N-Trimethylated Chitosan Chloride (TMC) Improves the Intestinal Permeation of the Peptide Drug Buserelin *In Vitro* (Caco-2 Cells) and *In Vivo* (Rats)

M. Thanou,[1] B. I. Florea,[1] M. W. E. Langemeÿer,[2] J. C. Verhoef,[1] and H. E. Junginger[1,3]

Received September 15, 1999; accepted October 9, 1999

Purpose. To evaluate N-trimethyl chitosan chloride (TMC) of high degrees of substitution as intestinal permeation enhancers for the peptide drug buserelin *in vitro* using Caco-2 cell monolayers, and to investigate TMCs as enhancers of the intestinal absorption of buserelin *in vivo*, in rats.

Methods. TMCs were tested on Caco-2 cells for their efficiency to increase the paracellular permeability of the peptide buserelin. For the *in vivo* studies male Wistar rats were used and buserelin was administered with or without the polymers intraduodenally. Both types of experiments were performed at pH 7.2.

Results. Transport studies with Caco-2 cell monolayers confirmed that the increase in buserelin permeation is dependent on the degree of trimethylation of TMC. In agreement with the *in vitro* results, *in vivo* data revealed highly increased bioavailability of buserelin following intraduodenal co-administration with 1.0% (w/v) TMCs. Intraduodenally applied buserelin resulted in 0.8% absolute bioavailability, whereas co-administrations with TMCs resulted in mean bioavailability values between 6 and 13 %. Chitosan HCl (1.0%; pH = 7.2) did not significantly increase the intestinal absorption of buserelin.

Conclusions. Both the *in vitro* and *in vivo* results indicate that TMCs are potent mucosal permeation enhancers of the peptide drug buserelin at neutral pH values.

KEY WORDS: N-trimethyl chitosan chloride; chitosan HCl; Caco-2 cells; intraduodenal administration; intestinal absorption *in vivo*; buserelin.

INTRODUCTION

The peroral route of administration is still considered the greatest challenge for peptide drug delivery. Poor membrane permeation and/or presystemic metabolism are the main reasons for the observed low plasma levels resulting in poor bioavailabilities after oral delivery of peptide drugs (1). A large number of peptide analogues which are resistant against enzymatic degradation have been synthesized over the last two decades and may be used in cases where the native peptide hormones are not suitable due to metabolism in the gastro-intestinal tract. Nevertheless, despite the stability over enzymatic degradation, the molecular size and the hydrophilicity of these analogues still remain important impeding factors for effective absorption through the intestinal epithelium (2).

Epithelial cells of the intestine have apical intercellular attachments (the most important being the tight junctions) which represent one of the main barriers to the passage of macromolecules through the intercellular space. Limited understanding of the physiology and regulation of the opening of the intestinal tight junctions has also limited the use of the peroral route for peptide drug delivery. Substances which are able to increase tight junctional permeability can be divided into two main classes: calcium chelators and surfactants. In case of chelators, extracellular Ca^{++} depletion induces global changes in the cells including disruption of actin filaments, while surfactants cause irreversible exfoliation of the intestinal epithelium. Both are nonspecific mechanisms of action which limit the use of these agents as permeation enhancers for hydrophilic macromolecules for chronic application (3).

A better approach for safe permeation enhancement is the use of functional, biocompatible and non-absorbable polymers, which have a specific and reversible effect on the tight junctions integrity. One of these polymers frequently explored as permeation enhancer (by means of opening the tight junctions) is chitosan (4–6). Chitosan, a natural origin polymer, has found a number of applications in the biomedical field because of its biocompatibility, biodegradability and absence of systemic and local toxicity (7,8). However, its poor solubility at pH values above 6.5 hinders chitosan to be used as permeation enhancer at intestinal sites of absorption. Chitosan is not able to open the tight junctions of intestinal epithelia at neutral pH values, lacking positive charge density, due to aggregation and precipitation phenomena (9). To overcome these drawbacks, N-trimethyl chitosan chloride (TMC) has been synthesized and evaluated at different degrees of substitution as safe and efficient permeation enhancer *in vitro* (10–12). Recently, TMC has proven to be a potent enhancer of both nasal and rectal insulin absorption in vivo in rats, especially at neutral pH values where chitosan salts are ineffective (13).

