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Mob: +92 300 3008585, Fax: +92 41 8815544
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Research Article
p-Aminobenzoic Acid-chitosan Conjugates for PABA Delivery to the Large Intestine

[1,2]Sirinporn Nalinbenjapun and [1,2]Chitchamai Ovatlarnporn

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, 90112 Songkhla, Thailand
[2]Drug Delivery System Excellence Center, Prince of Songkla University, Hat Yai, 90112 Songkhla, Thailand

Abstract

Background: The *p*-aminobenzoic acid (PABA) is an essential nutrient and important substrate for folic biosynthesis in human. The PABA deficiency can cause many symptoms and diseases which may related to folic acid insufficiency. In this study, chitosans with three different molecular weights (30, 80 and 300 kDa) were selected as a macromolecule carrier for the attachment of PABA for pharmaceutical and nutritional applications. **Materials and Methods:** The first step, amino groups of chitosan were substituted by *p*-nitrobenzoyl moiety resulting in *p*-nitrobenzoyl-chitosans (1a-c). The PABA-chitosan conjugates (2a-c) were finally obtained by sodium dithionite reduction process. They were characterized for their functional groups by FT-IR and PABA loading capacity by HPLC. **Results:** The products of the first step (1a-c) were obtained in good yields (82.49-90.67%) with high purity. The PABA-chitosan conjugates (2a-c) were achieved from the second step also in high yields (82.26-91.86%) and purity. The FT-IR results of 2a-c displayed the C=O stretching, amide II deformation of N-H group and the C-O stretching at 1632.58-1638.00, 1550.08-1559.93 and 1036.58-1040.09 cm^{-1}, respectively. The HPLC results demonstrated that PABA can be loaded onto chitosan in a range of 11.07-23.02% according to the MW of chitosans. **Conclusion:** These PABA-chitosan conjugates are suitable to delivery PABA to the large intestine and colon where the biodegradation process of chitosan and the cleavage of the attached PABA occur. The released PABA can be utilized as a substrate for folic acid synthesis and for the treatment of PABA deficiency syndrome.

Key words: Chitosan, PABA, conjugate, delivery, large intestine, colon, folic acid, biodegradable, polymer

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Corresponding Author: Chitchamai Ovatlarnporn, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, 90112 Songkhla, Thailand

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The *para*-aminobenzoic acid (PABA, Fig. 1) is an aromatic amino acid belongs to the vitamin B group[1,2]. The PABA has been known as a biosynthetic component of folic acid by bacteria including those found in human intestinal tract such as *Escherichia coli*[3,4] as well as biofidobacteria in the large intestine[5-7]. The PABA is thought to play a role in melanin formation[8]. It inhibits oxidative destruction of epinephrine and stilbestrol to block hair graying[9]. Moreover, PABA functions as a co-enzyme in the conversion of a number of precursors to purines[10]. Recently, it been discovered to be important in interferon synthesis and will assist the antiviral effect of antiviral agents[11,12]. In addition, many other important functions of PABA have been reported such as antioxidant[13] and anticoagulant[14]. The PABA deficiency in human may cause many symptoms such as fatigue, irritability, depression, nervousness and hair graying[15]. Potassium aminobenzoate or Potaba® is the most commonly prescribed to treat skin disorders (e.q., dermatomyositis, scleroderma and peyrenie's disease) cause by PABA deficiency[16] by orally administration with food 4-6 times daily. However, PABA is extremely and rapidly absorbed from the small intestine due to its lipophilicity[17]. It is therefore, a small amount of PABA of conventional administration method will reach at the large intestine where the folic acid biosynthesis occur[5].

Polymer-drug conjugates is one of among many successful approaches the deliver active drug to the target site especially at the large intestine or colon[18]. There were a number of polymer carriers have been extensively investigated for delivery of the drug to the large intestine or colon including polyethylene glycol[19], dendrimer[20] and cyclodextrin[21]. Recently, we reported the synthesis and characterization of PABA-hydroxypropyl cellulose (PABA-HPC) conjugates for pharmaceutical application[22].

