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Effects of Polyacrylic Polymers on the Degradation of Insulin and Peptide Drugs by Chymotrypsin and Trypsin

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Abstract

The purpose of this study was to determine whether carbopol polymers, polyacrylic acid polymers, can inhibit lumenal degradation of insulin, calcitonin and insulin-like growth factor I (IGF-I) by trypsin and chymotrypsin and to understand whether reducing the pH of the incubation medium by these polymers results in inhibition. Further, the effects of carbopol polymers on the in-situ absorption of insulin were studied in rats.

In saline, carbopol polymers at 1% and 4% (w/v%) inhibited close to 100% of trypsin and chymotrypsin activities against insulin. In 50 mM Tris buffer, carbopol polymers, including 934P, 974P and 971P, at 0·1% only weakly inhibited degradation of calcitonin and insulin by both enzymes; however, as the polymer concentration increased to 0·4%, degradation of insulin, calcitonin, and IGF-I by both enzymes was complete or almost complete. When the Tris buffer was increased to 100 mM, no inhibition was observed at 0·1%. Determination of the final pH of the incubation medium in the presence of polymers revealed that the inhibitory effects of carbopol polymers correlated with the final pH. When the incubation medium has no or low buffer capacity to buffer the protons released by carbopol polymers, these polymers are able to reduce the pH much lower than the optimum pH for the enzyme activities, and thus inhibit proteolytic degradation. When the buffer capacity of the incubation medium increases, the inhibitory effects of carbopol polymers weaken. In-situ absorption of insulin revealed that carbopol polymers improved insulin absorption and induced a significantly greater decline in blood glucose levels.

It is concluded that carbopol polymers with strong bioadhesive properties also can inhibit lumenal degradation of peptide hormones, offering multiple advantages for their uses in oral drug delivery.

Oral administration is most convenient in treating diseases. Insulin is used to treat Type I diabetes mellitus, and oral delivery is most ideal for the treatment of chronic diabetes. Further, oral small chronic doses of insulin has recently been shown to prevent diabetes in animals (Vlahos et al 1991). Currently, clinical trials are being conducted by The National Institutes of Health throughout the United States, testing whether oral insulin can prevent diabetes (Anon 1994). Calcitonin, a drug for treating senile and post-menopausal osteoporosis, has a wide therapeutic window, and is a good candidate for oral delivery. Oral delivery of insulin-like growth factor I, with potential uses in controlling insulin-resistant diabetes and stimulating bone growth, is also important.

It has long been recognized that lumenal degradation of peptide/protein hormones by pancreatic enzymes is one of the barriers to oral absorption. Co-administration of inhibitors, structural modification, and use of formulations such as nanocapsules seem to be prevailing approaches, in attempts to improve oral absorption (Lee et al 1991; Michel et al 1991; Morishita et al 1993). However, little success has been achieved. Further, absorbable inhibitors very likely cause systemic side effects. Recently, it has been reported that Carbopol 934P, a bioadhesive polyacrylic acid polymer, inhibits trypsin activities against its standard substrates and dDAVP, a vasopressin analogue, by depriving trypsin of Ca^{2+} (Lehr et al 1992; Leußen et al 1994). Carbopol 934P has been used in oral formulations (Gurny et al 1984; Smart et al 1984). Since polyacrylic polymers are bioadhesive large molecules and not absorbed through the intestinal epithelium, they are unlikely to cause any systemic toxicity, and can thus offer great advantages for improving oral absorption of peptide hormones.

Conceivably, carbopol polymers, which are polyacrylic polymers with numerous carboxylic acid groups, can release numerous protons in the intestinal lumen, creating a local temporary acidic shield and thus protecting peptide and protein drugs from being attacked by lumenal enzymes. In this acidic environment, activities of intestinal lumenal enzymes, pancreatic enzymes which usually have an optimum pH greater than 7·5 (Lee et al 1991), will be much lower or negligible. This report studies this proposed mechanism and summarizes the effects of carbopol polymers on the degradation of insulin, calcitonin, and insulin-like growth factor I by chymotrypsin and trypsin, and their effects on intestinal absorption of insulin.