In the present study, the effect of TMC polymers on the intestinal permeation was investigated. TMCs of two different degrees of substitution (40 and 60% degree of trimethylation; TMC40 and TMC60) were tested for their efficiency to increase the permeation of the peptide drug buserelin, a metabolically stable LHRH analogue, across intestinal mucosae both *in vitro* using the Caco-2 cell model and *in vivo* following intraduodenal administration in rats. In both cases the pH of application/administration was kept at 7.2, being a representative pH value for the intestinal environment.

MATERIALS AND METHODS

Materials

Chitosan (Seacure 244; 93% deacetylated; viscosity 40 mPas*sec) and buserelin acetate with its specific antiserum were generous gifts from Pronova AS (Drammen, Norway) and Hoechst AG (Frankfurt, Germany), respectively. Methyliodide and N-methylpyrrolidinone were obtained from Acros (Geel, Belgium). Costar Transwell® plates were purchased from Costar

[1] Division of Pharmaceutical Technology, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300RA Leiden, The Netherlands.

[2] Division of Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9503, 2300RA Leiden, The Netherlands.

[3] To whom correspondence should be addressed. (e-mail: junginge@chem.leidenuniv.nl)

0724-8741/00/0100-0027$18.00/0

Europe (Badhoevedorp, The Netherlands). Dulbecco's Modified Eagle's Medium (DMEM), benzyl-penicillin G, streptomycin sulfate, MES (2-[N-morpholino] ethane-sulfonic acid) and sheep-anti-rabbit-IgG were from Sigma (Bornem, Belgium). Foetal calf serum Hyclone® was from Greiner (Alphen a/d Rijn, The Netherlands). Hypnorm® (containing 0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone) was obtained from Janssen Pharmaceuticals (Groove, Oxford, England) and Dormicum® (5 mg/ml midazolam hydrochloride) from Hoffmann-La Roche (Mijdrecht, The Netherlands).

Preparation of TMC Polymers

N-trimethyl chitosan chloride (TMC) was synthesized in different degrees of substitution (D.S.) as described previously (11,14). Briefly, sieved chitosan with a particle size of 200–400 μm was mixed with methyliodide in an alkaline solution of N-methylpyrrolidinone at 60°C for 75 min. The product was isolated by precipitation with ethanol and subsequent centrifugation, and consisted of TMC20 (15–20% of trimethylation). This obtained product underwent a second step of reductive methylation, to yield the final products TMC40 and TMC60 iodide, dependent on the duration of this second reaction step. The product was precipitated by addition of ethanol and isolated by centrifugation. The purification step of the final products included the exchange of the counterion iodide with chloride in a NaCl solution and extensive washing with ethanol and diethylether. The products were dried in vacuo and measured for their degrees of quaternization by ^{1}H-NMR using a 600 MHz spectrometer (Bruker, Switzerland). In all experiments TMCs of two different D.S. were studied: TMC40 (D.S.39%) and TMC60 (D.S. 63%).

Caco-2 Cell Cultures

Caco-2 cell cultures of passage number 78 were used for the experiments. The cells were seeded on tissue culture polycarbonate membrane filters (pore size 0.4 μm, area 4.7 cm^2, not-coated) in Costar Transwell 6-well plates at a seeding density of 10^4 cells/cm^2 (10,15). DMEM, supplemented with 1% non essential amino acids, 10% foetal calf serum, benzyl-penicillin G (160 U/ml) and streptomycin sulfate (100 μg/ml) was used as culture medium, and added to both the donor and the acceptor compartment. The medium was changed every second day. The cell cultures were kept at a temperature of 37°C, in a humidified atmosphere of 5% CO_2 and 95% air. For all experiments cells were used 23–25 days after seeding. The transepithelial electrical resistance (TEER) was checked prior to the experiment by a Millicell® ERS meter (Millipore Corp., Bedford, MA, USA) connected to a pair of thin, side by side electrodes and the values ranged from 1000 to 1200 Ω*cm^2. Two hours before the experiments the medium was changed to DMEM buffered to pH 7.4 with 40 mM n-(2-hydroxyethyl) piperazine-N-(2-ethanosulfonic acid) (HEPES).

