Nasal Delivery of Insulin Using Novel Chitosan Based Formulations: A Comparative Study in Two Animal Models between Simple Chitosan Formulations and Chitosan Nanoparticles

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Received November 28, 2001; revised February 18, 2002; accepted April 2, 2002

Purpose. To investigate whether the widely accepted advantages associated with the use of chitosan as a nasal drug delivery system, might be further improved by application of chitosan formulated as nanoparticles.

Methods. Insulin-chitosan nanoparticles were prepared by the ionotropic gelation of chitosan glutamate and tripolyphosphate pentasodium and by simple complexation of insulin and chitosan. The nasal absorption of insulin after administration in chitosan nanoparticle formulations and in chitosan solution and powder formulations was evaluated in anaesthetised rats and/or in conscious sheep.

Results. Insulin-chitosan nanoparticle formulations produced a pharmacological response in the two animal models, although in both cases the response in terms of lowering the blood glucose levels was less (to 52.9 or 59.7% of basal level in the rat, 72.6% in the sheep) than that of the nasal insulin chitosan solution formulation (40.1% in the rat, 53.0% in the sheep). The insulin-chitosan solution formulation was found to be significantly more effective than the complex and nanoparticle formulations. The hypoglycaemic response of the rat to the administration of post-loaded insulin-chitosan nanoparticles and insulin-loaded chitosan nanoparticles was comparable. As shown in the sheep model, the most effective chitosan formulation for nasal insulin absorption was a chitosan powder delivery system with a bioavailability of 17.0% as compared to 1.3% and 3.6% for the chitosan nanoparticles and chitosan solution formulations, respectively.

Conclusion. It was shown conclusively that chitosan nanoparticles did not improve the absorption enhancing effect of chitosan in solution or powder form and that chitosan powder was the most effective formulation for nasal delivery of insulin in the sheep model.

KEY WORDS: chitosan; nanoparticles; complex; nasal delivery; insulin.

INTRODUCTION

In recent years there has been a great deal of interest in the exploitation of chitosan for agricultural and water purification purposes as well as utilization within the pharmaceutical industry for improved delivery of drugs (1). Hence, chitosan has been widely used as an excipient in oral drug formulations for increased dissolution of poorly soluble drugs, to obtain controlled and sustained release of drugs and for targeting of drugs to specific sites in the gastrointestinal tract (2–4).

Chitosan is a cationic polysaccharide produced by partial deacetylation of chitin that is derived from naturally occurring crustacean shells. The polymer comprises copolymers of glucosamine and N-acetyl glucosamine and the term chitosan embraces a series of polymers, which vary in molecular weight (from about 10,000 to 1 million dalton) and degree of deacetylation (in the range 50%–95%). Chitosan is insoluble at neutral and alkaline pH, but forms water soluble salts with inorganic and organic acids including glutamic acid, hydrochloric acid, lactic acid and acetic acid. The preferred salt form for nasal drug delivery is the glutamate salt due to its superior absorption enhancing ability (5). Upon dissolution in acid media, the amino groups of the polymer become protonated rendering the molecule positively charged. The properties of chitosan (eg pKa and solubility) can be modified by changing the degree of deacetylation and formulation properties such as the pH and ionic strength.

In the last few years it has been shown, mainly by our group, that chitosan, both as a solution and a powder formulation, is able to dramatically enhance the nasal absorption of polar molecules including peptides and proteins that otherwise are only poorly absorbed via the nasal route (1,6–9). Hence, it was shown in human volunteers that morphine given nasally in a chitosan solution formulation obtained a bioavailability of about 60% as compared to about 10% for a simple solution (9). Similarly, it was found in the sheep model that for the peptide goserelin, a bioavailability of 36% could be obtained with a powder chitosan formulation compared to less than 2% for a simple solution (10).

The absorption promoting effect of chitosan has been studied by us and by other research groups and found to be due to a combination of mucoadhesion and a transient opening of the tight junctions in the mucosal cell membrane (1,11–13). The mucoadhesive properties of chitosan are due to an interaction between the positively charged chitosan and negatively charged sialic acid groups on the mucin, and will provide a prolonged contact time between the drug and the absorptive surface and thereby promote the absorption (14). Furthermore, it has been shown in *in vitro* studies in Caco-2 cell mono-layers that chitosan is able to induce a transient opening of tight junctions thus increasing membrane permeability particularly to polar drugs, including peptides and proteins (6,12,13).

A variety of chitosan based colloidal delivery systems has been described in the literature for the mucosal delivery of polar drugs, peptides, proteins, vaccines and DNA (15–22). The colloidal nanoparticle systems have been produced by various methods to include crosslinking, desolvation, self-assembly or ionic interaction between the positively charged chitosan and a negatively charged polymer. One of the more interesting concepts, is that apart from complexation, chitosan is able to gel on contact with negatively charged tripolyphosphate ions by ionotropic gelation, thus facilitating instantaneous nanoparticle formation under very mild processing conditions. Nanoparticles are formed through inter and intra-

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0724-8741/02/0700-0998/0 © 2002 Plenum Publishing Corporation

molecular linkages created between tripolyphosphate anions and chitosan amino groups (15). It has been claimed by Fernandez-Urrusuna *et al.* that high nanoparticle yields were achievable provided that the conditions for their formation have been optimised (19,20). Additionally it was suggested that high amounts of proteins could be incorporated into such chitosan nanoparticles (19,20). For chitosan nanoparticles ionically crosslinked with tripolyphosphate pentasodium (TPP), the mechanism of association with proteins includes hydrophobic interactions, hydrogen bonding and other physiochemical forces, in addition to the physical entrapment associated with nanoparticle formation in the presence of drug (15). It has been reported in various publications that insulin-associated chitosan nanoparticles, prepared by the ionic gelation method, are able to enhance the nasal absorption of insulin, as expressed by the reduction in plasma glucose levels, to a greater extent than an insulin-chitosan solution following nasal administration to conscious rabbits (19,20). Interestingly, these workers also claimed that insulin-chitosan (chloride) solution (used as a control solution in the *in vivo* studies) induced only a minor decrease in plasma glucose levels in this animal model (20).