In this study, we would like to develop PABA-polymer conjugates by using chitosan as a carrier for delivery PABA to the large intestine. Chitosan is suitable polymer carrier for drug delivery system to the large intestine or colon, since it has biodegradability and biocompatibility with low oral toxicity (LD_{50} in rats of 16 g kg^{-1})[12]. It was reported that chitosan is not degraded in the upper GI tract but it was degraded by enzyme produced by microorganisms, located in the large intestine or colon[23]. Moreover, chitosan has many functional groups such as amino and hydroxyl groups which can be attached the drug for delivery system. Therefore, the aim of this study is to synthesize and characterize the PABA-chitosan conjugates using chitosan as a drug carrier.

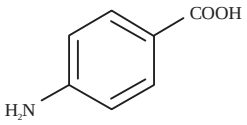

Fig. 1: Chemical structure of *p*-aminobenzoic acid (PABA)

Three different molecular weights of chitosan were utilized in this study in order to investigate the effect on drug loading capacity. The obtained PABA-chitosan conjugates were well characterized.

MATERIALS AND METHODS

Chitosan with molecular weight 30 kDa (80% degree of deaceylation (DD)), 80 kDa (85% DD), 300 kDa (80% DD) were obtained from Seafresh Chitosan (Lab) Co., Ltd., Thailand. The *p*-nitrobenzoyl chloride was analytical grade and purchased from Fluka, Germany. Sodium dithionite was analytical grade and purchased from Sigma-Aldrich, Germany. Dialysis bag (cellulose tubular membrane MW cut off 12000-14000) was purchased from Membrane Filtration Products, USA. The FT-IR spectra were acquired using a Perkin-Elmer spectrum one FT-IR spectrometer. High-performance liquid chromatography (HPLC) was carried out using a system based on an agilent 1100 series pump with photodiode-array (PDA) detection.

Preparation of PABA-chitosan conjugates: The PABA-chitosan conjugates (2a-c) were synthesized via two steps procedures (Fig. 2). First, *p*-nitrobenzoyl-chitosan (1a-c) were prepared by reaction between chitosan and *p*-nitrobenzoyl chloride. The *p*-aminobenzoyl-chitosan or PABA-chitosan conjugates (2a-c) were finally obtained by reduction of the nitro groups of *p*-nitrobenzoyl-chitosan using sodium dithionite.

Preparation of *p*-nitrobenzoyl-chitosan (1a-c): A solution of *p*-nitrobenzoyl chloride (11.13 g, 60 mmol) in CH_2Cl_2 (120 mL) was slowly added to a solution of chitosan (CS, MW ~30, 80 and 300 kDa) (5 g and 30 meq/GlcN) in 2% acetic acid (250 mL) at room temperature. An aqueous solution of KOH (8 g, 0.03 mol, 20 mL) was then added dropwise to the mixture and continued stirring at room temperature for 1 h. The emulsion formed was destroyed by heating to 50°C to remove methylene chloride. After the reaction, the mixture was dialyzed against distilled water for 2 days to remove any impurities and byproducts. The products were finally obtained by lyophilization and characterized by FT-IR.