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Materials and Methods

Materials

[^{125}I] Insulin-like growth factor (IGF-I) was obtained from New England Nuclear (Boston, MA). [^{125}I] Salmon calcitonin and [^{125}I] (A14)-human recombinant insulin was obtained from Amersham Corporation (Arlington Heights, IL). Chymotrypsin (from bovine pancreas), trypsin (from bovine

pancreas), trichloroacetic acid (TCA), sodium taurocholate, sodium glycodeoxycholate, sodium deoxycholate, sodium glycocholate, sodium taurodeoxycholate, and pentobarbitone were obtained from Sigma Chemical Co. (St Louis, MO). Dye reagent and bovine γ-globulin for the protein assay were obtained from Bio-Rad Laboratories (Richmond, CA). Insulin RIA kit was obtained from Diagnostic Products Corporation (Los Angeles, CA). Accu-Chek-III for testing blood glucose levels was obtained from Boehringer Mannheim (Indianapolis, IN). Zinc-free human recombinant insulin was a gift from Dr. John Wang (Scios Nova Inc., CA). Carbopol 974P, 934P, 971P were obtained from B.F. Goodrich (Cleveland, OH). All other chemical reagents and buffer components were of analytic grade. All chemicals were used as obtained.

Animals

Male Sprague-Dawley rats, 250–300 g, were used as the animal model for in-situ studies. The number of measurements was 3 for each experiment and the reported data represent mean ± s.e.

Experimental procedure

Effects of bile salts and carbopol polymers on the degradation of peptide hormones by chymotrypsin and trypsin. Effects of bile salts and carbopol polymers on the degradation of peptide hormones by chymotrypsin and trypsin were studied at 37°C using the TCA (trichloroacetic acid) method (Bai et al 1995). The Tris buffer consisting of 50 mM Tris/HCl buffer (pH 7·5 at 37°C), 1% BSA (w/v) and 125 mM NaCl was used. Each substrate solution containing insulin or calcitonin or insulin-like growth factor I and each enzyme solution containing chymotrypsin or trypsin were prepared in Tris buffer. Then, in controls, a substrate solution of 269 μL and and enzyme solution of 19 μL were mixed with 32 μL Tris buffer so that the final concentrations of substrate and enzyme were 30 pM ^{125}[I] (A14)-insulin or 45 pM [^{125}I] insulin-like growth factor I or 50 pM [^{125}I] calcitonin, and 0·1 mg mL^{-1} chymotrypsin or trypsin, respectively. In experimental groups, 32 μL inhibitor solution in Tris buffer was used to replace the 32 μL Tris buffer in the incubation mixture. The inhibitors studied included bile salts and carbopol polymers. Bile salts have been shown to inhibit proteolytic activities, and were thus tested to determine whether they extensively inhibit lumenal degradation in the small intestine (Bai 1994). Each bile salt was tested at 2 mM and each carbopol polymer was tested at 0·1, 0·4 and 0·5% (w/v). Hydrolysis was initiated by incubating the mixture in a 37°C water bath. Incubation periods were 0, 2, 10, 30, 60 min for insulin, 0, 2, 10, 30, 60, 90 min for IGF-I and 0, 0·5, 1·5, 3, 5 min for calcitonin. The 200 μL 15% TCA was added to 200 μL of incubation mixture to stop proteolysis. The final TCA concentration and pH in the mixture were 7·5% and less than 1, respectively. The resulting mixture was then centrifuged at 3000 *g* for 10 min. The degraded fragments of peptide hormones remained soluble in the TCA solution; the radioactivity of the supernatant was measured using a γ-counter. The supernatant radioactivity was transformed to the concentration of the degraded substrate in the incubation mixture, using the standard curve of that substrate, and then the concentration of the degraded substrate was plotted against time on a linear graph for linear regression. Each slope thus obtained was normalized for the enzyme concentration to determine the specific activity (pmol (mg protein)$^{-1}$ min^{-1}). The extent of activity left was calculated from ((specific activity of the experimental group/specific activity of the control group) × 100%), and the percent of inhibition was calculated by subtraction from 100%.

In separate experiments, 50 mM Tris buffer was replaced with 100 mM Tris buffer or saline to determine whether the final pH of the incubation mixture correlates with the inhibitory effect of carbopol polymers: in other words, whether the reduced degradation is attributed to the proposed decline in the pH of the incubation medium caused by carbopol polymers. Bovine serum albumin (1%) was included in the mixture when either saline or 100 mM Tris buffer was used. The final pH of each incubation mixture without enzymes was determined. To rule out the possibility that carbopol polymers may interfere with the TCA method by inducing precipitation of degraded peptide fragments resulting in an artifact of complete or extensive inhibition, in another set of experiments, the precipitated radioactivity, after TCA was added, of the incubation mixture containing no polymers was compared with that in which a carbopol polymer was added and before the addition of TCA. The results confirmed that these carbopol polymers did not interfere with the TCA method. Therefore, any change in the radioactivity of the supernatant, when carbopol polymers were used, were attributed to the changes of enzyme activities induced by carbopol polymers, not an artifact due to assay interference by polymers.

