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. Langemeijer,2 J. C. Verhoef,1 and H. E. Junginger1,3

Received September 15, 1999; accepted October 9, 1999

INTRODUCTION

The peroral route of administration is still considered the greatest challenge for peptide drug delivery. Poor membrane permeation and/or pre-systemic 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 administration was kept at 7.2, being a representative pH value of peptide analogues which are resistant against enzymatic degradation have been synthesized over the last two decades and administration was kept at 7.2, being a representative pH value.

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 Ca2+ 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 paracellular permeability of the peptide buserelin. For the in vitro 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.

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)
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 methyl iodide 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 counter ion 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 1H-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 cm², not-coated) in Costar Transwell 6-well plates at a seeding density of 10⁶ cells/cm² (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% CO₂ 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·cm². 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-ethanesulfonic 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 at 220nm for their content of buserelin (16). Isocratic elution was performed with K₂HPO₄ 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 Croma 5 10 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