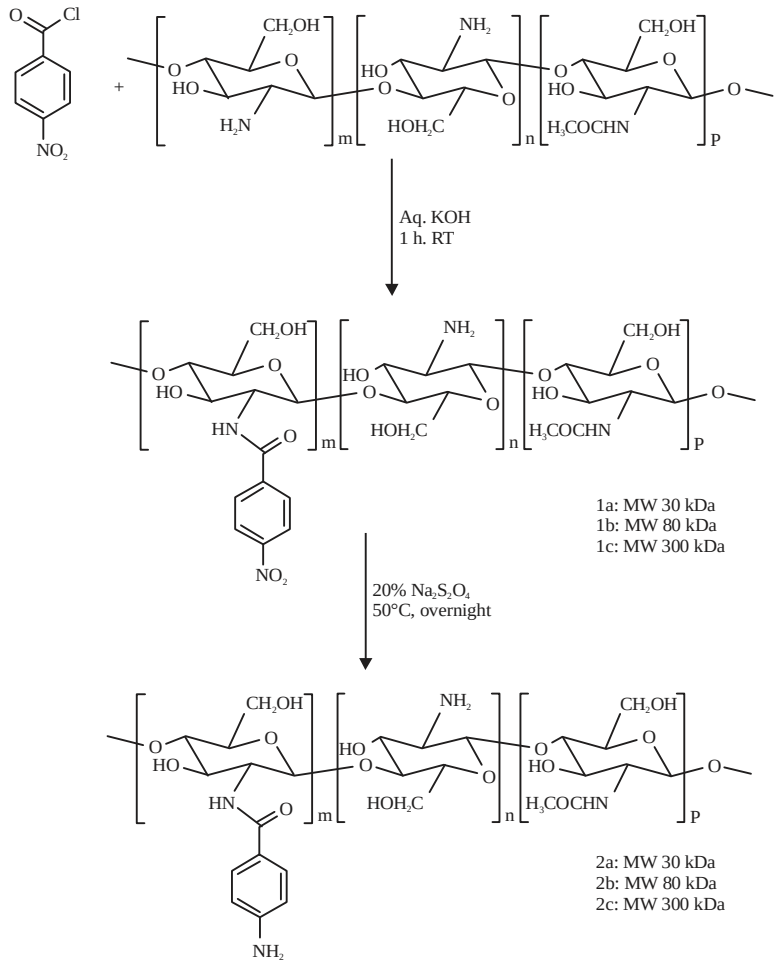

Fig. 2: Synthesis scheme of PABA-chitosan conjugates (2a-c)

Preparation of *p*-aminobenzoyl-chitosan conjugates (PABA-CS) (2a-c): The previously obtained *p*-nitrobenzoyl-chitosans (1a, from CS MW 30 kDa, 1b from CS MW 80 kDa and 1c from CS MW 300 kDa) (6 g, 35.5 meq/GlcN) were suspended in 20% $Na_2S_2O_4$ solution (360 mL). The resulting mixture was stirred at 50°C overnight. The resulting solution was dialyzed against distilled water for 2 days to remove byproducts and the remaining of the reducing agent. The products were finally obtained by lyophilization and characterized by FT-IR. Products (2a-c) were characterized by FT-IR and the PABA loading capacity was analyzed by HPLC as following procedure.

Determination of PABA loading capacity in PABA-chitosan conjugates (2a-c): The PABA content of each conjugate was measured by alkaline hydrolysis and the released PABA was analyzed by HPLC. Each conjugate (10 mg) was added to 1 N NaOH (10 mL) and the mixture was stirred at 90°C for 24 h. The sediment that formed on standing was discarded and the supernatant was collected and analyzed PABA by HPLC. The PABA content was calculated from the calibration curve of PABA prepared in similar process.

Analysis of PABA in PABA-chitosan conjugates (2a-c) by HPLC: The method for determination of PABA was performed by a reverse phase HPLC using a modified method of Dhananjeyan *et al.*[24]. The liquid chromatography system consisted of an agilent pump 1100 series and photodiode-array (PDA). The output signal was monitored and processed using Agilent Chemstation Plus. The HPLC column Phenomenex® C18 column, 250×4.6 mm containing Luna 5 µm packing was used for analysis of PABA. A mixture of acetonitrile and 10 mM ammonium acetate pH 4 (15:85) was used as a mobile phase. The flow rate of mobile phase was 1.0 mL min^{-1}. The sample injection volume was 20 µL and the detection wavelength was set at 290 nm.

RESULTS AND DISCUSSION

In this study, attempts to synthesis the polymer-PABA conjugation system for delivery of PABA directly to the large intestine by using chitosan as a carrier have been attempted. The attached PABA will be released from chitosan carriers by biodegradation process in the large intestine. The released PABA can be absorbed across the large intestine to play its biological effect as well as a precursor for the biosynthesis of folic acid by colonic microflora[4]. The preliminary experiment was performed by simple coupling reaction of PABA with chitosan by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as a coupling agent. However, no desired product was obtained, only self-coupling of PABA was detected. Another attempt was made by using amino group protected-PABA by benzaldehyde using the same coupling process also demonstrated that no desired product was obtained. That could be due to the phase separation during the coupling reaction.