Effects of carbopol polymers on in-situ intestinal absorption of insulin. Rats, fasted for 18 h with free access to water, were anaesthetized with phenobarbitone (60 mg kg^{-1}, i.p.) and placed on a heating pad with temperature maintained at 37°C. Portal vein cannulation was performed according to the method of Braillon & Brody (1988) with minor changes. Briefly, after a mid-line incision to expose the intestine and the portal vein, the portal vein was cannulated with a Jelco intravenous catheter (Critikon, Tampa, FL) which was connected to a PE 50 polyethylene tubing (Becton Dickinson, Parsippany, NJ). Before insertion, the cannula was filled with saline containing 10 units mL^{-1} heparin, and was attached to a 1-mL plastic syringe filled with heparinized saline. Microdissecting scissors (Iris scissors) were used to cut a tiny opening on the portal vein, and then a microdissecting forceps was used to guide the insertion of the cannula toward the liver. Thirty minutes after cannulation, 0·95 mL insulin solution (1 mg mL^{-1}) with or without Carbopol 974P (0·4%) was injected directly into the duodenum or ileum, and was allowed to flow distally. The insulin dose was 100 units kg^{-1}. The ileal injection site was approximately 20 cm proximal to the caecum.

Blood samples (250 μL) were withdrawn at 0, 1·5, 3, 5, 10, 15, 20, 30, 45, 60, 90, 120 min after dosing (Schilling & Mitra 1992). Immediately, using commercial assay kits, blood glucose and plasma insulin were determined.

Pharmacokinetic data analysis. Individual plasma insulin concentration and blood glucose level were plotted against

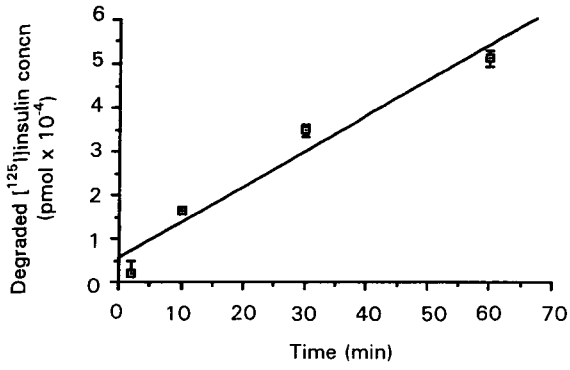

FIG. 1. The concentration–time profile of insulin.

time. Using the trapezoidal rule, the area under the curve, AUC, for each insulin concentration/time plot and the area above the blood glucose/time curve and below the blood glucose level at time zero, AAC, were calculated.

Results and Discussion

As shown in Fig. 1, linear regression of the degraded substrate concentration–time plot revealed zero order degradation. This indicates that the results were appropriately obtained for the evaluation of the effects of inhibitors. Since bile salts at 2 mM inhibit brush-border proteolytic activities (Bai 1994), their effects on the degradation of insulin by chymotrypsin and trypsin were tested. At high concentrations, bile salts form micelles, which protect insulin monomers from being exposed to enzymatic activities and degraded. The inhibition produced at concentrations higher than their critical micelle concentrations will not reveal any intrinsic inhibitory characteristics of bile salts; therefore, 2 mM was studied. As summarized in Table 1, glycodeoxycholate and deoxycholate inhibited chymotrypsin and trypsin to the greatest extent while taurocholate had the weakest inhibition. Since insulin was studied at 30 pM, which is well below its aggregation level (100 nM), it is unlikely that insulin aggregates were present in the incubation mixture. The results demonstrated that bile salts at a low concentration inhibited insulin degradation, though not to a large extent.

