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Preparation and *ex vivo* evaluation of TEC as an absorption enhancer for poorly absorbable compounds in colon specific drug delivery

PARISA YOUNESSI[1]
MOHAMMAD REZA AVADI[2,3]
KOOROUSH SHAMIMI[4]
ASSAL MIR MOHAMMAD SADEGHI[3]
LEILA MOEZI[4]
ELAHEH NAHID[1]
KHOSROW BAYATI[1,3]
AHMAD REZA DEHPOUR[4]
MORTEZA RAFIEE-TEHRANI[1]

[1] *Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

[2] *Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran Iran*

[3] *Hakim Pharmaceutical Company, Tehran Iran*

[4] *Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

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In previous studies, it was established that chitosan and its quaternized derivatives are potent enhancers of hydrophilic compounds absorption across intestinal epithelia. The aim of this study was to evaluate the application of a new quaternized chitosan, triethyl chitosan (TEC), in pharmaceutical approaches. TEC was synthesized by a one step process via a 2^2 factorial design to optimize the preparation conditions. In *ex vivo* experiments, everted rat colon sac was used to determine the effect of TEC on the penetration of hydrophilic compounds of different molecular masses (*e.g.*, sodium fluorescein and brilliant blue) through colonic epithelia in comparison with chitosan at pH 7.4. These studies indicated a significant increase in absorption of sodium fluorescein and brilliant blue in the presence of TEC compared to chitosan. TEC bearing positive charge is able to interact with the tight junctions of colon epithelia and hence increas the permeation of sodium fluorescein and brilliant blue through the tight junctions. This investigation has shown that triethyl chitosan could be used as a penetration enhancer for poorly absorbable compounds in the colon drug delivery system.

Keywords: triethyl chitosan, absorption enhancer, colon

Oral absorption of protein and peptide drugs is hindered by their high molecular mass, hydrophilicity and susceptibility to the proteolytic enzymes in the gastrointestinal tract (1). Therefore, various approaches, such as absorption enhancers, alternative routes and protease inhibitors, have been examined to overcome the delivery problems of these peptides and proteins through the gastrointestinal tract (2).

Deacetylation of chitin, the second most abundant biopolymer isolated from insects, crustaceans such as crab and shrimp as well as from fungi, leads to poly[β-(1,4)-D-glucosamine] or the so called chitosan (3) with excellent biodegradable and biocompatible

* Correspondence, e-mail: rafitehr@ams.ac.ir

characteristics. Because of their permeation enhancing effect, enzyme inhibitory capabilities, and mucoadhesive properties, chitosan and its derivatives are important excipients for peroral peptide delivery systems. Chitosan acts by opening tight junctions between epithelial cells to allow the paracellular transport of hydrophilic molecules (4).

Moreover, there has recently been an increasing interest in targeting peptide and protein drugs as well as oligonucleotides and vaccines to the colon because of the relatively low activity of proteolytic enzymes and their long residence time in colon. In addition, it is more effective to treat colonic diseases such as ulcerative colitis, colorectal cancer and Crohn's disease with a direct delivery of drugs to the affected area (2).

Chitosan is a polycation with an apparent pK_a 5.5 and thus, in neutral and basic environments as found in the large intestine, the chitosan molecules will lose their charge and precipitate from solution (5).

However, it has been shown that *N*-trimethyl chitosan chloride (TMC), a partially quaternized derivative of chitosan with superior solubility (6) could be a useful polymer for the drug delivery system in the large intestine and colon, where a neutral and basic environment prevails (7).

Different alkylated chitosans have been reported (8, 9). One of the relatively new derivatives of chitosan is *N*-triethylated chitosan (TEC), which has not yet been introduced into pharmaceutical approaches. In this investigation, TEC was synthesized and evaluated as a potential absorption enhancer of hydrophilic compounds including brilliant blue and sodium fluorescein. The present study is continuation of our *in vitro* and *ex vivo* studies with different alkylated chitosans as absorption enhancers.

EXPERIMENTAL

Materials

Chitosan (98% deacetylated, viscosity of 1% *m/V* solution is 264 mPa s) was a gift from Primex (Iceland). Ethyl iodide was obtained from Sigma (Austria). Sodium hydroxide, *N*-methyl pyrrolidone (NMP), sodium iodide, sodium fluorescein (M_r 332) and brilliant blue (M_r 792) were purchased from Merck (Germany). All chemicals and solvents were of pharmaceutical and analytical grade and were used as received.

Methods

Synthesis and identification of TEC. – TEC was synthesized as previously described (9). Briefly, chitosan was dispersed in 8 mL of *N*-methyl pyrrolidone and stirred with a magnetic stirrer for 4 hours at room temperature. Then, aqueous sodium hydroxide (20%, *m/V*) solution (1.2 mL), sodium iodide (480 mg) and 3 mL ethyl iodide were added. The mixture was heated at 60 °C for 6 hours under stirring. The product chitosan–$N^+(CH_2CH_3)_3I^-$ was precipitated with acetone and separated by centrifugation. To exchange I^- with Cl^-, the polymer was dissolved in 4 mL of 10% aqueous sodium chloride solution. The polymer was precipitated with acetone, centrifuged and dried to obtain a white water-soluble powder in quantitative yield.