The objective of the present work was to evaluate two different types of chitosan (glutamate) nanoparticle, produced by a complexation or by an ionic gelation method and to compare their absorption promoting effect by employing the nasal administration of a model drug (human zinc insulin) in rat and sheep models. The effect of the nanoparticles was compared to the absorption promoting effect of chitosan solution and chitosan powder formulations for nasally administered insulin.

MATERIALS AND METHODS

Materials

Chitosan glutamate (Protasan UP G213) was obtained from Pronova Biopolymer A/S (Oslo, Norway) and used as supplied. Chitosan dissolved as a 1% solution in acetic acid had a viscosity of 97 mPas and the molecular weight was quoted as 205,000 Da. The tripolyphosphate pentasodium (TPP) was purchased from Sigma (Aldrich, Poole, United Kingdom). Human zinc insulin (26.3 IU/mg) was obtained from Eli Lilly (Indianapolis, Indiana). All other materials used were of analytical or pharmaceutical grade.

Initial Nanoparticle Preparation Studies

Preparation of Chitosan Nanoparticles

Chitosan nanoparticles were prepared by the ionic gelation of tripolyphosphate pentasodium (TPP) and chitosan glutamate (CS) as described by Fernandez-Urrusuno *et al.* (19). Preliminary experiments were performed with the objective of identifying the concentrations of chitosan and TPP appropriate for nanoparticle formation (identified by the appearance of an opalescent suspension). Chitosan glutamate solutions (0.5, 1, 2, 3, 4, 5, 7 and 10 mg/ml) and TPP solutions (0.5, 1, 5 and 10 mg/ml) were prepared in ultrapure water (Elga) and a volume of TPP solution (0.25, 1, 2, 2.5 or 3 ml) was added dropwise to 5 ml of chitosan solution while stirring. The resultant mixtures were broadly characterized as either a clear solution, an opalescent suspension (nanoparticles), or as aggregates. Nanoparticle formation was confirmed quantitatively by Photon Correlation Spectroscopy using a Malvern S 4700 PCS System (Malvern Instruments Ltd., Malvern, United Kingdom).

The optimal initial polymer and polyphosphate salt solution concentrations were identified from these preliminary experiments as 2 mg/ml chitosan glutamate and 0.84 mg/ml TPP. These concentrations were subsequently used for the preparation of nanoparticle suspensions.

Drug Incorporation: Post-Loaded Insulin-Chitosan Nanoparticles

Chitosan nanoparticles were prepared by adding dropwise, 3.6 ml TPP solution (0.84 mg/ml) to 9 ml chitosan glutamate solution (2 mg/ml), while stirring using a magnetic stirrer. Aliquots of 10 ml of nanoparticle suspension were centrifuged in tared centrifuge tubes for 90 m at 3660 rpm and 20 °C using a MSE Mistral Centrifuge 3000i. The supernatant was decanted from each tube, the isolated solids freeze-dried using an Edwards Modulyo 4K freeze-drier (Edwards High Vacuum Int., Crawley, United Kingdom) and the chitosan nanoparticle yield determined. These data enabled calculation of the volumes of human zinc insulin solution (10 mg/ml) and ultrapure water (previously adjusted to pH 4 with 0.1 M HCl) required to re-suspend the centrifuged nanoparticles and to obtain insulin concentrations appropriate for the nasal administration of the formulations to rats and sheep. The nanoparticles were re-suspended using a vortex mixer and used without separating any free drug.

Drug Incorporation: Insulin-Loaded Chitosan Nanoparticles

The method of preparation of chitosan nanoparticles was as described previously, with the exception that the human zinc insulin was dissolved in the TPP solution (pH = 9.0) thus facilitating nanoparticle formation in the presence of drug. The insulin concentration used in the TPP solution was 1.25 mg/ml, since this concentration was previously reported as providing the highest association efficiency and loading capacity (20). Attempts to first dissolve the insulin in 0.01M sodium hydroxide solution prior to addition to the TPP solution as described by Fernandez-Urrusuno *et al.* (20) proved unsuccessful.

As before, aliquots of 10 ml of nanoparticle suspension were centrifuged in tared centrifuge tubes for 90 m at 3660 rpm using a MSE Mistral Centrifuge 3000i. The supernatant was decanted from each tube, the isolated solids freeze dried and the insulin-loaded chitosan nanoparticle yield determined. The concentration of insulin in the supernatant "free insulin" was determined using HPLC. Determination of the nanoparticle yield and the supernatant insulin concentration, enabled calculation of the appropriate volume of ultrapure water necessary to re-suspend centrifuged insulin-loaded chitosan nanoparticles to obtain the required insulin concentration. As before, nanoparticles were re-suspended using a vortex mixer.

Preparation of Formulations for the Rat Study

Preparation of Insulin Control Solution Formulation (F1)

For the insulin solution control formulation, human zinc insulin was dissolved in ultrapure water at pH 3.6, filtered

through a 0.45 μm Gelman Acrodisc membrane filter and diluted to 100 ml with filtered ultrapure water previously adjusted to pH 4.0. The final insulin concentration was 0.5 IU/ml as analyzed by HPLC.

Preparation of Insulin-Chitosan Solution Formulation (F2)

For the insulin-chitosan solution formulation, a chitosan glutamate solution (6.25 mg/ml) was added to a human zinc insulin solution (10 mg/ml). The final chitosan concentration was 5 mg/ml, the insulin concentration 20 IU/ml and the pH 4.0 for nasal administration in the rat study. The formulation was analyzed for insulin concentration by HPLC.