The results of carbopol polymers on the degradation of peptide hormones by chymotrypsin in 50 mM Tris buffer are summarized in Table 2. Carbopol 934P, 974P and 971P at 0·4% or 0·5% completely inhibited calcitonin degradation, and at 0·1% had only 49% inhibition. Carbopol 934P and 974P at 0·4% inhibited 80% and 100% of chymotrypsin activity against IGF-I, respectively. At 0·1%, Carbopol 934P and 974P only weakly inhibited insulin degradation, while at 0·4% Carbopol 934P, 974P and 971P achieved complete or almost complete inhibition. The effects of carbopol polymers on trypsin activities against peptide hormones are summarized in Table 3. Carbopol 934P and 974P at 0·1% inhibited 17 and 44% of trypsin activity against calcitonin, respectively; however, Carbopol 934P, 974P and 971P at 0·4 or 0·5% completely inhibited its degradation. Likewise, Carbopol 934P and 974P at 0·1% only partially inhibited insulin degradation, and these two polymers and Carbopol 971P at 0·4% completely or almost completely inhibited its degradation. Carbopol 934P and 974P at 0·4% achieved 90% inhibition of IGF-I degradation by trypsin.

The correlation of the inhibitory effects of carbopol polymers with the final pH of the incubation medium is summarized in Tables 4 & 5. In saline, the final pH of the incubation mixture in the presence of 0·1% and 0·4%

Table 1. Effects of bile salts on insulin degradation by chymotrypsin (1 mg mL^{-1}) and trypsin (1 mg mL^{-1}).

	Chymotrypsin	Trypsin
	Insulin-degrading activity left (%)	
Control	100	100
Taurocholate	77·6 ± 11·4	100
Glycodeoxycholate	26·5 ± 1·5	25·2 ± 0·6
Deoxycholate	30·2 ± 9·7	0
Glycocholate	49·3 ± 5·4	84·3 ± 13
Taudeoxycholate	29·1 ± 2·4	72·3 ± 12

Mean ± s.e. (n = 3). A Tris buffer (50 mM) containing NaCl (125 mM) and BSA (1%) was used as the incubation medium. The concentration of each bile salt was 2 mM.

Table 2. Effects of carbopol polymers on degradation of insulin, calcitonin and IGF-I by chymotrypsin.

Carbopol polymers	Insulin-degrading activity left (%)		
	Calcitonin	IGF-I	Insulin
Control	100	100	100
934P (0·1%)	–	–	75·4 ± 4·5
934P (0·4%)	0	20·6 ± 3·3	8·1 ± 2·1
974P (0·1%)	50·9 ± 7·3	–	84·8 ± 4·0
974P (0·4%)	0	0	11·8 ± 3·7
974P (0·5%)	0	–	–
971P (0·1%)	–	–	–
971P (0·4%)	0	–	–
971P (0·4%)*	–	–	0

Mean ± s.e. (n = 3), not determined. The incubation medium consisted of NaCl (125 mM), BSA (1%) and pH 7·5 Tris buffer (50 mM) unless specified otherwise. *Saline with BSA (1%) was used as the incubation medium.

Table 3. Effects of carpobol polymers on degradation of insulin, calcitonin and IGF-I by trypsin.

Carbopol polymers (w/v)	Insulin-degrading activity left (%)		
	Calcitonin	IGF-I	Insulin
Control	100	100	100
934P (0·1%)	82·6 ± 2·7	–	50·6 ± 0·6
934P (0·4%)	0	9·5 ± 4·4	6·6 ± 2·4
974P (0·1%)	55·8 ± 1·7	–	64·1 ± 2·0
974P (0·4%)	0	11 ± 5·1	10·1 ± 1·9
974P (0·5%)	1·57 ± 0·9	–	–
971P (0·1%)	–	–	–
971P (0·4%)	0	–	–
971P (0·4%)*	–	–	0

Mean ± s.e. (n = 3), not determined. The incubation medium consisted of NaCl (125 mM) and BSA 1%, and pH 7·5 Tris buffer (50 mM) unless specified otherwise. *Saline with BSA (1%) was used as the incubation medium.

Table 4. Correlation of the medium pH and inhibition of insulin degradation by chymotrypsin.

Medium	Polymer (w/v%)	934P		974P	
		pH[a]	Inhibition (%)	pH[a]	Inhibition (%)
saline	(0·4%)	2·8	100	2·9	99 ± 1·2
saline	(0·1%)	3·2	98 ± 1·3	3·2	100
Tris buffers					
50 mM	(0·4%)	5	92 ± 2·1	4·5	88·2 ± 3·7
50 mM	(0·1%)	6·8	24·6 ± 4·5	6·6	15·2 ± 4·0
100 mM	(0·1%)	7·4	0	7·4	0

Mean ± s.e. (n = 3). NaCl (125 mM) and BSA (1%) were included in each Tris buffer while only BSA was added to saline. The % inhibition was obtained by subtracting the activity left (%) in Table 2 from 100%.[a]Bai *et al*, (1995b).