The ^1H NMR spectrum was recorded in D_2O using a 500 MHz spectrometer (Brucker AC500, USA) and the degree of quaternization was calculated. The chemical shift (δ, ppm) was adjusted with external standard (dioxane in D_2O).

Ex vivo *evaluation*. – According to the requirement of the local Ethical Committee, healthy male Wistar rats weighing 200–250 g (three groups of animals, 3 animals in each group), were anesthetized with ether. Animals were kept under 12-h light/dark conditions at 22 ± 1 °C, and fasted for about 12 h prior to the experiment. After verification of the loss of the pain reflex, a midline incision was made and a segment of large intestine (about 3.5 cm) was quickly removed and washed gently with Krebs Ringer Bicarbonate (KRB) solution (sodium chloride 118 mmol L^{-1}, sodium hydrogencarbonate 25 mmol L^{-1}, potassium dihydrogenphosphate 1.19 mmol L^{-1}, calcium chloride 1.03 mmol L^{-1}, magnesium sulfate 1.2 mmol L^{-1}, glucose 5 mmol $L^{-1}$and albumin 1% pH = 7.4) and everted by means of a plastic rod inserted through the lumen. One end of the colon was tied to a needle and the opposite end was tied securely (10, 11). Then, 1 μmol sodium fluorescein or brilliant blue was added to 0.5% TEC in 100 mL KRB, the sac was placed into the solution and saturated with O_2 and CO_2 maintained at 37 °C. Control solutions were prepared without the dissolved TEC polymer. One mL of KRB solution was inserted with a needle to the inside compartment of the sac (12, 13).

Serial samples of an known volume were taken from the inside compartment by means of an insulin syringe every 10 minutes for 1.5 or 2 hours and each time, the volume was replaced with fresh solution (12). The absorbed amounts of sodium fluorescein and brilliant blue were detected by a spectrophotometer NovospecII (UK) at 494 and 634 nm, respectively.

Statistical analysis

The data are expressed as the mean ± SD. For statistical analysis, the two-way analysis of variance was used and followed by Tukey's HSD as *post-hoc* test to compare the means.

RESULTS AND DISCUSSION

A one step synthesis of TEC was carried out (9). The synthesis was based on nucleophilic substitution of chitosan's amine protons with ethyl groups of an ethyl iodide in the presence of sodium iodide and sodium hydroxide in water/NMP medium.

It was shown that a strong inorganic base, such as NaOH, was more suitable than organic bases (such as amines with similar alkalinity to that of chitosan) to neutralize the acidity of hydrophilic acid produced by the reaction. Studies have shown that sodium hydroxide and ethyl iodide are the two most effective reaction variables in the synthesis of triethyl chitosan (10). Table I shows four reaction conditions in triplicate based on the experimental design. Optimum amounts of the reactants were found to be 37.5 mmol of ethyl iodide (3.0 mL) and 6 mmol NaOH (1.2 mL, 20%) (formulation A_4, Table I), as the highest degree of quaternization (66%) was achieved.

Table I. The effect of reaction conditions on the degree of quaternization

Formulation	NaOH (%, *m*/*m*)/C_2H_5I (mL)	Degree of quaternization (%)
A1	20.0/1.5	60%
A2	10.0/1.5	36%
A3	10.0/3.0	46%
A4	20.0/3.0	66%

The ^1H NMR spectrum of TEC is shown in Fig. 1. The signal at 1.2 ppm was attributed to the CH_3 groups of the ethyl substituent, while H_2–H_6 protons of the polysaccharide backbone superimposed the CH_2 groups. The intense band at 4.8 ppm was related to HDO (a proton-exchanged species of D_2O with OH and amine). In this region, as observed more clearly from an extended spectrum, three anomeric protons (H_1S) appeared at 4.23, 4.98 and 5.1 ppm. They were attributed to the mono-*N*-acetylglucosamine unit, mono and di-*N*-alkyl substitued glucosamine units, respectively. The integral of these signals as well as the CH_3 of ethyl groups were used to calculate the degree of quaternization (11).

A model rat cannulated everted intestine could eliminate the possible variables due to blood flow and permit serial sampling from the serosal contents (13). Moreover, the everted intestinal sac has the advantage of simplicity and flexibility (12). Barthe *et al.* (14) noted that the values explained in caco-2 cell monolayers, which is one of the established *in vitro* models for intestinal epithelia, might exaggerate the potential of polymers for realistic absorption enhancement. The everted intestinal sac model may give more realistic values for intestinal function (15).

Using inverted sacs for absorption studies an O_2 and CO_2 mixture was bubbled into intestinal mucosa to increase or obtain peristaltic intestinal movement.