Preparation of Insulin-Chitosan Nanoparticles (F3)

Sixty milliliters of chitosan glutamate solution (2 mg/ml) was added to a small beaker and while stirring with a stirrer bar, 24 ml of TPP solution (0.84 mg/ml) containing 1.25 mg/ml human zinc insulin was slowly added. The nanoparticles were centrifuged in aliquots on a MSE Mistral Centrifuge 3000i, at 3660 rpm and 20°C for 90 min. The supernatant was decanted and 0.93 ml ultrapure water (previously adjusted to pH 4.0) was added to each tube and the tubes vortexed for 5 min. The pH of the formulation was 5.1. The nanoparticles were analyzed for insulin content by HPLC. The final insulin concentration was 33 IU/ml.

Preparation of Post-Loaded Insulin-Chitosan Nanoparticles (F4)

The nanoparticles were prepared as above for F3 but without the added insulin. The supernatant was decanted and to each tube was added 0.109 ml human zinc insulin solution (10 mg/ml) and 1.319 ml ultrapure water (previously adjusted to pH 4.0) and the tubes vortexed for 5 min. The pH of the final formulation was 4.7. The nanoparticles were analysed for insulin content by HPLC. The final insulin concentration was 15.6 IU/ml.

Preparation of Formulations for the Sheep Study

Preparation of Subcutaneous and Intranasal Insulin Control Solution Formulations (F1 and F2)

The insulin solutions were prepared as for F1 in the rat study. The formulations were analysed for insulin concentration by HPLC. The final insulin concentration was 166.6 IU/ml and the pH 3.9.

Preparation of Insulin-Chitosan Solution Formulation (F3)

The insulin-chitosan solution formulation was prepared as for the F3 formulation given to the rats. The formulation was analyzed for insulin concentration by HPLC. The final chitosan concentration was 5 mg/ml and the insulin concentration 166.6 IU/ml.

Preparation of Insulin-Chitosan Powder Formulation (F4)

For the insulin-chitosan powder formulation suitable quantities of the human zinc insulin (142 mg) and the chitosan glutamate (852 mg) were weighed and transferred into a mortar. The materials were carefully mixed with a pestle to provide a uniform powder blend (content uniformity was satisfactory with less than 10% deviation from the stated insulin content) as measured by HPLC. The final blend contained 14.3% w/w human zinc insulin and 85.7% w/w chitosan glutamate. The formulation was analysed for insulin concentration by HPLC and found to be 3.78 IU/mg.

Preparation of Insulin-Chitosan Complexes (F5)

A 4 mg/ml chitosan glutamate solution was prepared by dissolving 200 mg chitosan glutamate in 40 ml ultrapure water and adjusting the pH to 5.5 by adding 0.1 M NaOH and making up the volume to 50 ml with ultrapure water.

A 4 mg/ml human zinc insulin solution was prepared by dissolving 200 mg zinc insulin in 40 ml ultrapure water adjusted to pH 4.0 with 0.1 M NaOH. When the insulin was dissolved the pH was adjusted to 7.0 with 0.1 M HCl and the volume made up to 50 ml with water.

The complexes were prepared by adding dropwise 50 ml of 4 mg/ml insulin solution to 50 ml of the 4 mg/ml chitosan solution under vigorous stirring with a magnetic stirrer. The complexes were freeze dried on a Edwards Modulyo 4K freeze-drier overnight after adding 3 g of trehalose to the insulin-chitosan complex dispersion. Before the sheep testing, 1.077g of the freeze-dried complexes were suspended in 10 ml of ultrapure water to give a final insulin concentration of 166.7 IU/ml and a final pH of 5.8. The formulation was analyzed for insulin concentration by HPLC.

Preparation of Post-Loaded Insulin-Chitosan Nanoparticles (F6)

One hundred and eighty milli liters of chitosan glutamate solution (2 mg/ml) was added to a small beaker and during stirring with a stirrer bar, 72 ml of TPP solution (0.84 mg/ml) was slowly added. The nanoparticles were centrifuged in aliquots on a MSE Mistral Centrifuge 3000i, at 3660 rpm and 20°C for 90 min. The supernatant was decanted and 0.452 ml human zinc insulin solution (10 mg/ml) and 0.262 ml ultrapure water (adjusted to pH 4.0) were added to each tube and the tubes vortexed for 5 min. The final pH of the formulation was 4.7. The nanoparticles were analyzed for insulin content by HPLC. The final insulin concentration was 166.7 IU/ml.

Characterization of Nanoparticles and Complexes

The particle size of the nanoparticles and complexes were characterized by Photon Correlation Spectroscopy (PCS) using a Malvern S 4700 PCS System (Malvern Instruments Ltd., Malvern, United Kingdom) and the surface charge expressed as zeta-potential measured by Laser Doppler Anemometry (LDA) using a Malvern Zetasizer IV (Malvern Instruments Ltd., Malvern, United Kingdom). The effect on particle size of centrifuging and re-suspending nanoparticles in the presence and absence of insulin was determined. The measurements were performed in triplicate.

Analysis of Insulin Formulations

The insulin concentrations of the various formulations were analyzed using a Gilson HPLC system fitted with a Vydac C_{18} 5 μm pre-column and a Vydac reverse phase C_{18} 5 μm 150 × 4.6 mm column (Hichrom, Reading, United King-

dom). Gradient conditions and a flow rate of 1.0 ml/min (ambient temperature) were used. The mobile phase was composed of eluent A, containing 95% ethanolamine (0.6%, pH3 and 5% acetonitrile and eluent B, containing 40% ethanolamine (0.6%, pH3) and 60% acetonitrile. The injection volume was 50 μl. The ultraviolet detector was set at 210 nm. The analysis run time was 18 m. Samples were prepared for analysis by dissolving/diluting the formulation in acidified water (adjusted to pH 3.0 with orthophosphoric acid).