In Vitro Transport Studies

TMC (TMC40 or TMC60) and buserelin acetate were dissolved in DMEM-HEPES at concentrations of 1% (w/v) and 200 μg/ml, respectively. The pH of application was adjusted at 7.2. Apical applications of 2.5 ml containing the polymers and the peptide were applied on the cells and transport of the peptide was monitored by serosal sampling over 4 hours. Basolateral samples of 200 μl were added to 800 μl solution of phosphoric acid (pH = 2) and analysed by HPLC-UV_{220nm} for their content of buserelin (16). Isocratic elution was performed with K_2HPO_4 buffer (pH = 6.2) containing 35% acetonitril at a flow rate of 1 ml/min. A 250 × 4.6 mm Chromspher 5 C8 column equipped with a Cromspher 5 10 × 46 mm precolumn (Chrompack, Middelburg, The Netherlands) was used. In this system the retention time of buserelin was 5.6 min.

In Vivo Studies in Rats

The protocol for the animal studies was approved by the ethical committee of Leiden University. The experimental procedure was slightly different from previously reported studies (6). In brief, male Wistar rats SPF (average body weight 250 g) were obtained from Harlan (Zeist, The Netherlands). The animals were fasted for 18 h prior the experiment, with free access to water. The animals were anaesthetised with Hypnorm® (1.5 ml/kg body weight) and Dormicum® (500 μg midazolam/kg body weight). Body temperature was monitored rectally and kept at 36.5–37° C. After buserelin administration (intraduodenally or intravenously), blood sampling was performed through a cannula previously inserted into the right carotic artery. Samples of 200 μl were withdrawn at predetermined time points and 200 μl of heparinized physiological saline (25 anti-Xa U/ml) were subsequently administered to the rat through the same cannula to prevent blood clotting and to compensate for blood loss during sampling. Blood samples were centrifuged (13000 rpm for 15 min) and serum samples were collected and stored at −20° C until analysis.

In order to determine the pharmacokinetic parameters of buserelin a group of 6 animals received buserelin intravenously (i.v.). The femoral vein was cannulated (6) and a bolus of 100 μg of buserelin acetate dissolved in 100 μl of sterile physiological saline was injected into the cannula. To ensure complete dosing the injection cannula was flushed afterwards with 200 μl physiological saline. Blood samples were taken from the same cannulated vein and treated as described above.

At the end of the experiment the animals were sacrificed and segments of the intestine were removed and checked macro- and microscopically (using a Zeiss IM 35 inverted light microscope; Carl Zeiss, Oberkochen, Germany) for possible damage of the intestinal epithelium.

Formulations and Intraduodenal Administration

TMC40 and TMC60 polymer solutions and chitosan hydrochloride dispersions were prepared at concentrations of 1% (w/v) in 50 mM MES/KOH buffer, (pH = 7.2) containing 250 mM mannitol. An amount of 250 μg buserelin acetate was dissolved per ml of the control (MES/KOH buffer) and the different polymer preparations. The pH of the formulations was readjusted (if needed) with 0.1 M KOH and 0.1 M HCl to values of 7.2.

In order to administer the buserelin formulations intraduodenally, a teflon tube connected to a syringe was inserted by a small incision into the corpus of the stomach and guided through the pylorus about 5 to 10 mm into the duodenum. Then 2 ml of the control or polymer containing formulations were administered slowly. Afterwards the tube was removed and the

incision in the stomach was closed. The stomach was subsequently washed with physiological saline at the site of incision and the abdomen was closed.

Buserelin Analysis in Serum

The analysis of serum samples on buserelin concentrations was performed by radioimmunoassay as previously described (6,17). The peptide was labelled with $Na^{125}I$ (Amersham, s'Hertogenbosch, The Netherlands) using iodogen beads (Iodobeads®; Pierce Rockford, IL, USA) as an oxidizing agent. Radiolabelled buserelin was separated from non-bound ^{125}I, using Seppak ® C18 solid phase extraction cartridges (Waters; Etten-Leur, The Netherlands). A specific antiserum for buserelin raised in rabbits was used for the first 48 h of incubation and sheep-anti-rabbit-IgG was used as the second antibody for precipitation purposes. To avoid inter-assay variations, all samples were analyzed in one assay.

Pharmacokinetic Analysis of Data

The serum profiles of buserelin after i.v. bolus injection were fitted using WiNnonlin program (Scientific Consulting Inc., Palo Alto, CA, U.S.A.). The serum concentration-time profiles were fitted according to:

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

In which C equals the serum concentration of buserelin at time t and A_1, A_2, α_1, α_2 are the coefficients and exponents of this equation. The i.v. pharmacokinetic parameters were calculated according to Gibaldi and Perrier (18).The areas under the individual concentration-time curves (AUC) were calculated with the linear trapezoidal rule. Absolute bioavailability values 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 data were evaluated for statistically significant differences by one way analysis of variance (ANOVA).