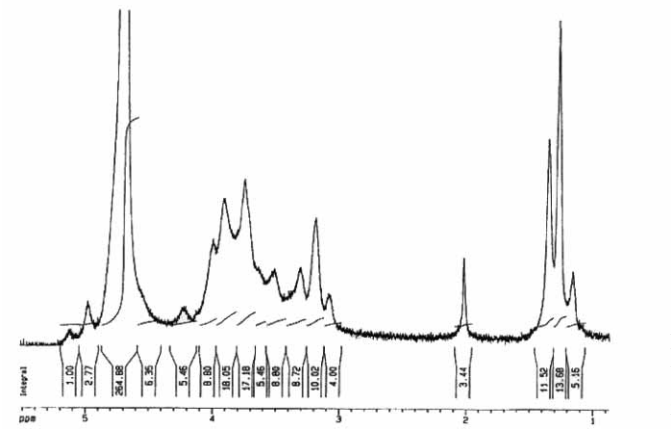

Fig. 1. ^1H NMR spectrum of triethyl chitosan chloride (degree of quaternization 66%) in D_2O.

In the present study we have shown that TEC, which has better solubility than chitosan and does not need an acidic environment for its activity, has significantly increased the intestinal absorption of both sodium fluorescein and brilliant blue compared to chitosan (Figs. 2a and 2b). TEC, with the involvement of actin filaments of the intestinal epithelium, was able to increase the permeation of the hydrophilic compounds through the tight junctions. It has been reported that chitosan induces a redistribution of cytoskeletal F-actin and the ZO-1 protein, thereby altering the integrity of tight junctions (16). Confocal laser scanning microscopy has also confirmed that chitosan and TMC are able to open the tight junctions to allow the paracellular transport of large hydrophilic compounds (17, 18).

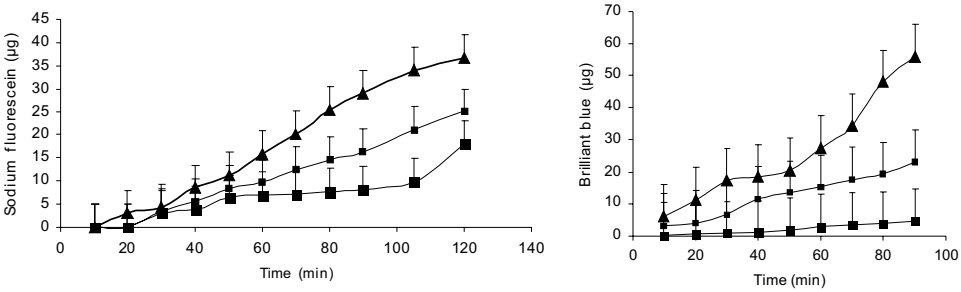

Fig. 2. a) Sodium fluorescein alone (■), added chitosan (▪), added TEC (▲) (*n* = 3); b) brilliant blue alone (■), added chitosan (▪) added TEC (*n* = 3). Bars denote SD values.

In addition, TEC effectiveness as permeation enhancer is more pronounced in the case of fluorescein compared to brilliant blue probably due to the lower molecular mass of fluorescein (19).

CONCLUSIONS

It has been shown that TEC with superior water solubility could be a potential absorption enhancer of hydrophilic compounds, including sodium fluorescein and brilliant blue, in colon and is promising for colon-specific drug delivery.

It has been also concluded that the potential use of TEC can be an important contribution to the development of effective delivery systems for hydrophilic compounds such as peptide drugs, especially in the more neutral and basic environment of the large intestine and colon where the efficacy of chitosan as an absorption enhancer is not so strong. However, more *in vitro* studies in caco-2 cell and *in vivo* experiments are required to confirm TEC as an enhancing agent.

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S A Ž E T A K

Priprava i *ex vivo* evaluacija TEC kao promotora apsorpcije tvari u koloni

PARISA YOUNESSI, MOHAMMAD REZA AVADI, KOOROUSH SHAMIMI, ASSAL MIR MOHAMMAD SADEGHI, LEILA MOEZI, ELAHEH NAHID, KHOSROW BAYATI, AHMAD REZA DEHPOUR i MORTEZA RAFIEE-TEHRANI

U ranijim istraživanjima utvrđeno je da su kitozan i njegovi kvaternizirani derivati snažni promotori apsorpcije hidrofilnih spojeva kroz intestinalnu sluznicu. Cilj rada bio je evaluirati novi kvaternizirani kitozan, trietilkitozan (TEC). TEC je sintetiziran u jednom stupnju. U *ex vivo* eksperimentima na kolonu štakora praćen je učinak tog polimera na penetraciju hidrofilnih spojeva različitih molekulskih masa (fluorescein natrija i briljant plavila). Rezultati su uspoređivani s učinkom kitozana pri pH 7,4. Primijećeno je da TEC značajno povećava apsorpciju ispitivanih tvari u odnosu na nemodificirani kitozan. TEC svojim pozitivnim nabojem dolazi u interakciju s epitelom kolona i tako povećava njegovu permeabilnost. Ispitivanja ukazuju da se trietilkitozan može upotrijebiti kao promotor penetracije za spojeve koji se slabo apsorbiraju u kolonu.

Ključne riječi: trietilkitozan, promotor apsorpcije, kolon

Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Hakim Pharmaceutical Company, Tehran, Iran

Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran