In Vivo Evaluation of Thiolated Chitosan Tablets for Oral Insulin Delivery

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ABSTRACT: Chitosan–6-mercaptonicotinic acid (chitosan–6-MNA) is a thiolated chitosan with strong mucoadhesive properties and a pH-independent reactivity. This study aimed to evaluate the *in vivo* potential for the oral delivery of insulin. The comparison of the nonconjugated chitosan and chitosan–6-MNA was performed on several studies such as mucoadhesion, release, and *in vivo* studies. Thiolated chitosan formulations were both about 80-fold more mucoadhesive compared with unmodified ones. The thiolated chitosan tablets showed a sustained release for 5 h for the polymer of 20 kDa and 8 h for the polymer of 400 kDa. Human insulin was quantified in rats' plasma by means of ELISA specific for human insulin with no cross-reactivity with the endogenous insulin. *In vivo* results showed thiolation having a tremendous impact on the absorption of insulin. The absolute bioavailabilities were 0.73% for chitosan–6-MNA of 20 kDa and 0.62% for chitosan–6-MNA 400 kDa. The areas under the concentration–time curves (AUC) of chitosan–6-MNA formulations compared with unmodified chitosan were 4.8-fold improved for the polymer of 20 kDa and 21.02-fold improved for the polymer of 400 kDa. The improvement in the AUC with regard to the most promising aliphatic thiomer was up to 6.8-fold. Therefore, chitosan–6-MNA represents a promising excipient for the oral delivery of insulin. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3165–3170, 2014

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INTRODUCTION

Insulin was introduced in clinical practice in 1922, and since then, numerous attempts have been made to find alternative routes of its administration. At the moment, to maintain the blood glucose level at a near-physiological level, treatment of insulin-dependent diabetes requires up to four subcutaneous injections per day.[1] Generally, the parenteral routes are satisfactory in terms of efficacy in the majority of cases. However, a subcutaneous administration of insulin can result in peripheral hyperinsulinemia, the stimulation of smooth muscle cell proliferation, and the incorporation of glucose into the lipid arterial walls leading to diabetic micro- and macroangiopathy.[2] Moreover, daily injections have the disadvantage of very low patient compliance, physiological stress, pain, inconvenience, high risk, cost, and risk of infections. Modern delivery technology, being patient friendly, has shown a great interest in researching noninvasive routes of administration for insulin delivery. Among the alternative routes of administration, oral, nasal buccal, pulmonary, transdermal, rectal, and ocular drug delivery systems have been studied. The oral route is the preferred one. A big advantage of the oral route is that fate of insulin orally given is similar to the physiological route, and could therefore provide a better glucose homeostasis. However, when orally administered insulin is degraded in the acidic environment of the stomach and by digestive enzymes,especially in the small intestine. Furthermore, the epithelial surfaces of the gastrointestinal (GI) tract itself present an effective barrier to the absorption of insulin.[3] Therefore, the delivery system should protect insulin from the harsh environment of the GI tract. Additionally, an optimal delivery system for the oral administration of insulin should prolong its intestinal residence time, and reveal no toxicity.[4] Thiomers fulfill both these criteria. Thiolated polymers have been developed as a category of mucoadhesive polymers with reactive thiol groups immobilized on the polymer backbone. They have been shown to protect peptides from enzymatic degradation.[5] Furthermore, they have been shown to possess high mucoadhesive properties. The thiol groups present on the polymer backbone can form disulfide bonds with the thiol groups present on the mucus substructures.[6] Recently, a thiolated chitosan named chitosan–6-mercaptonicotinic acid (chitosan–6-MNA) has been synthesized. The polymer showed high mucoadhesive properties and showed not to be toxic.[7] The particularity of this polymer is its pH-independent reactivity of the thiol groups, rendering it active through the whole range of GI pH values. The aim of the study was to develop an oral insulin delivery system by exploiting the mucoadhesive and protective effect of chitosan–6-MNA. The formulation was tested *in vitro* regarding its mucoadhesive properties and drug release and *in vivo* by measuring the insulin plasma level.

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MATERIALS AND METHODS

Materials

6-Mercaptonicotinic acid (6-MNA), dioxane, N-3(dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDAC), human insulin and fluorescein isothiocyanate-dextran (FD4) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Chitosan medium molecular mass (400 kDa) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were obtained from Fluka (Buchs, Switzerland). ELISA kits for human insulin were purchased from Mercodia, Uppsala, Sweden. WHO Insulin Standard (66/304) used for ELISA measurements was purchased from NISBC, United Kingdom. Rat citrate plasma from fasted rats used for ELISA measurements was obtained from Harlan-Winkermann, Borchen, Germany.

Synthesis of Chitosan–6-MNA

Chitosan–6-mercaptonicotinic acid was synthesized as described previously.[7] Briefly, 2 g of chitosan with 20 and 400 kDa molecular mass was hydrated in 16 mL 1 M HCl and dissolved in water to obtain a 1% (m/v) solution. Afterward, 5 g of 6-MNA previously dissolved in 200 mL of a dioxane–water mixture (160 + 40 mL) was slowly added under continuous stirring. Afterward, EDAC was added in a final concentration of 12.5 mM, and the pH was adjusted to 6. The reaction was allowed to proceed for 7 h. TCEP was then added in a final concentration of 10 mM and let to react for 30 min. The resulting reduced thiomer was dialyzed in tubings against an acidic and saline media. The polymer was then frozen and freeze dried.