Finally, two-step synthesis process was acquired for the synthesis of PABA-chitosan conjugates. The first step was the substitution reaction of chitosan by using *p*-nitrobenzoyl chloride as a substrate. The reaction was performed in the presence of solution of KOH at room temperature for 1 h. The KOH was added to the reaction in order to promote the reaction go forward by removing HCl byproducts. The products (1a-c) were obtained as pale yellow and white powders. The FT-IR spectrum of *p*-nitrobenzoyl-chitosan conjugates (1a-c) are shown in Fig. 3 depicted prominent board bands at 3405.25-3422.61 cm^{-1} due to OH and NH_2 groups of chitosan. The peak at 1726.74-1728.82 cm^{-1} corresponded to the C=O stretching of ester groups which may be formed between the reaction of OH groups of chitosan with *p*-nitrobenzoyl chloride. The absorption bands at 1654.00-1655.42 and 1546.34-1550.69 cm^{-1} are corresponded to the amide I of C=O stretching and amide II deformation of N-H group, respectively[25]. The absorption bands at 1523.39-1524.82 and 1348.22-1350.45 cm^{-1} attributed to the vibrational mode of symmetric and asymmetric stretching from the NO_2 group ($ArNO_2$). The absorption bands at 844.66-845.09 cm^{-1} are relative to the C-N stretching of $ArNO_2$ and the bands at 719.60-720.56 cm^{-1} belong to the deformation of NO_2 group. The FT-IR characteristics were in the same region to the previously reported data of *p*-nitrobenzyl-hydroxypropyl cellulose[26], *p*-nitrobenzyl cellulose[27], *p*-nitrobenzoyl cellulose[28] and 2, 3, 4-nitrobenzoylated cellulose[29]. The three peaks at 1110.13-1111.53, 1068.30-1070.59 and 1019.51-1031.05 cm^{-1} are corresponded to the symmetric stretching of C-O-C which involved skeletal vibration of the C-O stretching. The yields of the obtained products (1a-c) were 82.49, 87.55 and 90.67%, respectively (Table 1).

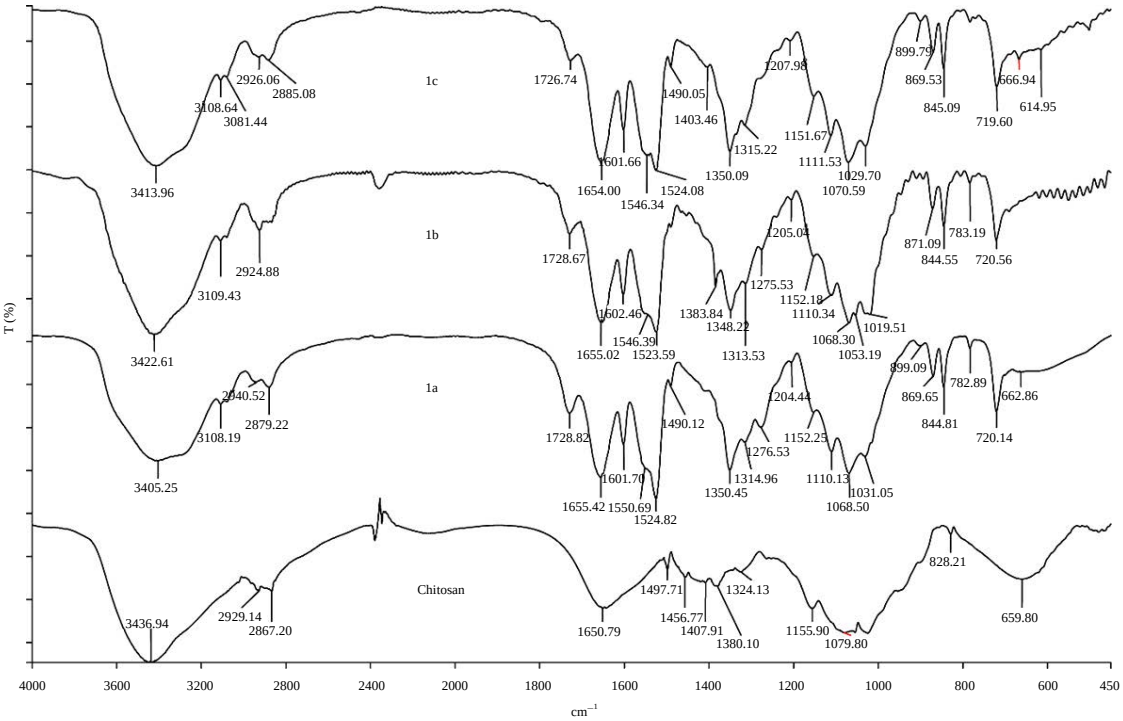

Fig. 3: FT-IR spectra of chitosan and *p*-nitrobenzoyl-chitosans (1a-c)