In Vivo Studies in the Rat Model

Twenty-one male Wistar rats, weighing 273 ± 12 g (Charles River, United Kingdom), were acclimatized for one week before the study. The animals were fasted overnight before the study with free access to water and terminal (non-recovery) anaesthesia induced and maintained by the intravenous injection of a solution of Hypnorm (Janssen Pharmaceuticals, Beerse, Belgium) and Hypnovel (Roche Products Ltd, Hertfordshire, United Kingdom) via a tail vein cannula. The rats were placed in a supine position on a heated working surface to prevent hypothermia. The rats were surgically prepared by cannulation of the trachea to maintain patency of the airway and carotid artery to facilitate blood sample collection. The study was performed under a valid Home Office (United Kingdom Government) Project Licence and had received approval by the Ethical Review Committee at University of Nottingham.

The rats were randomly allocated (GraphPad™, StatMate®) to one of the four treatment groups each containing five or six animals (Table I). Insulin was dosed at 2 IU/kg nasally and 0.5 IU/kg by subcutaneous injection into the scruff of the neck. Dose selection was based on the expected pharmacodynamics of insulin be the respective routes. The nasal formulations (0.1 ml/kg body weight) were administered using a microsyringe (Hamilton Bonaduz AG, Switzerland) attached via a needle to a short polyethylene tubing inserted approximately 0.7 cm into one nostril.

Blood samples (140 μl) were collected in glucose fluoride blood tubes (Sarstedt Ltd, United Kingdom) at 10 and 5 m prior to dosing and serially for up to 4 h post dosing (11 samples) constituting 12%–13% of the total blood volume. Plasma glucose analysis was performed using a GLUCOTREND® 2 Blood Glucose monitor (Roche Diagnostics, Germany). The minimum blood glucose concentration (C_{min}), time to reach C_{min} (T_{min}) were noted and area over the curve (AOC) calculated using a Microsoft Excel spreadsheet. The blood glucose data was used to estimate the pharmacodynamic availability of nasally administered insulin F_{dyn}. Mean values of C_{min}, T_{min}, AOC and F_{dyn} with standard deviations (SD) were calculated for each dose group.

In Vivo Studies in the Sheep Model

Six female crossbred sheep (Suffolk & Texel), weighing 61 +/– 2 kg, were allowed to acclimatize to the environmental conditions within the School of Biomedical Sciences, Sutton Bonington Campus, University of Nottingham, United Kingdom, for at least six days before commencing the study. Prior to the study day, a cannula was implanted into the jugular vein of each sheep under local anaesthesia (3 ml of 2% lignocaine hydrochloride subcutaneously) by a Seldinger technique to enable blood sample collection. The study was performed under a valid Home Office (United Kingdom Government) Project Licence and had received approval by the Ethical Review Committee at University of Nottingham.

The study was of a nonrandomized crossover design in six sheep, with a minimum washout period of 3 days between successive doses. Food was withdrawn 1 h prior to dosing and for the duration of each study leg. Water was available *ad libitum*. The sheep were dosed a total of six times. To restrain the animals during nasal dosing, sheep were administered 2.25 mg/kg ketamine hydrochloride (100 mg/ml) (Ketaset®, Fort Dodge Animal Health Ltd, Southampton, United Kingdom), via the cannulated jugular vein, providing sedation for about 3 m.

Insulin was administered at a fixed nominal dose of 100 IU nasally and 10 IU by subcutaneous injection into a shaved region in the upper neck. The treatment schedule is shown in Table II. Dose selection was based on the expected pharmacodynamics of insulin by the respective routes. The nasal doses were divided equally between the two nostrils. The nasal liquids were administered from a 1 ml syringe via a modified CB-18 spray actuator. (Valois, France) and the nasal powder formulation using a Blueline siliconised tracheal tube (Portex, United Kingdom) containing the pre-weighed dose and one-way bellows. The nasal administration devices were inserted approximately 7 cm into the nasal cavity.

Blood samples (5.7 ml) were collected from the cannulated jugular vein of each sheep into serum tubes at 20 and 10 m prior to dosing and serially for up to 5 h post dosing (10

Table I. Summary of Insulin Formulations and Doses Administered to Rats

Formulation	Insulin content[e] (IU/ml)	Insulin dose (IU/kg)	Chitosan glutamate (mg/kg)	Chitosan base (mg/kg)	Dose volume (ml/kg)
INS Sol SC[a]	0.4	0.5	N/A	N/A	1.25
INS CHI Sol IN[b]	19.3	2.0	0.52	0.27	0.104
INS CHI NP IN[c]	33.0	2.0	0.31	0.16	0.061
INS CHI NP PL IN[d]	15.6	2.0	0.64	0.33	0.128

Note: N/A Not applicable.

[a] Insulin solution, administered by subcutaneous (SC) injection (F1).

[b] Insulin chitosan solution, administered intranasally (IN) (F2).

[c] Insulin chitosan nanoparticles loaded with insulin during production, administered IN (F3).

[d] Insulin chitosan nanoparticles post-loaded with insulin, administered IN (F4).

[e] As measured by HPLC.