Table 5. Correlation of the medium pH and inhibition of insulin degradation by trypsin.

Medium	Polymer (w/v%)	934P		974P	
		pH[a]	Inhibition (%)	pH[a]	Inhibition (%)
saline	(0·4%)	2·8	100	2·9	96·9 ± 1·9
saline	(0·1%)	3·2	94·4 ± 1·2	3·2	94·9 ± 1·7
Tris buffers					
50 mM	(0·4%)	5	93·4 ± 2·4	4·5	89·9 ± 1·9
50 mM	(0·1%)	6·8	49·4 ± 0·6	6·6	36 ± 2·0
100 mM	(0·1%)	7·4	0	7·4	0

Mean ± s.e. (n = 3). NaCl (125 mM) and BSA (1%) were included in each Tris buffer while only BSA was added to saline. The % inhibition was obtained by subtracting the activity left (%) in Table 3 from 100%. [a] Bai *et al* (1995b).

Carbopol 934P or 974P was 3·0; importantly, at this pH both polymers completely or almost completely inhibited insulin degradation by chymotrypsin and trypsin. Apparently, saline does not have any buffer capacity to reverse the reduced pH resulting from the protons released by carbopol polymers; enzymatic activities were, therefore, inhibited at the acidic pH induced by these polyacrylic acid polymers. When 50 mM Tris buffer was used, Carbopol 934P and 974P at 0·1% reduced the final pH only to 6·7, resulting in a weak inhibition; as the concentration of both polymers increased to 0·4%, the final pH was 5·2, resulting in a more than 88% inhibition. When 100 mM Tris was used, 0·1% Carbopol 934P or 974P had no effects on the final pH, and did not inhibit insulin degradation by either or both enzymes. Interestingly, the inhibitory effects of carbopol polymers seemed to correlate with the final pH of the incubation mixture: carbopol polymers at 0·1% and 0·4% had strong inhibition when the incubation medium had no buffer capacity, but their inhibition at 0·1% completely disappeared when the incubation medium was able to maintain the pH close to 7·5. In 50 mM Tris buffer, Carbopol 971P at 0·4% reduced the final pH of the incubation mixture to 5, and it completely inhibited the degradation of calcitonin and insulin by chymotrypsin and trypsin (Tables 2 and 3),

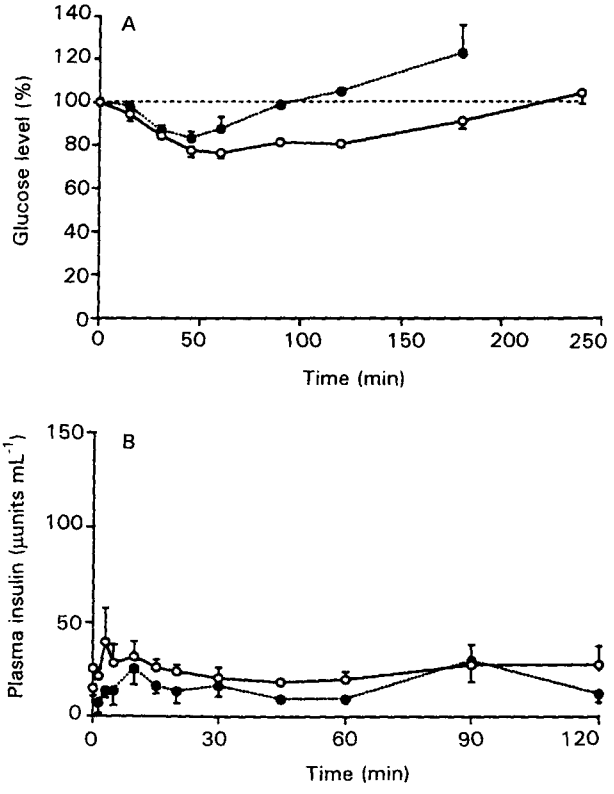

FIG. 2. A, The blood glucose level–time profile and B, the plasma insulin concentration–time profile after an insulin solution was injected into the ileum (100 units kg^{-1}). ○ Experimental group 0·4% Carbopol 974P; ● the control group.

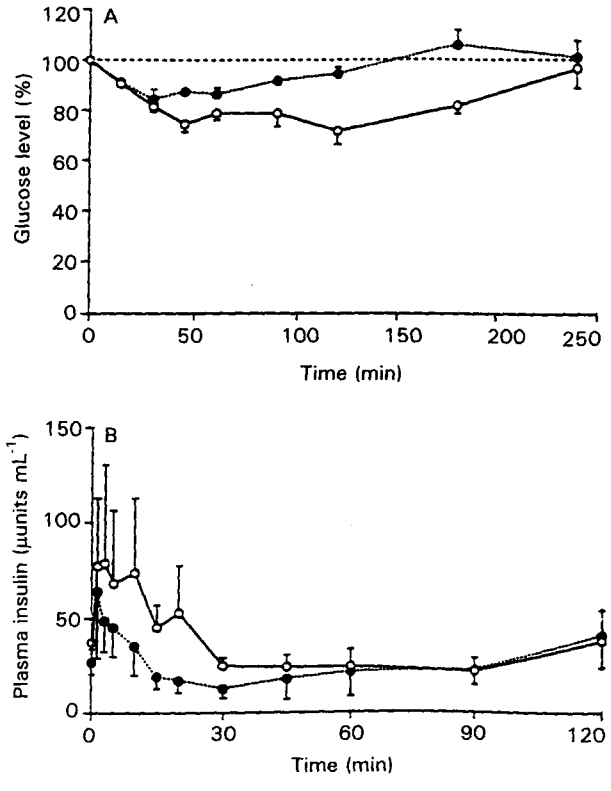

FIG. 3. A, The blood glucose level–time profile and B, the plasma insulin concentration–time profile after an insulin solution was injected into the duodenum (100 units kg^{-1}). ● Experimental group 0·4% Carbopol 974P; ○ the control group.

illustrating again that reducing the final pH of the incubation medium indeed contributed to their inhibitory effects. The pH of 50 mM Tris buffer, when 0·4% of carbopol polymers was used, was more than 2 pH units lower than the optimum pH of chymotrypsin and trypsin, therefore, activities of trypsin and chymotrypsin were negligible. Importantly, in addition to their bioadhesive properties which prolong the residence time of drugs in the intestine, these polymers are also capable of protecting peptide and protein hormones from being cleaved by pan-creatic enzymes, through lowering the pH of the incubation medium, as suggested by this study, and through depriving trypsin of Ca^{2+} (Lehr et al 1992; Lueßen et al 1994).

Dosed with insulin to the ileum, Carbopol 974P increased the AUC_{0-120} of plasma insulin 3 times, from 78 ± 35 to 240 ± 83 μunits mL^{-1} min^{-1} (Fig. 2A), and increased the AAC_{0-200} more than 4 times, from 788 ± 249 to 3318 ± 490 (Fig. 2B). The increase in insulin AUC was not significant due to large standard errors, but the increase in AAC was statistically significant with $P < 0{\cdot}005$. When injected with insulin to the duodenum, Carbopol 974P increased the AUC_{0-120} of plasma insulin more than 7 times, from 157 ± 21 to 1227 ± 780 μunits mL^{-1} min^{-1} (Fig. 3A), and increased the AAC_{0-200} more than twice, from 1717 ± 568 to 4216 ± 483 (Fig. 3B). Likewise, the increase in insulin AUC was not significant due to large variation while the increase in the AAC was significant ($P < 0{\cdot}01$). In the intestine, bicarbonate and carbonate ions, constantly secreted to maintain a lumenal pH of 7 to 7·5, will buffer the protons released by polymers, resulting in a short-term reduction of enzymatic activity by polymers. This may explain why the effects of carbopol polymers on insulin absorption were not higher than observed. It is expected, however, that the higher the concentration of polymers used, the longer the local acidic environment created by polymers can be maintained. Hence, the increases in AUC and AAC will be higher if a higher concentration of Carbopol 974P is used.

In summary, in-vitro studies suggest that carbopol polymers, including 971P, 934P and 974P, inhibit lumenal degradation of insulin, IGF-I and calcitonin by reducing the pH of the incubation medium below the optimum pH values of pancreatic enzymes. In-situ absorption of insulin into the portal vein and pharmacological responses of blood glucose levels confirm that carbopol polymers are useful in improving intestinal absorption of peptide hormones.

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