i.v.								
0.25	–	3	–	199.7 ± 3.5	–	302.2 ± 10.1	–	0.25
0.5	4	3	198.5 ± 3.7	205.0 ± 7.6	89.6 ± 5.6	326.2 ± 32.5	0.5	0.5
1.0	4	3	202.8 ± 3.0	200.8 ± 5.6	85.7 ± 6.6	291.5 ± 29.7	1.0	1.0
1.5	3	–	206.0 ± 2.1	–	87.3 ± 3.6	–	1.5	–
p.o.								
Control	8	7	212.5 ± 3.5	220.0 ± 9.4	94.9 ± 3.2	367.2 ± 27.5	–	–
IMS without inhibitor	8	7	211.3 ± 4.2	202.9 ± 5.2	94.8 ± 6.3	349.0 ± 19.4	20.0	20.0
IMS containing TI	8	7	210.8 ± 3.2	196.0 ± 4.3	90.7 ± 2.9	374.5 ± 34.1	20.0	20.0
IMS containing CS	8	7	213.8 ± 4.2	190.7 ± 3.9	84.2 ± 1.9	312.8 ± 27.8	20.0	20.0
IMS containing AP	8	7	206.4 ± 4.7	206.4 ± 8.3	98.8 ± 4.0	324.7 ± 21.4	20.0	20.0
IMS containing BBI	8	7	201.3 ± 5.3	205.6 ± 6.3	99.1 ± 4.1	340.4 ± 34.6	20.0	20.0

Results represent means ± S.E.

The insulin i.v. doses were 0.5, 1.0 and 1.5 U/kg body weight to normal rats and 0.25, 0.5 and 1.0 U/kg body weight to diabetic rats. A 0.2 ml aliquot of blood sample was collected from the jugular vein on the opposite side to the injection before and at 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 h after dosing. Serum was separated by centrifugation at 3000 rpm for 3 min and kept frozen until analysis. Serum glucose level was determined enzymatically using the glucose B-Test kit.

The absorption of intact biologically active insulin was evaluated by measuring the hypoglycemic effect. The hypoglycemic response to insulin was characterized as follows: serum glucose levels after insulin administration were expressed as a percentage of the initial level. The percentage of change in serum glucose was taken as the percentage of the initial level subtracted from 100. From the percentage of change vs time curve for 0–10 h, the cumulative percentage of change was calculated by summing the areas below baseline levels (negative values) using the trapezoidal method. In this experiment, the areas above baseline levels (positive values) were not included in counting.

Statistical analysis

Each value was expressed as the mean ± S.E. of the mean. For group comparisons, an analysis of variance (ANOVA) with one-way layout was applied followed by Student's unpaired *t*-test. A p value of 0.05 or less was considered significant.

Results and Discussion

Hypoglycemic effect of IMS administered orally

Average serum glucose level vs time profiles after oral administration of these microspheres to normal rats are shown in Fig. 1. In control rats administered insulin-free microspheres, the serum glucose levels were higher than the initial level during the experimental period. This increase is considered to be due to stress provoked by force-feeding and blood sampling under non-anesthetized conditions (Kahn and Shechter, 1990).

The profiles of serum glucose levels after the administration of IMS without inhibitor were similar to those of the control microspheres, suggesting that IMS without inhibitor could not protect insulin from degradation by proteolytic enzymes in the gastrointestinal tract. Moreover, administration of IMS containing TI or CS produced no hypoglycemic response. On the other hand, a small but significant decrease in levels was brought about by the administration of IMS containing AP. The hypoglycemic effect was maintained during the experimental period and serum glucose levels did not return to the baseline levels in six out of eight rats. In two rats, serum glucose levels returned to the baseline levels at 8 h after administration. The continuous hypoglycemic effect may be explained by the progressive arrival of IMS from the stomach in the gut. In another dissection experiment, we found that administered IMS could remain in the stomach after 6 h. The profiles of serum glucose levels after the oral administration of IMS containing BBI were simi-

