Mucoadhesive Polymers in Peroral Peptide Drug Delivery. VI. Carbomer and Chitosan Improve the Intestinal Absorption of the Peptide Drug Buserelin *In Vivo*

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Purpose. To evaluate the effect of the crosslinked poly(acrylate) carbomer 934P (C934P) and its freeze-dried neutralized sodium salt (FNaC934P) as well as chitosan hydrochloride on the intestinal absorption of the peptide drug buserelin.

Methods. Buserelin was applied intraduodenally in control buffer, 0.5% (w/v) C934P, 0.5% (w/v) FNaC934P, 1.5% (w/v) chitosan hydrochloride or FNaC934P/chitosan hydrochloride (1:1 (v/v)) mixture in rats.

Results. All polymer preparation showed a statistically significant improvement of buserelin absorption compared to the control solution. The absolute bioavailabilities for the different polymer preparations were: control, 0.1%; 0.5% FNaC934P, 0.6%; 0.5% C934P, 2.0%; chitosan hydrochloride, 5.1% and FNaC934P/chitosan hydrochloride (1:1 (v/v)) mixture, 1.0%. The higher bioavailability with chitosan hydrochloride compared to C934P and FNaC934P indicates that for buserelin the intestinal transmucosal transport enhancing effect of the polymer plays a more dominant role than the protection against proteases such as α-chymotrypsin.

Conclusions. The mucoadhesive polymers carbomer 934P and chitosan hydrochloride are able to enhance the intestinal absorption of buserelin *in vivo* in rats, and may therefore be promising excipients in peroral delivery systems for peptide drugs.

KEY WORDS: poly(acrylates); carbomer; chitosan; peroral peptide drug delivery; buserelin; intraduodenal application; intestinal absorption *in vivo*.

INTRODUCTION

Successful peroral peptide drug delivery has for a long time been regarded as almost impossible. The difficulties accompanying researchers in this field have been expressed by Borchardt as: "That to date most of the success in achieving oral delivery of peptides by passive diffusion has resulted from serendipity" (1). However, recent years have shown strong progress in peptide drug delivery to other absorption sites besides the parenteral route. A number of nasal formulations showed therapeutical plasma levels of derivatives of oligopeptide hormones, such as commercially available products of the vasopressin analogue desmopressin and the LHRH-superagonists buserelin and leuprorelin (2–4). These nonapeptides are stabilized against metabolic degradation, allowing them already to be absorbed passively without use of absorption enhancers. Their nasal bioavailabilities are usually in the range of 2 to 10% in man (4).

Nevertheless, the most convenient route of administration for patients would be peroral ingestion of a suitable dosage form. Unfortunately, the gastro-intestinal tract has built up very efficient barriers against absorption of peptides and proteins. Besides the mucus layer, which hampers diffusion of various macromolecules such as peptides (5), the metabolic and the epithelial absorption barriers can be regarded as the main hurdles against gastro-intestinal peptide drug absorption (6). In previous studies we demonstrated that two different types of polymers have promising properties to overcome these absorption barriers. The crosslinked poly(acrylic acid) derivatives polycarbophil and carbomer were able to inhibit the activities of the proteolytic enzymes trypsin, α-chymotrypsin, carboxypeptidase *A* and *B* as well as cytosolic aminopeptidase (7,8). Moreover, carbomer as well as the poly(2-deoxy-2-amino glucan) polymer chitosan showed a clear effect on opening of intercellular junctions, thereby enhancing the paracellular permeability for hydrophilic macromolecules (9, 10). This was also demonstrated *in vivo*, where the glutamate salt of chitosan enhanced the nasal absorption of insulin in sheep (11). Both types of polymers display strong mucoadhesive properties (12, 13), and may therefore be able to localize their enzyme inhibiting and absorption enhancing activities to a confined area in the intestinal tract.

The aim of the present study was to evaluate the enhancing effect of the poly(acrylate) carbomer and its freeze-dried neutralized derivative as well as of chitosan hydrochloride on the intestinal absorption of the peptide drug buserelin in rats *in vivo*.