Table II. Summary of Insulin Formulations and Doses Administered to Sheep

Formulation	Nominal Insulin content (IU/ml or IU/mg†)	Insulin content (% of nominal)[g]	Insulin dose (IU)	Chitosan glutamate (mg)	Chitosan base (mg)	Dose volume or weight (ml or mg†)
INS Sol SC[a]	16.67	94.0	10	N/A	N/A	0.6
INS Sol IN[b]	166.67	100.4	100	N/A	N/A	0.6
INS CHI Sol IN[c]	166.67	99.1	100	3.0	1.6	0.6
INS CHI PWD IN[d]	3.78†	93.7	128 ± 13	29 ± 3	15 ± 2	34 ± 4†
INS CHI COMPL IN[e]	166.67	91.2	100	3.0	1.6	0.6
INS CHI NP PL IN[f]	166.67	N/D	100	3.0	1.6	0.6

Note: N/A Not applicable. N/D Not determined.
[a] Insulin solution, administered by subcutaneous (SC) injection (F1).
[b] Insulin solution, administered intranasally (IN) (F2).
[c] Insulin chitosan solution, administered IN (F3).
[d] Insulin chitosan powder, administered IN (F4).
[e] Insulin chitosan complex, administered IN (F5).
[f] Insulin chitosan nanoparticles post-loaded with insulin, administered IN (F6).
[g] As measured by HPLC (all formulations were within ± 10% of nominal insulin content).

samples) equal to 2% of total blood volume in each study leg. Serum glucose and insulin analyses were performed at the Clinical Chemistry Department, Queens Medical Centre, University of Nottingham, United Kingdom. Post-dose blood glucose concentrations (mmol/l) were expressed as percentages of the average basal (pre-dose) concentration (% of basal). Values of C_{min}, T_{min} and AOC were calculated as previously described.

Insulin Serum Sample Analysis

Insulin serum sample analysis was performed using a Coat-A-Count® insulin assay kit (DPL Division, EURO/DPC Ltd, Gwynedd, United Kingdom). The Coat-A-Count® insulin procedure is a solid-phase radioimmunoassay, in which ^{125}I-labelled insulin competes with insulin in a test sample for sites on an insulin-specific antibody. Because the antibody is immobilized to the wall of a polypropylene tube, simply decanting the supernatant of the incubation mixture suffices to terminate competition and to isolate the antibody-bound fraction of the radiolabelled insulin. The radioactivity counts are obtained by means of a gamma counter. The insulin concentration of the samples was calculated from an insulin standard curve using RIA-CALC software. The maximum blood insulin concentration (C_{max}) and time to reach C_{max} (T_{max}) were noted for each animal and values of area under the insulin curve (AUC) calculated using a Microsoft Excel spreadsheet. The bioavailability of nasal insulin (F_{rel}) to subcutaneous injection was calculated, see Table VI. Mean values of C_{max}, T_{max}, AUC and F_{rel} with standard deviations (SD) were calculated for each dose group.

Statistical Analysis

Statistical analysis was performed on the data obtained in the *in vivo* studies by one-way analysis of variance (ANOVA) with Tukey-Kramer Multiple Comparisons Post test using GraphPad InStat™ software (GraphPad Software, Inc., San Diego, California). Throughout the level of significance was chosen as less than 0.05 (i.e., $P < 0.05$). The Post test was performed only if findings of the ANOVA were significant.

RESULTS AND DISCUSSION

Nanoparticle Preparation

Preliminary experiments were performed with the objective of identifying the optimal chitosan glutamate and TPP concentrations for the achievement of ionic gelation and nanoparticle formation. The results are presented in Fig. 1.

The zone of the opalescent suspension corresponding to a suspension of nanoparticles was associated with a formulation containing a final chitosan glutamate concentration in the range 1–3 mg/ml and a final TPP concentration in the range 0.2–0.5 mg/ml. These findings were comparable to the results reported previously by Calvo *et al.* (18), who found the optimal final chitosan (Seacure® 123) and TPP concentrations to be in the order of 1–3 mg/ml and 0.2–1.0 mg/ml, respectively. The optimal chitosan/TPP ratio on a weight-to-weight basis in the Calvo *et al.* study was found to be between 3 and 5, whereas in the present study the ratio was about 6 to 7. The difference in optimal ratio is probably due to use of the chitosan hydrochloride salt in the Calvo *et al.* study which contains approximately 82% chitosan base as compared to the chitosan glutamate which contains about 55% chitosan base. From these results the use of initial concentrations of TPP and chitosan glutamate solution concentrations of 0.84 mg/ml and 2 mg/ml, respectively for preparation of the nanoparticles were selected, which gave a chitosan/TPP weight to weight ratio of 6/1.

To achieve the desired chitosan glutamate concentrations and insulin concentrations in chitosan nanoparticles, it was necessary to concentrate the chitosan nanoparticle formulation by centrifuging, prior to drug loading for the post-loaded nanoparticles and after preparation for the nanoparticles loaded with insulin during production. The theoretical yield from 10 ml of nanoparticle suspension was between 17 mg and 20 mg (equivalent to about 0.2% w/v nanoparticle suspension). Following centrifuging, a yield of 35%–50% w/w was obtained (e.g. 50.1% w/w, $n = 9$, %RSD = 7.6). The low yields were similar to previously reported (19). The release of insulin from both types of nanoparticles was found to be complete within 1 h (data not shown). These release rates were similar to the ones obtained by Fernandez-Urrusuna (19,20).