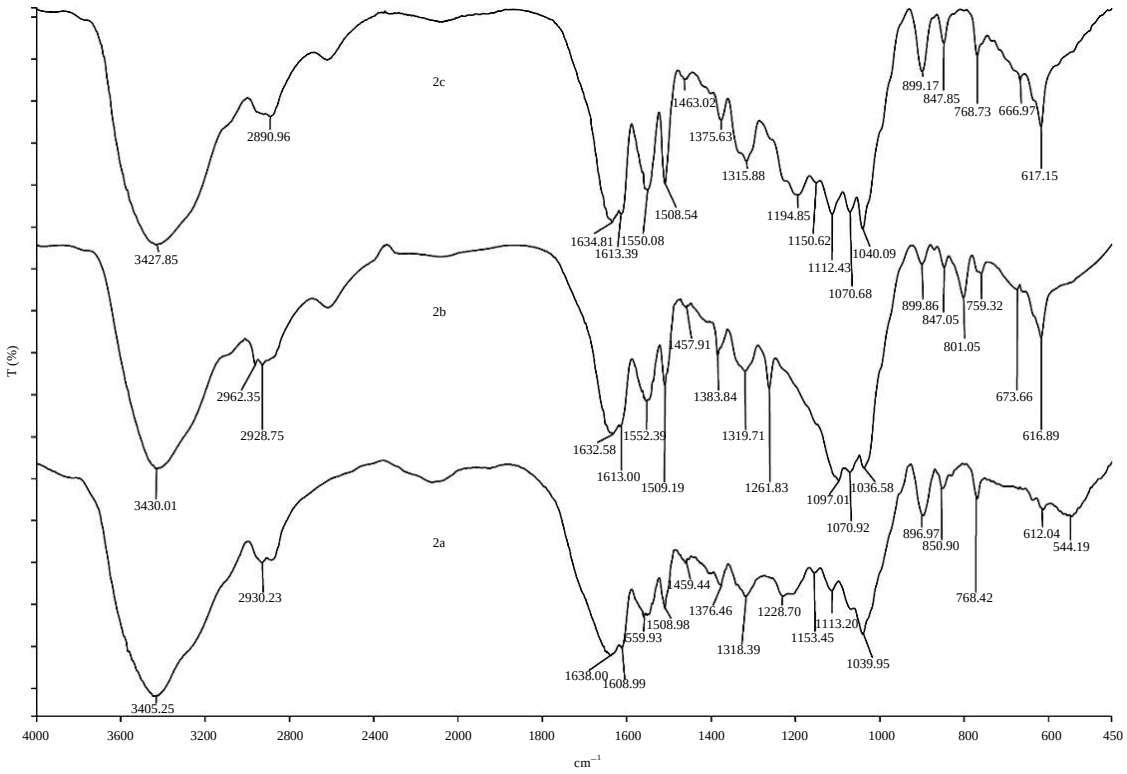

Fig. 4: FT-IR spectra of PABA-chitosan conjugates (2a-c)

Table 1: Percentage yields of the obtained products 1a-c and 2a-c

Compounds	Product yield (%)
1a	82.49
1b	87.55
1c	90.67
2a	89.26
2b	90.88
2c	91.86

Table 2: Percentage of PABA loading capacity of PABA-chitosan conjugates (2a-c)

Compounds	PABA loading capacity (% w/w)
2a	11.07±0.38
2b	22.32±1.78
2c	23.02±1.03

The *p*-aminobenzoyl-chitosan conjugates (2a, from CS MW 30 kDa, 2b from CS MW 80 kDa and 2c from CS MW 300 kDa) were achieved by reduction of *p*-nitrobenzoyl-chitosan (1a-c) using sodium dithionite as a reducing agent at 50°C for 24 h[25]. The *p*-aminobenzoyl-chitosan conjugates (2a-c) were obtained as white powders after lyophilization. The FT-IR spectrum of *p*-aminobenzoyl-chitosan (2a-c) are depicted in Fig. 4 displayed a broad absorption band at 3405.25-3430.01 cm^{-1} is corresponding to the O-H and N-H stretching[22,26]. The peaks at 1632.58-1638.00 and 1550.08-1559.93 cm^{-1} are corresponded to the amide I of C=O stretching and amide II deformation of N-H group, respectively. Absorption band at 1036.58-1040.09 cm^{-1} is corresponded to the C-O stretching. These FT-IR informations were similar to the previous reported values[30,31], however, different methods of preparation were utilized. The absorption band of NO_2 group of 2a-c at 1523.39-1524.82, 1348.22-1350.45 cm^{-1} was observed to decrease in intensity in comparison to that of *p*-nitrobenzoyl-chitosan (1a-c, Fig. 3) and disappearance of C-N peak of $ArNO_2$ at 847.05-845.90 cm^{-1} and NO_2 deformation at 759.32-768.73 cm^{-1} was observed indicating that the NO_2 group was reduced to NH_2. The product yields of 2a-c were 89.26, 90.88 and 91.86%, respectively (Table 1). The amounts of the loading content of PABA on the PABA-chitosan conjugates (2a-c) were determined by alkali hydrolysis (Fig. 5) and subsequently analyzed the released PABA by HPLC method. The amount of released PABA of each conjugates were calculated from the calibration curves of PABA (retention time = 6.7 min). The standard curve of PABA displayed linearity (Y = 126.8x-18.793) in the range of 2-10 μg mL^{-1}. The percentage loading capacities of 2a-c are summarized in Table 2. The results demonstrated that using chitosan with high MW (80000 or 300000) provided higher PABA loading than using chitosan with low MW (30000).