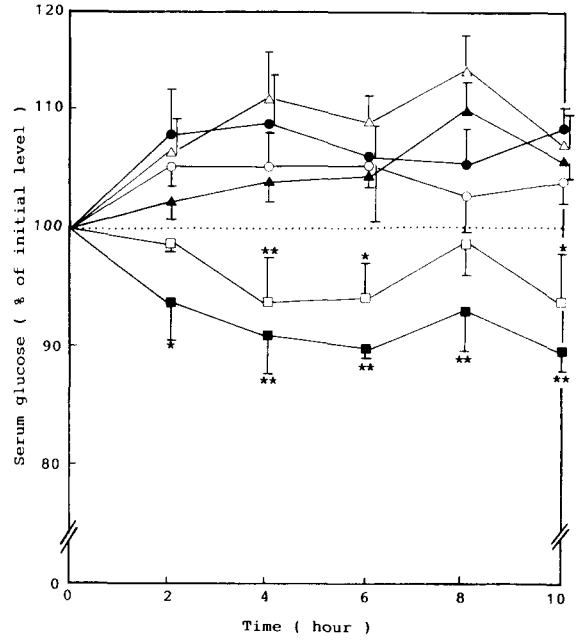

Fig. 1. Hypoglycemic effect of insulin microspheres administered orally to normal rats. (●) Control; (○) IMS without protease inhibitor; (▲) IMS containing TI; (△) IMS containing CS; (■) IMS containing AP; (□) IMS containing BBI. Each point represents the mean ± S.E. of 8 animals. Comparisons calculated at each period against controls: * $p < 0.05$, ** $p < 0.01$.

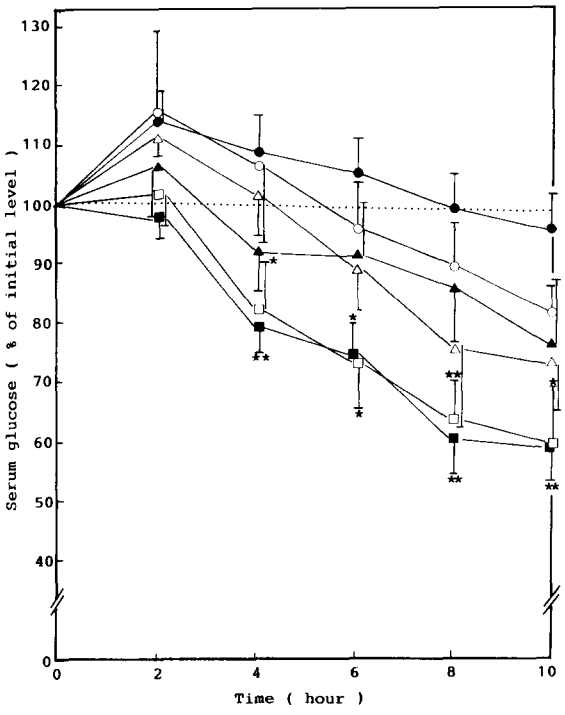

Fig. 2. Hypoglycemic effect of insulin microspheres administered orally to diabetic rats. (●) Control; (○) IMS without protease inhibitor; (▲) IMS containing TI; (△) IMS containing CS; (■) IMS containing AP; (□) IMS containing BBI. Each point represents the mean ± S.E. of seven animals. Comparisons calculated at each period against controls: * $p < 0.05$, ** $p < 0.01$.

lar to those of IMS containing AP. However, the intensity of the hypoglycemic effect was lower than that of IMS containing AP.

Fig. 2 shows average serum glucose level vs time profiles after oral administration to diabetic rats. In diabetic rats, the mean serum glucose levels of IMS without inhibitor tended to be lower than control levels. Also, IMS containing TI or CS caused a slight decrease in serum glucose levels without significant difference compared to the control. Similar to the results in normal rats, IMS containing AP and BBI displayed significant hypoglycemic effects. Continuous decreases in serum glucose to the control were observed from 2 h after administration of both preparations. At the end of the experimental period, the percentages of decrease in serum glucose levels of IMS without inhibitor, and IMS containing TI, CS, BBI and AP were -18.6 ± 3.0, -23.5 ± 11.7, -25.8 ± 9.3, -38.9 ± 8.8 and $-39.1 \pm 6.3\%$, respectively.