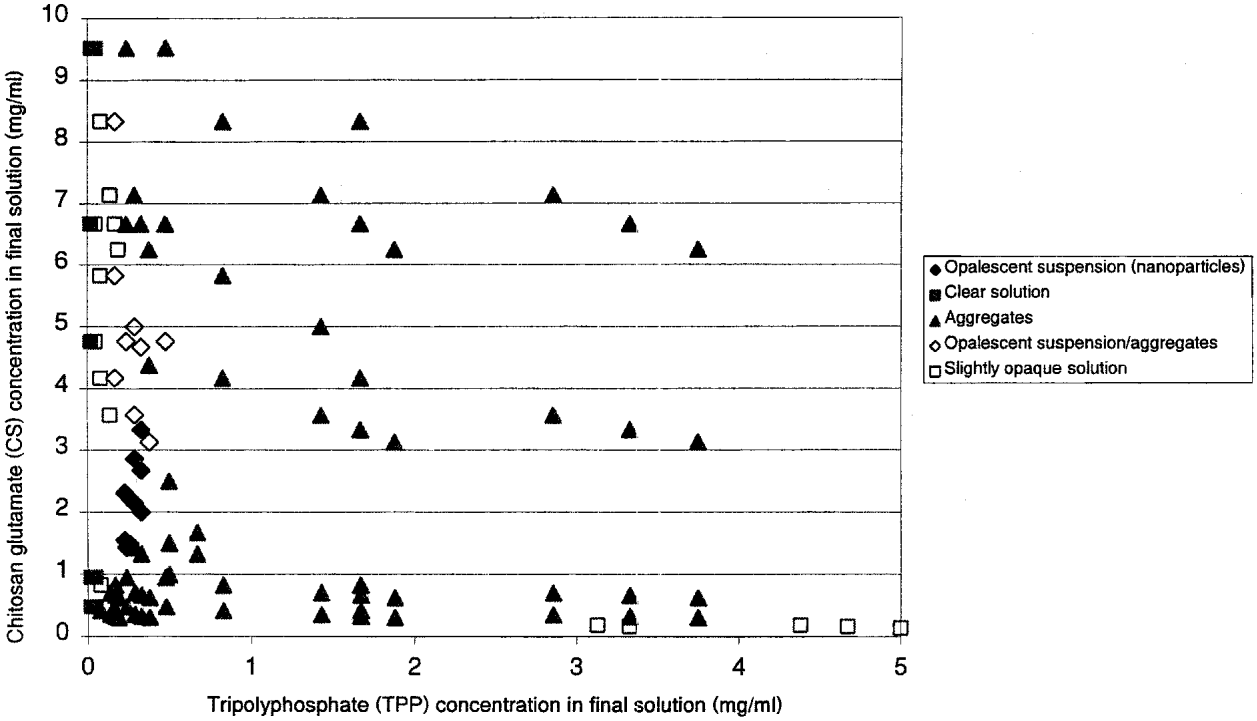

Fig. 1. Identification of chitosan and TPP concentrations appropriate to nanoparticle formation.

It is recognized that the effectiveness of chitosan in enhancing drug absorption is attributable to the ionic interaction between the positively charged amino groups in chitosan and the negatively charged sialic acid residues in mucin and on the epithelial cells is dependent upon the polymer retaining its cationic charge (1). Previous workers have shown that increasing the concentration of TPP relative to chitosan, although it causes an increase in nanoparticle yield, also not surprisingly, causes a reduction in the positive charge of the nanoparticles (19). However, a systematic study of the effect of surface charge on the absorption promoting ability of the chitosan nanoparticles has not been reported in the literature. In the present work, the potential for increased nanoparticle yield was balanced against a potential reduction in the positive charge of chitosan; hence the preferred chitosan glutamate/TPP w/w ratio was selected as 6/1, leaving a surplus of positive charges on the nanoparticles. In addition, whilst it may be possible to manufacture appropriate volumes of this product for *in vivo* administration to animals, the rather low nanoparticle yield raises the question about the suitability of these nanoparticles for clinical use and subsequent commercialisation.

Characterization of Nanoparticles

The various types of nanoparticles were characterized in terms of particle size and zeta potential by PCS and LDA, respectively (Table III). The nanoparticles not loaded with insulin were shown to have a mean particle size around 250 nm and a positive zeta potential of 28.9 mV.

An increase in mean particle size was noted following centrifuging and re-suspending of chitosan nanoparticles in insulin solution. For post-loaded insulin-chitosan nanoparticles, the mean particle size was independent of both the insulin and the chitosan concentrations whereas for the zeta potential a significant increase in positive charge could be seen for an increase in chitosan concentration from 5mg/ml to

Table III. Characterization of Chitosan Nanoparticles

Type of nanoparticle	Insulin concentration (IU/ml)	Chitosan concentration (mg/ml)	Particle size (mean ± SD) (nm)	Zeta potential (mV)
CHI NP (before centrifuging)		1.4	246.5 ± 38.2	28.9 ± 0.4
CHI NP (after centrifuging)		5	575.9 ± 93.7	33.5 ± 0.2
INS CHI NP PL[a]	20	5	751.8 ± 109.6	37.2 ± 1.2
INS CHI NP PL[a]	166	5	860.1 ± 124.4	39.3 ± 0.7
INS CHI NP PL[a]	166	7.5	713.5 ± 227.4	33.4 ± 0.5
INS CHI NP PL[a]	166	10	717.3 ± 73.0	43.7 ± 0.3
INS CHI NP (before centrifuging)[b]	9.5	1.4	347.5 ± 142.0	30.2 ± 0.7
INS CHI NP (after centrifuging)[b]	34	5	472.6 ± 43.3	25.0 ± 0.9

[a] INS CHI NP PL: Insulin chitosan nanoparticles post-loaded with insulin.
[b] INS CHI NP: Insulin chitosan nanoparticles loaded with insulin during production.

10 mg/ml. As a control, chitosan nanoparticles were re-suspended in ultra pure water with the objective of establishing a possible explanation for the apparent increase in particle size. After centrifuging, the mean size increased from 247 to 576 nm, probably due to some agglomeration. For the nanoparticle systems loaded with insulin during the production process, the mean particle size was not significantly affected by centrifugation and re-dispersion. It can be concluded that the inter-particle association of nanoparticles may partly be occurring as a consequence of the presence of insulin, but is mostly attributable to the process of centrifugation followed by re-dispersion. For all nanoparticle systems the mean particle size was below 1 μm.