MATERIALS AND METHODS

Materials

Carbomer (Carbopol® 934P, C934P) and chitosan hydrochloride (chitosan-HCl) were kindly donated by BF Goodrich (Cleveland, OH, USA) and Pronova AS (Drammen, Norway), respectively. Buserelin acetate (Suprecur®) and its specific antiserum were obtained as generous gifts from Hoechst AG (Frankfurt, Germany). 2-[N-morpholino]ethane-sulfonic acid] (MES) and sheep-anti rabbit IgG were purchased from Sigma (Bornem, Belgium). $Na^{125}I$ was from Amersham (s'Hertogenbosch, The Netherlands), Seppak® C18 cartridges from Waters (Etten-Leur, The Netherlands) and iodogen beads (Iodobeads®) from Pierce (Rockford, IL, USA). Freeze-dried neutralized carbomer (FNaC934P) was prepared as described previously (14). Hypnorm® (containing 0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone) was obtained from Janssen Pharmaceuticals Ltd. (Grove, Oxford, England) and Dormicum® (5 mg/ml midazolam-HCl) from Hoffmann-La Roche B.V. (Mijdrecht, The Netherlands).

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METHODS

Animals

The protocol for these experiments has been approved by the Leiden University ethical committee for animal experimentation. Male Wistar rats, SPF status and weighting between 190 and 230 g, were obtained from Harlan (Zeist, The Netherlands) and housed at least one week before the experiments in our Centers animal facilities. They were fasted for 16 h prior to the experiment, having free access to tap water. Between 8:30 and 9:00 a.m. the rats were anaesthesized with Hypnorm® (1.5 ml/kg body weight) and Dormicum® (500 μg midazolam/kg body weight) by intramuscular injection into the regio femoris posterior and anaesthesia was maintained by injecting 500 μl Hypnorm®/kg body weight and 250 μg midazolam/kg body weight every 2 h of the experiment. Body temperature was monitored rectally and kept at 37°C. The right carotic artery was cannulated with a polyvinylchloride tube (Med-PVC tube C1E, ø 0.5 mm I.D. × 1.0 mm O.D., Medica B.V., s'Hertogenbosch, The Netherlands), filled with heparinized physiological saline (20 IU/ml), for blood sampling. Blood samples of about 150 μl were withdrawn at predetermined time points and collected in 1.5 ml polypropylene reaction tubes (Eppendorf, Hamburg, Germany). To avoid blood clotting, the cannula was carefully flushed with heparinized physiological saline (20 IU/ml) after taken blood samples. Samples were centrifuged immediately, serum was collected and stored at −20°C until analysis.

Formulations

For intravenous (*i.v.*) bolus injection 1 mg buserelin acetate was dissolved in 1 ml physiological saline (0.15 mM). For intraduodenal application, the following polymer dispersions were prepared in 50 mM MES/KOH buffer (pH 6.7) containing 250 mM mannitol: 0.5% (w/v) C934P, 0.5% (w/v) FNaC934P and 1.5% (w/v) chitosan hydrochloride. Furthermore, an 1:1 (v/v) mixture of the mentioned FNaC934P and chitosan hydrochloride dispersions was prepared. After dispersion of the polymers in the buffer solutions the pH was adjusted with either 1 M NaOH or 1 M HCl to values of 6.7. An amount of 250 μg buserelin acetate was dissolved per ml of the control (MES/KOH buffer) and the different polymer preparations.

Intravenous Injection of Buserelin

The *i.v.* pharmacokinetics of buserelin in rats were determined by injecting a bolus of 100 μg buserelin acetate in 100 μl physiological saline into the femoral vein. Therefore, the vein was cannulated with a polyethylene tube (ø, 0.28 I.D. × 0.61 O.D., Talas B.V., Ommen, The Netherlands). To ensure complete dosing the injection cannula was flushed afterwards with 200 μl physiological saline. Blood samples of approximately 150 μl were withdrawn at 0, 0.5, 2, 10, 20, 30, 45, 60, 90, 120, 150, and 180 min after application, and treated as described above.

Intraduodenal Application of Buserelin

A teflon tube (ø, 0.5 mm I.D. × 1.0 mm O.D., Talas B.V., Ommen, The Netherlands) was introduced by an incision into the corpus of the stomach and carefully forwarded through the pylorus approximately 5 to 10 mm into the duodenum. After application of 500 μg buserelin, dissolved in 2 ml of the control and the different polymer preparations, respectively, the tube was removed from the GI-tract and the incision in the stomach was closed immediately. Blood samples of about 150 μl were taken at 0, 20, 40, 60, 90, 120, 150, 180, 240, and 300 min after application, and treated as described above. Animals were only used for one single application of either control or polymer formulation and immediately sacrified by decapitation at the end of the experiment.

Analysis of Buserelin

Serum concentrations of buserelin were determined in triplicate by a specific radioimmunoassay as described previously (15). The peptide was labelled with ^{125}I using iodogen beads as an oxidizing agent. ^{125}I-buserelin was extracted from the reaction medium by solid phase separation over Seppak® C18 cartridges as described previously (16). The labelled peptide in tracer solution was stored at −20°C until use. A specific antiserum for buserelin raised in rabbits was used for the first 48 h incubation period and sheep-anti-rabbit-IgG served as the second antibody for precipitation purposes.

Pharmacokinetic Analysis of Data

For each animal, the serum profiles of buserelin after i.v. injection were fitted using the non-linear least squares regression program Siphar (Simed SA, Creteil, France). The serum concentration-time profiles were fitted according to:

$$C_t = A_1 \times e^{-\alpha_1 t} + A_2 \times e^{-\alpha_2 t}$$

in which C_t equals the serum concentration of buserelin at time t, and A_1, A_2, α_1, and α_2 are the coefficients and exponents of this equation. The *i.v.* pharmacokinetic parameters were calculated as described by Gibaldi and Perrier (17) and determined for each individual animal. The areas under the individual concentration-time curves (*AUC*) were calculated with the linear trapezoidal rule. Absolute bioavailabilities after intraduodenal administration of buserelin were calculated according to:

$$F = \frac{AUC_{i.d.} \times D_{i.v.}}{AUC_{i.v.} \times D_{i.d.}} \times 100\%$$

in which F is the absolute bioavailability and D is the administered dose. The $AUC_{i.v.}$ was the mean of a separate group of 6 rats (Table I). Absorption profiles were extracted from the concentration-time profiles by numerical deconvolution, and

Table I. Pharmacokinetic Parameters of i.v. Administered Buserelin in Rats (n = 6)

Parameters	Mean ± S.D.
body weight (kg)	0.192 ± 0.006
$t_{1/2}$ dist. (min)	3.3 ± 0.7
$t_{1/2}$ elim. (min)	36.7 ± 8.2
Cl (ml/min*kg)	3.8 ± 0.5
V_d (ml/kg)	185 ± 21

Note: $t_{1/2}$ distr. = distribution half-life; $t_{1/2}$ elim. = elimination half-life; Cl = clearance; V_d = volume of distribution.

T_{90} values (time to 90% of total absorption) were calculated by the above mentioned software program Siphar. All data were evaluated for statistically significant differences by one way analysis of variance (ANOVA). The probability level was set at 0.5%. All data are presented as mean ± S.D.

RESULTS

Intravenous Pharmacokinetics of Buserelin

Figure 1 shows the mean serum levels of buserelin after *i.v.* injection of this peptide (100 μg/rat), and the pharmacokinetic parameters are given in Table I. The serum levels of buserelin displayed a bi-exponential decline, with a mean initial half-life of 3.3 min followed by an elimination half-life of 36.7 min. The average clearance was 3.8 ml/(min × kg) and the volume of distribution was 185 ml/kg. As also demonstrated by Hoogstraate *et al.* in pigs (21), initial pilot experiments revealed that the anaesthesia did not affect the pharmacokinetics of buserelin in rats.

Intestinal Absorption of Buserelin

The mean serum buserelin concentrations after intraduodenal administration of buserelin (500 μg/rat) in buffer solution as well as in the presence of 0.5% (w/v) of the poly(acrylates) C934P and FNaC934P are depicted in Fig. 2. Table II lists the corresponding pharmacokinetic parameters, including the variabilities observed between the experimental animals. Intraduodenal application of buserelin in buffer solution resulted in low serum peptide levels, leading to an absolute bioavailability of 0.1 ± 0.1%. Both poly(acrylates) improved the intestinal peptide absorption remarkably, and C934P appeared to be more potent than FNaC934P (Fig. 2). Increased mean serum peak concentrations of buserelin of 45.8 and 112.1 ng/ml were observed after co-administration with FNaC934P and C934P, respectively. Compared to the control solution, FNaC934P and C934P showed a 6- and 19-fold increase in the intestinal buserelin bioavailability, with absolute bioavailabilities of 0.6 ± 0.2% and 1.9 ± 1.3%, respectively.

The enhancing effect of 1.5% (w/v) chitosan hydrochloride on the intestinal buserelin absorption was even more pronounced than compared to the poly(acrylic acid) derivatives. The mean serum buserelin concentrations after intraduodenal administration of the peptide (500 μg/rat) in the chitosan preparation are shown in Fig. 3, and their corresponding pharmacokinetic parameters are presented in Table II. Moreover, a mixture (1:1 (v/v)) of FNaC934P/chitosan was included in this study, and the resulting mean serum buserelin concentrations are also given in Fig 3. The intraduodenal buserelin administration in the chitosan preparation resulted in highly increased serum peak concentrations of 364.0 ± 140.0 ng/ml, whereas the FNaC934P/chitosan mixture showed only serum peak concentrations of 139.4 ± 108.5 ng/ml. Absolute bioavailabilities of buserelin were 5.1 ± 1.5% for the chitosan preparation and 1.0 ± 0.4% for the FNaC934P/chitosan mixture, showing in both cases a significantly improved intestinal buserelin absorption compared to the control solution.

Numerical deconvolution revealed a statistically significant prolonged absorption phase in the case of C934P compared to chitosan hydrochloride ($P < 0.05$ in one way ANOVA). T_{90} values rose from 76 (chitosan hydrochloride) to 165 min (C934P). No statistically significant difference between FNaC934P and either C934P or chitosan hydrochloride could be demonstrated. Absorption values for the mixture did not differ from those of C934P and FNaC934P.

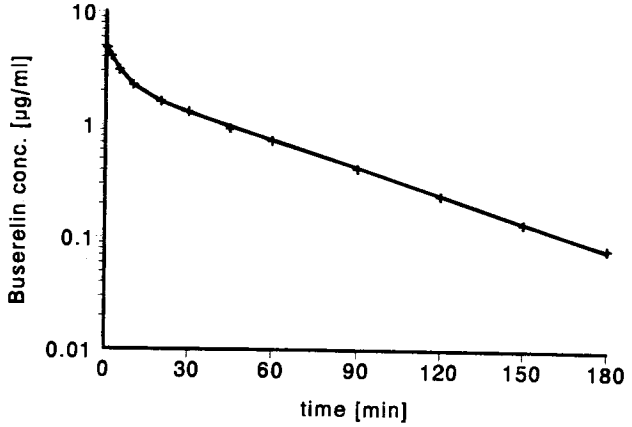

Fig. 1. Serum concentrations after i.v. administration of buserelin (100 μg/rat). +, average of 6 rats; solid line, fitted curve (two-compartement model; $R = 0.9999$).

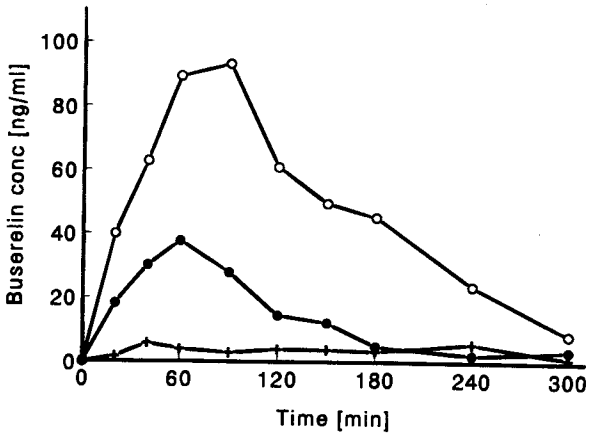

Fig. 2. Serum concentrations after intraduodenal application of buserelin (500 μg/rat). +, control (MES/KOH buffer pH 6.7); ●, 0.5% (w/v) FNaC934P; ○, 0.5% (w/v) C934P. Data are presented as the mean of 5 or 6 rats (Table II).

DISCUSSION

The nonapeptide buserelin is stabilized against metabolic degradation by elimination of Gly^{10}, N-ethylation of Pro^9 and replacement of Gly^6 with [6-D-SER(BU^t)] in comparison to the parent peptide LHRH (luteinizing hormone-releasing hormone). These chemical modifications lead to prolonged binding of buserelin to the LHRH receptor which allows a three times daily dosing to establish a so-called superagonistic action (18). The continuous stimulation of the LHRH receptor causes subsequently a supression of LH and FSH release from the anterior pituitary gland by a feed-back mechanism (19).