Generally, the serum glucose levels vs time profiles of these preparations observed in diabetic rats differed compared to those of normal rats. In normal state, the phenomenon of autoregulation of insulin secretion by exogenous insulin is well known. Moreover, the fall in blood glucose induced significant responses of counter-regulatory hormones such as glucagon, growth hormone and cortisol (Paquot et al., 1988). In particular, glucagon plays a major role in the recovery from hypoglycemia in the normal state. In contrast, these phenomena do not occur in diabetic rats. On the assumption that the intestinal absorption of insulin differs little between normal and diabetic rats, the marked change in serum glucose levels observed in diabetic rats may be attributed to the absence of these phenomena. In normal rats, even when the same amount of insulin is absorbed as in diabetic rats, it is presumably not enough to produce dramatic changes.

TI (Birk, 1985) and CS (Umezawa et al., 1970) are specific trypsin and α-chymotrypsin inhibitors, respectively. Recent studies have reported their promoting effects on the intestinal absorption of insulin (Kidron et al., 1982; Fujii et al., 1985; Ziv et al., 1987). In fact, it had been observed that among the preparations used in this study, IMS containing TI or CS showed the most potent insulin protective efficiency against trypsinic or α-chymotrypsinic degradation, respectively (Morishita et al., 1992). Nevertheless, we failed to observe any marked hypoglycemic effect of oral IMS containing TI or CS in both normal and diabetic rats. On the other hand, BBI (Birk, 1985) and AP (Trautschold et al., 1967) inhibit both trypsin and α-chymotrypsin. In this study, only IMS containing AP or BBI promoted the hypoglycemic effect clearly in both normal and diabetic rats. Since the dose of each protease inhibitor was equal in this experiment, the results suggest that trypsin-chymotrypsin inhibitors such as AP or BBI can efficiently protect insulin from proteolytic degradation in the gastrointestinal tract.

Relationship between i.v. insulin dose and hypoglycemic effect

Accurate assessment of the efficacy of insulin requires definition of the dose/response profile by a standard administration route. We have assessed the efficacy of oral insulin administration compared to that of the i.v. route using response measurement. Fig. 3 shows average serum glucose level vs time profiles after intravenous administration of insulin at several doses to normal and diabetic rats. In normal rats, with increasing insulin doses, the maximum hypoglycemic effects increased. The times of the nadir were observed to occur in the first 15–30 min for all doses. In diabetic rats, the time of the nadir were retarded and the maximum hypoglycemic effect was greater than in the case of the same doses in normal rats. The relationship between the logarithm of intravenous insulin doses and the average values of cumulative percentages of change in serum glucose levels is shown in Fig. 4. The cumulative percentages of change in serum glucose levels were calculated by summing the area below the baseline from the results demonstrated in Fig. 3. Equations of linear regression of the average values of cumulative percentages of change vs logarithm of doses in normal and diabetic rats are shown in Fig. 4. The equations were rearranged so that the intravenous dose giving an equivalent hypoglycemic response to that after the oral administration could be calculated. Then, relative efficacy (% of i.v.) could be obtained from the percentage ratio of calculated dose to actual oral dose.

Relative efficacy of oral IMS to i.v. insulin

To determine the relative efficacy of each preparation to i.v. insulin in normal and diabetic rats, the cumulative percentages of change in serum glucose levels were calculated from the