To have a nanoparticle system that was simpler to prepare and could be manufactured with a higher yield, we investigated the possibility of forming nanoparticles by co-precipitation of insulin and chitosan in the absence of TPP. By carefully selecting the process parameters, especially the pH of the insulin and chitosan solutions it was possible to prepare complexes between the positively charged chitosan and negatively charged insulin. The charge carried by insulin will depend upon pH. Below the isoelectric point (5.3–5.35) insulin carries a net positive charge and is soluble below pH 4.5. Above the isoelectric point the insulin carries a net negative charge and is soluble above pH 6.3. It was found that a mixture of a 2 mg/ml chitosan solution at pH 5.5 and a 2 mg/ml insulin solution at pH 7.0 gave an opalescent solution indicating that nanoparticles had formed. The insulin-chitosan complex particles were characterized in terms of particle size and zeta potential. It can be seen that freshly made, the mean size of the complexes were 751.8 +/– 74.7 nm and the zeta potential was +45.9 +/– 0.9 mV. The complexes were concentrated by freeze-drying and re-suspending in a smaller volume of water. After freeze-drying and re-suspending, the particle size increased to 1402.9 +/– 285.7 nm indicating that some agglomeration had taken place. The zeta potential was found to be 41.2 +/– 0.8 mV, slightly lower than before freeze drying.

In Vivo Studies

The pharmacodynamic parameters after nasal application of the various insulin formulations in the rat are given in Table IV. Following subcutaneous injection of 0.5 IU/kg insulin, values of C_{min} and T_{min} were in the region of 67% of basal glucose concentration and 81 min., respectively. The insulin-chitosan solution formulation appeared to perform better than both of the insulin-chitosan nanoparticle formulations, with values for C_{min} in the region of 40% and T_{min} of 90 min, respectively and the F_{dyn} of about 48%. The values for F_{dyn} for the insulin-loaded chitosan nanoparticles and the post-loaded insulin-chitosan nanoparticles were 38% and 37%, respectively. The insulin-loaded chitosan nanoparticles and the post-loaded insulin-chitosan nanoparticles showed similar effects on the plasma glucose levels. However, decreases in blood glucose concentrations were observed after the nasal administrations were not statistically significant ($P > 0.05$) (Table IV). Hence, these data showed that in the rat model the insulin-chitosan nanoparticle formulations offered no advantage to the nasal insulin-chitosan solution formulation in terms of the nadir and overall hypoglycaemic response.

The nasal absorption of insulin was also investigated in the conscious sheep model since it has been shown that the use of anaesthetised animals, such as the rat model in the present study, most often overestimates the nasal absorption of drugs such as insulin. This is due to the partial impairment of the mucociliary clearance mechanism (23,24). Furthermore, the sheep model has been shown to be very predictive of the nasal absorption of drugs in man (9). Due to the amount of insulin needed to be administered in this larger animal model, it was not possible to investigate the insulin-loaded chitosan nanoparticles as opposed to the post-loaded insulin-chitosan nanoparticles, where the concentration of insulin could be significantly increased. Based on the previous results by Fernandez-Urrusuno *et al.* (19,20) and the results in the present rat studies, it was expected that there would be no major difference in insulin absorption whether the insulin was incorporated during production or post-loaded. The pharmacodynamic parameters obtained in the sheep model after nasal administration of the various formulations are given in Table V and serum insulin concentrations and pharmacokinetic parameters are shown in Table VI and Fig. 2, respectively.

Table IV. The Pharmacodynamics of Insulin in Rats

Formulation	n	T_{min} (min)	C_{min} (% basal glucose)	AOC (% glucose.min)	F_{dyn} (%)
INS Sol SC[a]	5	81.0 ± 91.6	67.2 ± 11.4	3788.5 ± 2659.1	100.0 ± 70.2
INS CHI Sol IN[b]	5	93.0 ± 67.4	40.1 ± 6.1	7262.8 ± 2933.9	47.9 ± 19.4
INS CHI NP IN[c]	6	120.0 ± 111.7	59.7 ± 28.8	5714.9 ± 5736.4	37.7 ± 37.9
INS CHI NP PL IN[d]	5	107.5 ± 81.8	52.9 ± 14.4	5477.9 ± 2035.4	36.1 ± 13.4
One-way ANOVA[e]		$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
One-way ANOVA[f]		$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$

Note: NA Not applicable.

Fdyn* = (individual $AOC_{IN\ or\ SC} \times Dose_{SC}$/mean $AOC_{SC} \times Dose_{IN\ or\ SC}$) × 100. Calculated for each individual animal following IN or SC administration relative to mean AOC following SC administration.

Data given as mean ± SD (sample size, n, is given in the Table).

[a] Insulin solution, administered by subcutaneous (SC) injection (F1).

[b] Insulin chitosan solution, administered intranasally (IN) (F2).

[c] Insulin chitosan nanoparticles loaded with insulin during production, administered IN (F3).

[d] Insulin chitosan nanoparticles post-loaded with insulin, administered IN (F4).

[e] Comparisons made of all formulations (F1–F4).

[f] Comparisons made only of the nasal formulations (F2–F4).

Table V. The Pharmacodynamics of Insulin in Sheep

Formulation	T_{min} (min)	C_{min} (% basal glucose)	AOC (% glucose.min)
INS Sol SC[a]	85.0 ± 29.5	43.1 ± 3.8[a,b,c]	10472.4 ± 1209.0[j,k,l,m]
INS Sol IN[b]	63.3 ± 63.9	79.5 ± 8.6[a,d,e,aa,bb]	2117.2 ± 1028.0[j,n,o,gg,hh]
INS CHI Sol IN[c]	67.5 ± 12.5	53.0 ± 10.4[d,f,g,aa,cc,dd]	4631.8 ± 1176.6[k,n,p,q,gg,ii,jj,kk]
INS CHI PWD IN[d]	90.0 ± 9.5	38.1 ± 5.4[e,h,i,bb,ee,ff]	8662.7 ± 1634.3[o,p,r,s,hh,ii,ll,mm]
INS CHI COMPL IN[e]	51.3 ± 23.9	73.3 ± 13.5[b,f,h,cc,ee]	2543.0 ± 1414.6[l,r,jj,ll]
INS CHI NP PL IN[f]	46.3 ± 12.0	72.6 ± 9.4[c,g,i,dd,ff]	2301.5 ± 588.4[m,q,s,kk,mm]
One-way ANOVA[g]	$P > 0.05$	$P < 0.0001$	$P < 0.0001$
One-way ANOVA[h]	$P > 0.05$	$P < 0.0001$	$P < 0.0001$