As shown in the present study, after *i.v.* injection buserelin was eliminated from the circulation with a half-life of 37 min, which was considerably shorter than that reported in man (75 min, (20)) and pigs (101 min, (21)). In consequence, it is

Table II. Pharmacokinetic Parameters After Intraduodenal Administration of Buserelin (500 μg/rat)

Polymer	T_{max} (min)	T_{90} (min)	C_{max} (ng/ml)	F (%)	n
control	60–90	n.d.	6.7 ± 1.7	0.1 ± 0.1	6
poly(acrylates) FNaC934P gel (0.5%)	40–60	95 ± 25	45.8 ± 20.8[b]	0.6 ± 0.2[b]	5
C934P gel (0.5%)	40–90	165 ± 80	112.1 ± 53.4[b,c]	1.9 ± 1.3[b,c]	5
chitosan Chitosan-HCl gel (1.5%)	40–90	76 ± 29[a]	364.0 ± 140.0[b,d]	5.1 ± 1.5[b,d]	6
combination Combi gel	20–40	61 ± 19	139.4 ± 108.5[b,e]	1.0 ± 0.4[b,f]	4

Note: Data are presented as mean ± S.D. for the number of animals (n) indicated; T_{max}: time to reach serum peak concentration; T_{90}: time to reach 90% of total amount absorbed; C_{max}: serum peak concentration; F: absolute bioavailability; n.d.: not determined.
[a] Significantly different from C934P/FNaC934P ($P < 0.05$).
[b] Significantly different from control ($P < 0.01$).
[c] Significantly different from FNaC934P ($P < 0.05$).
[d] Significantly different from C934P ($P < 0.005$).
[e] Significantly different from chitosan-HCl ($P < 0.05$).
[f] Significantly different from chitosan-HCl ($P < 0.01$).

expected that the pharmacokinetic parameters of buserelin in rats differ from those in man and that extrapolation to the human situation is not directly possible. The longer half-life in man, presuming comparable absorption kinetics, may lead to prolonged increase of buserelin plasma levels and also to longer-lasting pharmacological effects compared to rats.

Intraduodenal application of buserelin displayed an improved systemical absorption in presence of all poly(acrylate) and chitosan polymers tested compared to the control buserelin solution. The mechanism of the high buserelin absorption can be explained by previous *in vitro* results obtained with these polymers (7, 9, 10). It has been reported that the proteolytic activity of α-chymotrypsin, which is also responsible for degradation of buserelin in the intestinal lumen, was inhibited by carbomer in concentrations of 0.5% (w/v) (7). Besides its enzyme inhibitory action, carbomer also showed an additional effect on the permeability of the intestinal epithelium by opening of intercellular junctions (9). Chitosans, however, did not seem to have any inhibitory effect on proteolytic enzyme activities such as trypsin, α-chymotrypsin and carboxypeptidase B (10). It has therefore been suggested that chitosans can only improve mucosal peptide absorption by opening of intercellular junctions.

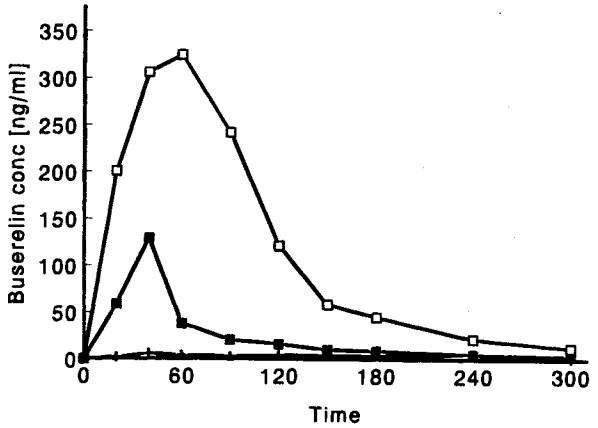

Fig. 3. Serum concentrations after intraduodenal application of buserelin (500 μg/rat). +, control (MES/KOH buffer pH 6.7); ■, 0.5% (w/v) FNaC934P/1.5% (w/v) chitosan-HCl mixture (1:1); □, 1.5% (w/v) chitosan-HCl. Data are presented as the mean of 5 or 6 rats (Table II).