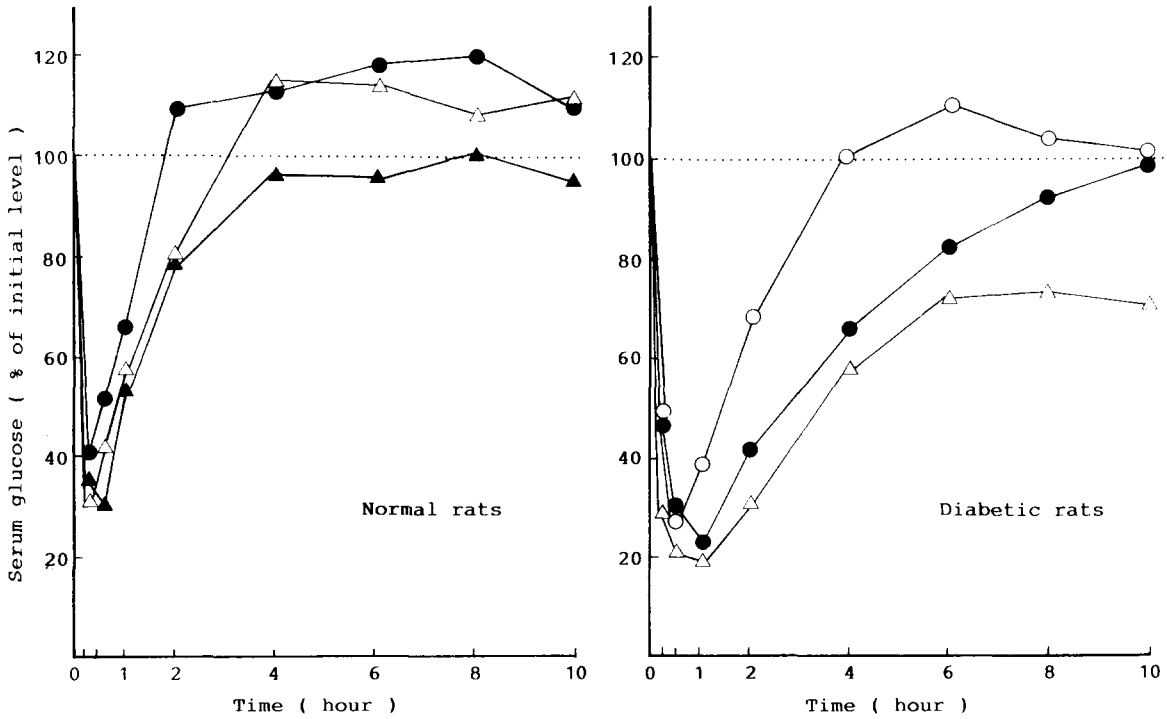

Fig. 3. Serum glucose levels normalized to percentage of the initial level after i.v. administration of insulin in doses of 0.25 U/kg (○), 0.5 U/kg (●), 1.0 U/kg (△) or 1.5 U/kg (▲) in normal and diabetic rats. Each point represents the mean of 3–5 animals for normal rats and 3–4 animals for diabetic rats.

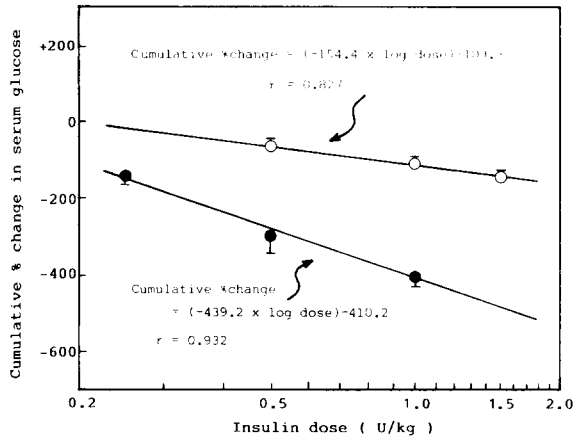

Fig. 4. Relationship between i.v. insulin dose and efficacy, expressed as the cumulative percentage of change in serum glucose level. The cumulative percentage of change was calculated by summing the areas below baseline levels. Results are means ± S.E. from normal rats (○———○, $n = 3$–4) and diabetic rats (●———●, $n = 3$).

results demonstrated in Figs 1 and 2, respectively. Rats with values of overall positive cumulative percentage of change were assigned relative efficacy values of zero. The results are given in Table 2. As shown in Figs 1 and 2, apparently, greater hypoglycemic effects were observed in diabetic rats than in normal rats. However, it should be noted that the degrees of efficacy did not differ between normal and diabetic states (Table 2). The mean relative efficacy values of IMS containing AP in diabetic rats were lower than those determined in normal rats, however, the difference did not reach a statistically significant level.

As shown in Table 2, it is clearly evident that the presence of protease inhibitor, AP or BBI, enhanced the relative efficacy of oral insulin administration. In particular, IMS containing AP tended to be more effective than IMS containing BBI in normal rats. These results agree well with those in the preceding article in which it is demonstrated that the potency of IMS containing AP against trypsin and α-chymotrypsin is higher than that of IMS containing BBI (Morishita et al., 1992). In diabetic rats, there was no apparent difference between the mean relative efficacy values of IMS containing AP and BBI. However, a large inter-individual variation was noted in the hypoglycemic responses of IMS containing BBI.

The strategy which utilizes protease inhibitors such as AP or BBI to circumvent insulin degradation by pancreatic enzymes may be useful for enhancing the efficacy of oral insulin administration. A concern with using protease inhibitors to promote the oral absorption of insulin is their possible effect on the process of digestion of dietary proteins. As much as 200–800 μg of trypsin and chymotrypsin is present in the human duodenum shortly after feeding and contributes to digestion (Lee et al., 1991). The approach of coadministration with protease inhibitors, of course, must be safely achieved without modifying such a physiological digestive process.

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TABLE 2

Efficacy of oral administration of insulin microspheres as a percentage of i.v. insulin efficacy

Treatment	Efficacy relative to i.v. (%)	
	Normal rats	Diabetic rats
IMS without inhibitor	0.9 ± 0.3 (0 –2.1)	1.1 ± 0.2 (0.7–2.1)
IMS containing TI	0.5 ± 0.2 (0 –1.1)	1.2 ± 0.4 (0 –3.2)
IMS containing CS	0.6 ± 0.2 (0 –1.3)	1.4 ± 0.5 (0 –3.8)
IMS containing AP	3.4 ± 0.6 [b] (2.0–7.1)	2.1 ± 0.4 [b] (1.0–3.6)
IMS containing BBI	2.1 ± 0.4 [a] (1.0–3.7)	2.0 ± 0.6 [a] (0.8–4.6)

Each value represents the means ± S.E. The range of efficacy is indicated in parentheses. Asterisks denote significant difference from IMS without protease inhibitor ([a] $p < 0.05$, [b] $p < 0.01$).

References

Birk, Y., The Bowman-Birk inhibitor. *Int. J. Peptide Protein Res.*, 25 (1985) 113–131.

Fujii, S., Yokohama, T., Ikegawa, K., Sato, F. and Yokoo, N., Promoting effect of the new chymotypsin inhibitor FK-448 on the intestinal absorption of insulin in rats and dogs. *J. Pharm. Pharmacol.*, 37 (1985) 545–549.

Goriya, Y., Bahoric, A., Marliss, E.B., Zinman, B. and Alibisser, A.E., Glycemic regulation and insulin clearance in unrestrained diabetic dogs portally infused with a portable insulin delivery system. *Diabetologia*, 19 (1980) 452–457.

Kahn, C.R. and Shechter, Y., Insulin, oral hypoglycemic agents, and the pharmacology of the endocrine pancreas. In Gilman, A.G., Rall, T.W., Nies, A.S. and Taylor, P. (Eds), *The Pharmacological Basis of Therapeutics*, 8th Edn, Pergamon, New York, 1990, pp. 1463–1495.

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

Lee, V.H.L., Dodda-Kashi, S., Grass, G.M. and Rubas, W., Oral route of peptide and protein drug delivery. In Lee, V.H.L. (Ed.), *Peptide and Protein Drug Delivery*, Dekker, New York, 1991, pp. 691–738.

Liu, J.C., Sun, Y., Siddiqui, O., Chien, Y.W., Shi, W.M. and Li, J., Blood glucose control in diabetic rats by transdermal iontophoretic delivery of insulin. *Int. J. Pharm.*, 44 (1988) 197–204.

Morishita, I., Morishita, M., Machida, Y. and Nagai T., Controlled release microspheres based on Eudragit L100 for the oral administration of erythromycin. *Drug Des. Delivery*, 7 (1991) 309–319.

Morishita, M., Morishita, I., Takayama, K., Machida, Y. and Nagai, T., Novel oral microspheres of insulin with protease inhibitor protecting from enzymatic degradation. *Int. J. Pharm.*, 78 (1992) 1–7.

Paquot, M., Scheen, A.J., Franchimont P. and Lefebvre, P. J., The intra-nasal administration of insulin induces significant hypoglycemia and classical counterregulatory hormonal responses in normal man. *Diabetes Metab.*, 14 (1988) 31–36.

Ritschel, W.A. and Ritschel, G.E., Rectal administration of insulin. *Methods Find. Exp. Clin. Pharmacol.*, 6 (1984) 513–529.

Trautschold, I., Werle, E. and Zickgraf-Rüdel, G., Trasylol. *Biochem. Pharmacol.*, 16 (1967) 59–72.

Umezawa, H., Aoyagi, T., Morishima, H., Kunimoto, S., Matsuzaki, M., Hamada, M. and Takeuchi, T., Chymostatin, a new chymotrypsin inhibitor produced by actinomycetes. *J. Antibiot.*, 23 (1970) 425–427.

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