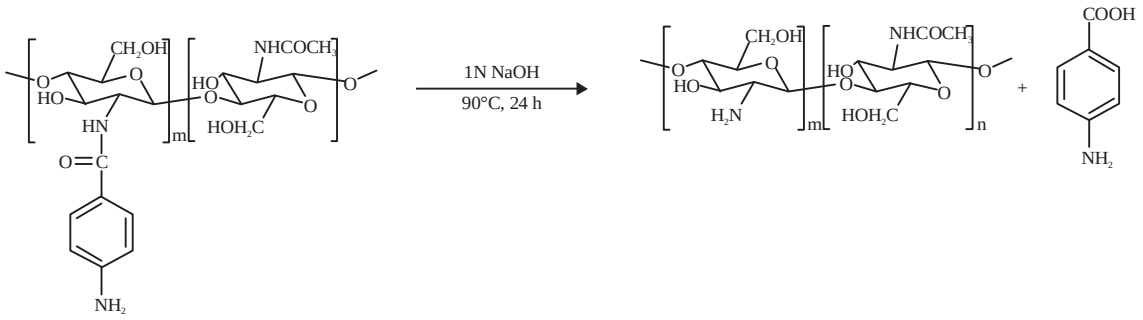

Fig. 5: Scheme of alkali hydrolysis of PABA-chitosan conjugates (2a-c)

This could be due to higher MW chitosan has longer chain and has more active functional groups which can be substituted by *p*-nitrobenzoyl moiety better than the shorter chain one.

The obtained PABA-chitosan conjugates (2a-c) could be utilized for deliver PABA by oral administration. This system will protect the release of PABA in stomach and will specifical release PABA at the large intestine where the biodegradation process occurs. At the large intestine, chitosan can be biodegraded by enzymes produced by colonic microflora resulting in small units of chitosan[32]. A number of enzymes in large intestine that can degrade chitosan are lysozyme, β-*N*-acetylhexosaminidase, chitosanase, chitinase and chitin deacetylase[33,34]. Moreover, chitosan can also be fermented by bifidobacteria[35] resulting in chitosan oligosaccharide. The resulting PABA-small units of chitosan will be consequently cleaved by amidase in the large intestine[36] to give free PABA. The released PABA can be absorbed via colon and PABA can also be a substrate for folic biosynthesis.

CONCLUSION

In this study PABA-chitosan conjugates having three different MWs were successfully synthesized by using three different MWs of chitosan (30, 80 and 300 kDa). They can be obtained by two steps process. The *p*-nitrobenzoyl-chitosan were prepared by substitution reaction between chitosan and *p*-nitrobenzoyl chloride in the presence of base. The nitro groups of the obtained products were further reduced by using sodium dithionite at 50°C to give *para*-amino substituted aromatic moiety. The resulting PABA-chitosan conjugates (2a-c) were finally obtained in high yields with high purity and were well characterized. The obtained PABA-chitosan conjugates has potential to be utilized for delivering PABA to the large intestine and colon to provide PABA at the target site for further absorbed or as a substrate in folic biosynthesis.

SIGNIFICANT STATEMENTS

Clear explanation of the importance of this study in a process of preparation PABA-chitosan conjugates and relevance of the study to the PABA deficiency related diseases which can be treated by the developed system are explained in the introduction part.

ACKNOWLEDGMENTS

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