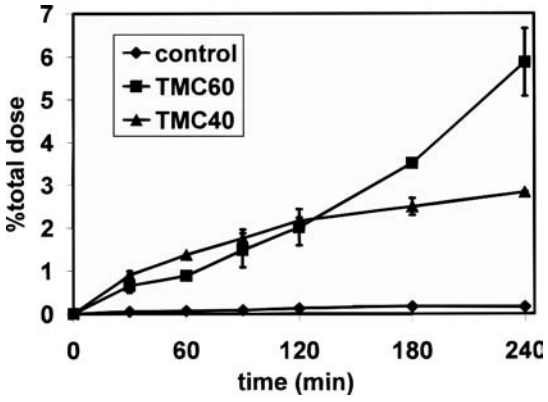

Fig. 1. Transport of buserelin acetate across Caco-2 cell monolayers, without co-application of the polymers (control) and with co-application of 1.0% TMC40 and TMC60 (pH = 7.2; n = 3; mean ± SE).

Table I. Apparent Permeability Coefficient (P_{app}) and Enhancement Ratio (R) Values for Buserelin Acetate Across Caco-2 Cell Monolayers, Without Coapplication of the Polymers (Control) or with Coapplication of 1.0% TMC40 and TMC60

Application	P_{app}* 10^{-6} (cm/sec)	R
Control	0.04 ± 0.01	1
TMC40	0.85 ± 0.02	21.2 ± 1.3
TMC60	2.40 ± 0.27	60 ± 5.7

Note: pH = 7.2; n = 3; mean ± SE.

RESULTS

In Vitro Transport Studies

Caco-2 cell monolayers were used as an intestinal epithelium model to investigate the ability of the two different TMC polymers to increase the paracellular transport of the peptide drug buserelin. Since chitosan HCl does not alter the permeability of Caco-2 cell monolayers at pH 7.2 (10), it was not included in this study. The transport profiles of buserelin across the Caco-2 cell monolayers are depicted in Fig. 1. Both TMCs managed to significantly increase the transport of buserelin compared to the control. However, the effect of TMC60 was more prominent. TMC60 increased the transported amount of the peptide up to 6% of the apically applied buserelin. These results are in agreement with previously reported results using Caco-2 cells and ^{14}C-mannitol as a hydrophilic marker for paracellular transport (11). The P_{app} and the enhancement ratios R (R defined as $P_{app\ polymer}/P_{app\ control}$) are presented in Table 1. TMC40 and TMC60 induced a 17- and 46-fold enhancement of the transport of buserelin. Buserelin transport across monolayers without co-application of the polymers was found to be very minor, indicating that buserelin per se poorly permeates the Caco-2 monolayers. At the end of the experiments all monolayers were routinely checked for viability using trypan blue nucleic stain (11). Exclusion of the dye from all monolayers in the present study demonstrated their viability.

In Vivo Absorption Studies

The mean serum concentrations of buserelin after i.v. bolus injection were analyzed in terms of a two-compartment model and were found to be similar as previously reported in rats and pigs (6,19). The calculated i.v. pharmacokinetic parameters are presented in Table 2.

Table II. Pharmacokinetic Parameters of i.v. Administered Buserelin (100 μg/rat)

Parameters	mean ± SE
body weight (g)	247 ± 13
$t_{1/2}$ dist (min)	11.6 ± 4.1
$t_{1/2}$ elim (min)	145.0 ± 68.9
V_d (ml/kg)	231 ± 35
Cl (ml/min*kg)	3.3 ± 0.7

Note: Data are presented as mean ± SE of 6 rats. Abbreviations: $t_{1/2}$ dist., distribution half-life; $t_{1/2}$ elim., elimination half-life; Cl, clearance; V_d, volume of distribution.

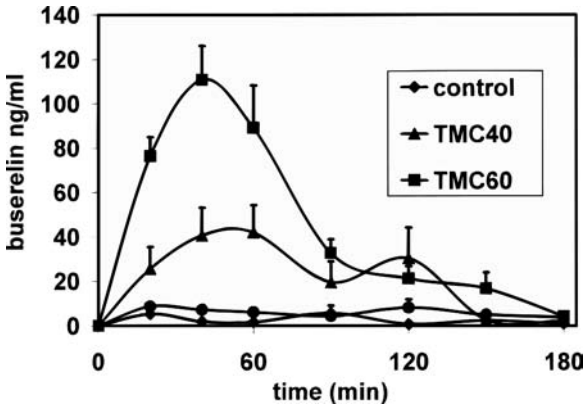

Fig. 2. Buserelin serum levels after intraduodenal administration of buserelin acetate (500 μg/rat) alone (control) and after co-administration of buserelin with 1.0% TMC40, 1.0% TMC60 or 1.0% chitosan HCl. (pH of administered formulations 7.2; n = 6; mean ± SE).

Figure 2 shows the serum buserelin levels after intraduodenal application of buserelin in buffer solution (control) and in the presence of 1.0% TMC40 or 1.0% TMC60. A remarkable increase in buserelin serum concentrations was observed after co-administration of the peptide with both polymers, whereas buserelin alone was poorly absorbed. In agreement with the present *in vitro* results, TMC60 demonstrated better intestinal absorption enhancement of buserelin than TMC40.

Figure 2 also depicts the buserelin serum levels after co-administration of the peptide drug with 1% chitosan HCl. This chitosan HCl dispersion showed a slight increase in buserelin absorption compared to the control, but it did not manage to increase the buserelin concentrations to the levels achieved with both TMCs. The pharmacokinetic parameters of intraduodenally administered buserelin with or without the polymers are presented in Table 3. TMC60 reached the highest C_{max} and the highest peptide bioavailability. The absolute bioavailabilities of buserelin after co-administration with TMC40 and TMC60 were 6.3 and 13.0% respectively, indicating 8- and 16-fold increases in comparison with intraduodenal administration of buserelin as control.

At the end of the experiments macro- and microscopical investigation of segments of the intestine did not show the occurrence of bleeding or mucosal damage.

DISCUSSION

TMCs represent a novel group of polymeric mucosal permeation enhancers (10–13). In this study two TMCs were evaluated as potent enhancers of the absorption of a particular peptide drug from the small intestine. For this reason, the Caco-2 system was used as an *in vitro* model of intestinal epithelia. The use of the Caco-2 cell cultures as model to predict oral absorption of different types of drugs *in vivo* has been reviewed extensively (20,21) and Caco-2 cells are widely used to study the paracellular permeabilities of hydrophilic compounds. Previously reported data demonstrated that chitosan HCl and TMC with a low degree of substitution (D.S. 20%) are not able to increase the paracellular permeability of ^{14}C-mannitol when applied at pH 7.2 (10). The positive charge density of the chitosan polymers has been found to play an important role in the effect on the tight junction regulation (9,10). With respect to TMCs, TMC60 shows higher permeability enhancement ratios than TMC40, indicating a specific interaction of the quaternized polymers with the negatively charged tight junctions (11). These effects on intestinal permeability could to be clearly differentiated from possible cytotoxicity and cell membrane disruption (12). In the present study the D.S.-dependent effect of TMC on facilitating the paracellular permeation *in vitro* in Caco-2 cells could also be demonstrated *in vivo* in rats. TMC60 in concentrations of 1.0% shows increased intestinal absorption of buserelin when compared with TMC40 as well. Hence, Caco-2 cell monolayers indeed are suitable to predict similar effects of TMC polymers *in vivo*. These observations are in accordance with previously reported studies that the Caco-2 model is a reliable *in vitro* system for screening compounds as candidates for intestinal absorption enhancers of peptide drugs (22,23).

Lueßen *et al.* (6) also studied the intestinal absorption of buserelin after co-administration with chitosan HCl as an absorption enhancer, and reported increased buserelin bioavailability (5.1%). The pharmacokinetic parameters reported in the present study for the same application are lower than those reported by Lueßen *et al.*, but these differences can be explained by the differences in the pH value of the administered formulations and the concentrations. Whereas Lueßen *et al.* have administered a gel of 1.5% chitosan HCl at a pH value of 6.7, 1.0% chitosan HCl at pH 7.2 precipitated to a dispersion. It is well possible, that chitosan HCl, or another chitosan salt, prepared at lower pH value (for instance pH of 6 in which chitosan has higher positive charge density than at pH of 7.2) would lead to similar or even higher increase of the buserelin absorption through the intestine. Nevertheless, the TMC polymers have been synthesized to overcome the disadvantage of chitosan salts, being soluble and effective exclusively at lower pH values.