Of all polymers tested, chitosan hydrochloride resulted in the highest absolute bioavailability for intraduodenally administered buserelin in rats (5.1 ± 1.5%). This bioavailability value was even higher than reported for the commercial nasal formulation Suprecur® (3.3%) in men (22). Regarding the higher buserelin bioavailibility obtained after co-administration with chitosan hydrochloride compared to the poly(acrylates), it is suggested that the intestinal transmucosal transport enhancing effect plays a more dominant role for intestinal buserelin absorption than the protection against proteolytic degradation. This may be different for different peptides drugs which are more susceptible to enzymatic degradation. On the other hand, the duration of absorption of buserelin after application with the C934P preparation was longer compared to the chitosan hydrochloride preparation. Numerical deconvolution revealed a 2-times longer period until 90% of the totally absorbed amount of buserelin was taken up into the systemic circulation. This indicates that C934P was able to prolong the survival time of the intact peptide drug in the intestinal lumen by inhibition of buserelin-degrading enzymes such as α-chymotrypsin. Previously, it was found that the inhibitory effect of poly(acrylic acid) derivatives on proteolytic enzymes is time-dependent (7, 8, 23). Inhibition was even more pronounced when the protease was preincubated in the poly(acrylate) preparation for about 20 min before the substrate was added to the incubation medium. This time-dependency of enzyme inhibition has also been demonstrated using Caco-2 cell monolayers, where the apical medium, besides control buffer, 1.5% (w/v) FNaC934P or 1.5% (w/v) chitosan hydrochloride, also contained α-chymotrypsin (24). When buserelin was added 30 min after preincubation, buserelin transport from the apical to the basolateral side was the same for both polymers, whereas peptide transport was about 2-times higher in the chitosan preparation without α-chymotrypsin (24). Thus, the protection of buserelin against proteolytic degradation by carbomer may be further improved, if the peptide drug is released in a burst 20 to 30 min after formation of a swollen poly(acrylate) environment in the gut. To achieve sufficient fast swelling of the poly(acrylate),

the freeze-dried sodium salt of carbomer (FNaC934P) was prepared, showing fast dispersing properties in a mixture with suitable disintegrants or after microdispersion in microparticles of polyglycerol esters of fatty acids (14). Moreover, one unit/two phase capsule formulations with FNaC934P were developed, which showed pronounced protease inhibiting properties (23). The presence of large amounts of sodium in FNaC934P, which acts as a counter ion for the dissociated carboxylic groups, seemed to reduce the binding affinity of the poly(acrylate) to bivalent cations, such as Ca^{2+} and Zn^{2+}, and by that reducing its potential to inhibit proteolytic enzyme activities in comparison to carbomer (C934P) (23). Such a decrease of binding affinity towards Ca^{2+} may also reduce the efficacy to open intercellular junctions and, consequently, reduce paracellular permeability. This would explain the lower intestinal bioavailability of buserelin co-administered with FNaC934P compared to C934P. Because of the properties of the polymers, it is expected that a combination of the strong peptide transport enhancement effect of chitosan together with the potent enzyme inhibitory effects of C934P or FNaC934P would be an optimized system for intestinal buserelin absorption. Therefore, a mixture of FNaC934P and chitosan hydrochloride was also included in the present absorption studies. However, no pronounced improvement in the intestinal buserelin bioavailability was found compared to the FNaC934P preparation. This may be due to ionic interactions between the two different types of polymers, which reduce the charge density and hence reduce the absorption enhancing properties of the single polymers.

CONCLUSIONS

From the present results, it is concluded that mucoadhesive polymers of the poly(acrylate)- and chitosan-type are able to substantially improve the intestinal absorption of the peptide drug buserelin *in vivo*. Based on previous *in vitro* results (7–10, 23, 24), the development of an optimized dosage form with a fast-swelling polymer part and a time-controlled burst release of buserelin may even further increase the intestinal absorption of the peptide drug. The proven safety of the polymers used and the previously reported bioavailability data of the commercial nasal buserelin formulation (Suprecur®) may imply that peroral application of buserelin has promising prospects for therapeutical use.

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