Quantification of Thiol Groups

The total amount of thiol groups present was measured as follows. 6-MNA exhibits an absorption maximum in the range of 260–280 nm in the UV–vis range. The freeze-dried conjugates were dissolved in a 0.1 M acetate buffer pH 3.3–dioxane mixture (1 + 1) in a final concentration of 0.2 mg/mL. The amount of ligand bound to chitosan was calculated from the corresponding calibration curve made with 4-mercaptobenzoic acid (0–0.16 μmol/mL) dissolved in 0.1 M acetate buffer pH 3.3–dioxane mixture (1 + 1) with absorbance values at 268 nm (Beckman DU 650 spectrophotometer; Brea, Carlifornia, USA) from 0 to 0.9 giving origin to the following equation: $y = 7.3574x - 0.003$; $R^2 = 0.9987$.

The amount of thiol groups in their reduced form was measured via iodometric titration.[8] Briefly, different concentrations of the conjugate have been prepared (0.5–0.05 mg/mL) in 0.5 M acetate buffer pH 2.7. Then, 1 mL starch aqueous solution (1%, m/v) was added to 2 mL of each sample concentration. Afterward, 0.1 M iodine was added until the majority of the samples of each conjugate displayed a blue-violet color. The oxidation reaction was let to proceed for 24 h, protected from light. A calibration curve was elaborated under the same conditions with cysteine–HCl solution in a concentration of 10–40 μmol/L. The excess iodine that reacts with starch was measured at 560 nm. The following equation was obtained: $y = -54.648x + 2.0563$; $R^2 = 0.9974$.

Preparation of the Oral Dosage Form

The polymers and insulin in a ratio of polymer:insulin of 1:4 were mixed together in an aqueous solution for 30 min. The mixture was then frozen and lyophilized. Lyophilized thiolated chitosan conjugates and unmodified chitosan were compressed into 10 mg flat-faced tablets with a single punch eccentric press (Paul Weber, Remshalden, Germany). The compaction pressure of 5 kN was kept constant during the preparation of all tablets. The composition was polymer without any other excipients and drug.

Thirty milligrams of the lyophilized polymer–insulin mixture was compressed into 5 mm diameter flat-faced discs.

Preparation of Insulin Solution for Intravenous and Subcutaneous Injection

Intravenous injection served to calculate the absolute bioavailability of the orally given tablets. 27.5 μg of human insulin was dissolved in 0.2 mL of a sterile phosphate buffer saline at pH 7.5, then filtered through a cellulose filter unit (pore size 0.22 μg) and subsequently injected. Subcutaneous injections served to calculate the relative bioavailability. 0.55 μg of human insulin was dissolved in 0.2 mL of a sterile phosphate buffer saline at pH 7.5, then filtered through a cellulose filter unit (pore size 0.22 μg) and a subcutaneous injection was performed.

In Vitro Mucoadhesion Studies

Thirty milligrams of the lyophilized polymer–insulin mixture was compressed into 5 mm diameter flat-faced discs. Afterward, the tablets were placed on a freshly excised intestinal porcine mucosa, which was previously attached to a stainless cylinder. The cylinder was immersed in the dissolution apparatus containing 1 L of 0.1 M phosphate buffer pH 6.8 at 37°C. The cylinder was rotated with 100 rpm. The detachment of the tablets was visually recorded. In order to evaluate whether the incorporation of the drug has an impact on the integrity of the tablet and the mucoadhesive properties of the polymer, tablets composed of 100% polymer were tested as well.

In Vitro Release Studies from the Polymer–Insulin Tablets

The *in vitro* release rate of insulin out of the prepared tablets was determined in 10 mL of the release medium consisting of 0.1 M phosphate buffer pH 6.8 containing 30% dimethyl sulfoxide (DMSO). The samples were incubated in an oscillating water bath at 37°C. Aliquots of 1 mL were withdrawn at 1 h intervals and replaced with an equal volume of release medium pre-equilibrated at 37°C. The amount of insulin released was evaluated by HPLC. The concentrations were quantified from integrated peak areas and calculated by interpolation from an according standard curve.

HPLC Analysis

The mobile phase consisted of eluent A:acetonitrile and eluent B:0.1% (v/v) trifluoroacetic acid. A gradient elution was performed with a flow rate of 1 mL/min over 0–22 min (linear gradient from 91% A/9%B to 39% A/61% B). Insulin was analyzed quantitatively at a wavelength of 254 nm. For determining the linearity of the system, eight concentrations in the range from 2 to 0.03 mg/mL of insulin were prepared and analyzed.

In Vivo Evaluation of the Delivery Systems

The protocol for the study on animals was approved by the Animal Ethical Committee of Vienna, Austria and adhered to the principle of Laboratory Animal Care. Healthy male Wistar rats with a mean body weight of 250 g were used. Rats