Buserelin is a peptide drug stabilized against enzymatic degradation. To date, buserelin is administered as a monthly subcutaneous depot formulation or daily intranasal and subcutaneous administrations for suppression of prostate cancer and

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

Polymer	T_{max}(min)	C_{max}(ng/ml)	AUC (ng/ml*min)	F(%)
Control	90	5.8 ± 3.3	510 ± 152	0.8 ± 0.2
Chitosan HCl	20, 120	8.2 ± 3.7	1070 ± 235	1.7 ± 0.4
TMC40	40–60	41.9 ± 12.3[a]	4018 ± 1037[a]	6.3 ± 1.6[a]
TMC60	40	110.9 ± 15.3[a,b]	8199 ± 392[a,b]	13.0 ± 0.6[a,b]

Note: Data are presented as mean ± SE of 6 animals. Abbreviations: T_{max}, time to reach serum peak concentration; C_{max}, serum peak concentration; F, absolute bioavailability. Statistical evaluation: [a], significantly different from the control ($p < 0.005$); [b], significantly different from TMC40 ($p < 0.005$).

treatment of endometriosis and precocious puberty (24). However, the oral route of administration is undoubtedly preferred due to its high patient comfort. To develop solid dosage forms for peroral delivery of drugs is both a challenge for industry and academia, and polymer-based oral delivery systems were as first in sales in the U.S.A. in 1997 (25). As also evident from this study, TMCs are novel polymers with permeation enhancing properties. The absence of cytotoxicity (10–12) in combination with their polymeric features make TMCs, particularly TMC with high degree of substitution, promising excipients for delivery systems of peptide and protein drugs.

CONCLUSIONS

TMCs are a novel class of polymeric permeation enhancers, which are able to open the paracellular pathway in a reversible way. From the present study, it is evident that both TMC40 and TMC60 are able to increase the permeation of the peptide drug buserelin across intestinal epithelia *in vitro* (Caco-2 cell monolayers) and *in vivo* (rats). The type of interactions of the TMCs with the components of the tight junctions requires further investigation.

ACKNOWLEDGMENTS

The financial support of I.K.Y. (State Scholarship Foundation of Greece), Lohmann Therapie Systeme (Andernach, Germany) and Capsugel (Basel, Switzerland) is gratefully acknowledged.

REFERENCES

1. B. J. Aungst, H. Saitoh, D. L. Burcham, S.-M. Huang, S. A. Mousa, and M. A. Hussain. Enhancement of the intestinal absorption of peptides and non-peptides. *J. Contr. Rel.* **41**:19–31 (1996).
2. G. M. Pauletti, S. Gangwar, T. J. Siahaan, J. Aube, and R. T. Borchardt. Improvement of oral peptide bioavailability: Peptidomimetics and prodrug strategies. *Adv. Drug Del. Rev.* **27**:235–256 (1997).
3. A. Fasano. Innovative strategies for the oral delivery of drugs and peptides. *Trends Biotechnol.* **16**:152–157 (1998).
4. P. Artursson, T. Lindmark, S. S. Davis, and L. Illum. Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). *Pharm. Res.* **11**:1358–1361 (1994).
5. L. Illum, N. F. Farraj, and S. S. Davis. Chitosan as a novel nasal delivery system for peptide drugs. *Pharm. Res.* **11**:1186–1189 (1994).
6. H. L. Lueßen, B. J. de Leeuw, M. W. Langemeÿer, A. G. de Boer, J. C. Verhoef, and H. E. Junginger. Mucoadhesive polymers in peroral peptide drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug buserelin in vivo. *Pharm. Res.* **13**:1668–1672 (1996).
7. O. Felt, P. Buri, and R. Gurny. Chitosan: A unique polysaccharide for drug delivery. *Drug Dev. Ind. Pharm.* **24**:979–993 (1998).
8. L. Illum. Chitosan and its use as a pharmaceutical excipient. *Pharm. Res.* **15**:1326–1331 (1998).
9. A. F. Kotzé, H. L. Lueßen, A. G. de Boer, J. C. Verhoef, and H. E. Junginger. Chitosan for enhanced intestinal permeability: Prospects for derivatives soluble in neutral and basic environments. *Eur. J. Pharm. Sci.* **7**:145–151 (1999).
10. A. F. Kotzé, M. M. Thanou, H. L. Lueßen, A. G. de Boer, J. C. Verhoef, and H. E. Junginger. Enhancement of paracellular drug transport with highly quaternized N-trimethyl chitosan chloride in neutral environments: In vitro evaluation in intestinal epithelial cells (Caco-2). *J. Pharm. Sci.* **88**:253–257 (1999).
11. M. Thanou, A. F. Kotzé, T. Scharringhausen, H. L. Lueßen, A. G. de Boer, J. C. Verhoef, and H. E. Junginger. Effect of degree of quaternization of N-Trimethyl chitosan chloride for enhanced transport of hydrophilic compounds across intestinal Caco-2 cell monolayers. *J. Contr. Rel.* (1999). *In Press.*
12. M. M. Thanou, J. C. Verhoef, S. G. Romeijn, J. F. Nagelkerke, F. W. H. M. Merkus, and H. E. Junginger. Effects of N-trimethyl chitosan chloride, a novel absorption enhancer, on Caco-2 intestinal epithelia and the ciliary beat frequency of chicken embryo trachea. *Int. J. Pharm.* **185**:73–82 (1999).
13. A. F. Kotzé, M. Thanou, J. C. Verhoef, and H. E. Junginger. Chitosan and N-trimethyl chitosan chloride as absorption enhancers for nasal and rectal delivery of insulin. *Proceed. Intern. Control. Rel. Bioact. Mater.* **25**:479–480 (1998).
14. A. B. Sieval, M. Thanou, A. F. Kotzé, J. C. Verhoef, J. Brussee, and H. E. Junginger. Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride. *Carbohydr. Polym.* **36**:157–165 (1998).
15. G. Borchard, H. L. Lueßen, A. G. de Boer, J. C. Verhoef, C.-M. Lehr, and H. E. Junginger. The potential of mucoadhesive polymers in enhancing intestinal peptide drug absorption .3. Effects of chitosan-glutamate and carbomer on epithelial tight junctions *in vitro*. *J. Contr. Rel.* **39**:131–138 (1996).
16. A. F. Kotzé, B. J. de Leeuw, H. L. Lueßen, A. G. de Boer, J. C. Verhoef, and H. E. Junginger. Chitosans for enhanced delivery of therapeutic peptides across intestinal epithelia: in vitro evaluation in Caco-2 cell monolayers. *Int. J. Pharm.* **159**:243–253 (1997).
17. H. M. Behre, J. Sandow, and E. Nieschlang. Pharmacokinetics of the gonadotropin-releasing hormone agonist buserelin after injection of a slow release preparation in normal men. *Arz.-Forsch./Drug Res.* **42**:80–84 (1992).
18. M. Gibaldi and D. Perrier. Pharmacokinetics. J. Swarbrick (ed), *Drugs and the pharmaceutical sciences.* Vol. 1, Marcel Dekker, New York: 409–424 (1975).
19. A. J. Hoogstraate, J. C. Verhoef, A. Pijpers, L. A. van Leengoed, J. H. M. Verheijden, H. E. Junginger, and H. E. Boddé. *In vivo* buccal delivery of the peptide drug buserelin with glycodeoxycholate as an absorption enhancer in pigs. *Pharm. Res.* **13**:1233–1237 (1996).
20. P. Artursson and R. T. Borchardt. Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond. *Pharm. Res.* **14**:1655–1658 (1997).
21. F. Delie and W. Rubas. A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model. *Crit. Rev. Ther. Drug Carr. Syst.* **14**:221–286 (1997).
22. H. Lennernäs, K. Palm, U. Fagerholm, and P. Artursson. Comparison between active and passive drug transport in human intestinal epithelial (Caco-2) cells *in vitro* and human jejunum *in vivo*. *Int. J. Pharm.* **127**:103–107 (1996).
23. T. Lindmark, N. G. Schipper, L. Lazorova, A. G. de Boer, and P. Artursson. Absorption enhancement in intestinal epithelial Caco-2 monolayers by sodium caprate: assessment of molecular weight dependence and demonstration of transport routes. *J. Drug Target.* **5**:215–223 (1998).
24. P. M. Conn and W. F. Crowley. Gonadotropin-releasing hormone analogues. *Annu. Rev. Med.* **45**:391–405 (1994).
25. R. Langer. Drug delivery and targeting. *Nature* **392**:5–10 (1998).