Note: Only significant relations are indicated, for all other comparisons made $P > 0.05$. Data given as mean ± SD (n = 6).
NA Not applicable.
[a] Insulin solution, administered by subcutaneous (SC) injection (F1).
[b] Insulin solution, administered intranasally (IN) (F2).
[c] Insulin chitosan solution, administered IN (F3).
[d] Insulin chitosan powder, administered IN (F4).
[e] Insulin chitosan complex, administered IN (F5).
[f] Insulin chitosan nanoparticles post-loaded with insulin, administered IN (F6).
[g] Comparisons made of all formulations (F1–F6). Tukey-Kramer Multiple Comparisons Test following ANOVA: [a,b,c,d,e,h,i,j,k,l,m,o,p,r,s]: $P < 0.001$, [f,g]: $P < 0.01$, [n,q]: $P < 0.05$.
[h] Comparisons made only of the nasal formulations (F2–F6). Tukey-Kramer Multiple Comparisons Test following ANOVA‡: [aa,bb,ee,ff,hh,ii,ll,mm]: $P < 0.001$, [cc,dd,gg,jj,kk]: $P < 0.05$.

After the subcutaneous injection of insulin (dose 10 IU) serum blood glucose values declined to about 45% (C_{min}) of basal levels with a T_{min} of 85 min (Table V). Corresponding insulin data showed values of C_{max} and T_{max} of about 191 μIU/ml and 14 min respectively (Table VI). Serum insulin levels remained elevated for the duration of the study and for some animals, in contrast to nasally administered insulin, had not returned to basal concentrations by 300 min. The absorption of insulin from the control nasal insulin solution (100 IU dose) was poor with C_{max} in the region of 53 μIU/ml and the relative bioavailability only 0.5%. The pharmacokinetics and pharmacodynamics of insulin were significantly improved ($P < 0.05$) relative to nasal control after nasal administration of the chitosan based solution and powder formulations. For the insulin-chitosan solution formulation (100 IU dose) values of C_{max}, T_{max} and F_{rel} were about 179 μIU/ml, 28 min. and 3.6%, respectively (Table VI). The T_{max} occurred at 28 min. but the insulin serum levels 5 min after dosing, were comparable to those obtained at T_{max} for the chitosan nanoparticles and complex formulations. The improved insulin absorption after nasal administration of the insulin-chitosan solution formulation was supported by the coresponding glucose data.

Table VI. The Pharmacokinetics of Insulin in Sheep

Formulation	T_{max} (min)	C_{max} (μIU/ml)	AUC (μIU/ml.min))	Frel (%)
INS Sol SC[a]	14.2 ± 9.2[a]	190.5 ± 102.5[g]	18657.5 ± 6579.1[l,m,n,o,p]	100
INS Sol IN[b]	11.7 ± 5.2[b,c,aa,bb]	52.6 ± 27.0[h,ff]	917.2 ± 491.7[l,q,jj]	0.5 ± 0.1[nn]
INS CHI Sol IN[c]	27.5 ± 14.7[b,d,aa,cc]	179.1 ± 65.5[i,gg]	6581.3 ± 2575.0[m,r,kk]	3.6 ± 0.8[oo]
INS CHI PWD IN[d]	40.0 ± 7.7[a,c,e,f,bb,dd,ee]	743.1 ± 259.0[g,h,i,j,k,ff,gg,hh,ii]	38201.4 ± 13286[n,q,r,s,t,jj,kk,ll,mm]	17.0 ± 6.6[nn,oo,pp,qq]
INS CHI COMPL IN[e]	11.7 ± 5.2[d,e,cc,dd]	66.9 ± 24.1[j,hh]	2308.7 ± 817.5[o,s,ii]	1.8 ± 0.9[pp]
INS CHI NP PL IN[f]	15.0 ± 0.0[f,ee]	106.2 ± 98.9[k,ii]	2249.4 ± 1985.6[p,t,mm]	1.3 ± 0.8[qq]
One-way ANOVA[g]	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	N/A
One-way ANOVA[h]	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

Note: Only significant relations are indicated, for all other comparisons made $P > 0.05$. Data given as mean ± SD (n = 6).
NA Not applicable
[a] Insulin solution, administered by subcutaneous (SC) injection (F1).
[b] Insulin solution, administered intranasally (IN) (F2).
[c] Insulin chitosan solution, administered IN (F3).
[d] Insulin chitosan powder, administered IN (F4).
[e] Insulin chitosan complex, administered IN (F5).
[f] Insulin chitosan nanoparticles post-loaded with insulin, administered IN (F6).
Frel* = $(AUC_{IN} \times Dose_{SC}/AUC_{SC} \times Dose_{IN}) \times 100$. Calculated for each individual animal relative to SC data from the same animal.
[g] Comparisons made of all formulations (F1–F6). Tukey-Kramer Multiple Comparisons Test following ANOVA: [a,c,e,f,g,h,i,j,k,l,n,p,q,r,s,t]: $P < 0.001$, [o]: $P < 0.01$, [b,d,m]: $P < 0.05$.
[h] Comparisons made only of the nasal formulations (F2–F6): Tukey-Kramer Multiple Comparisons Test following ANOVA: [bb,dd,ee,ff,gg,hh,ii,jj,kk,ll,mm,nn,oo,pp,q]: $P < 0.001$, [aa,cc]: $P < 0.05$.

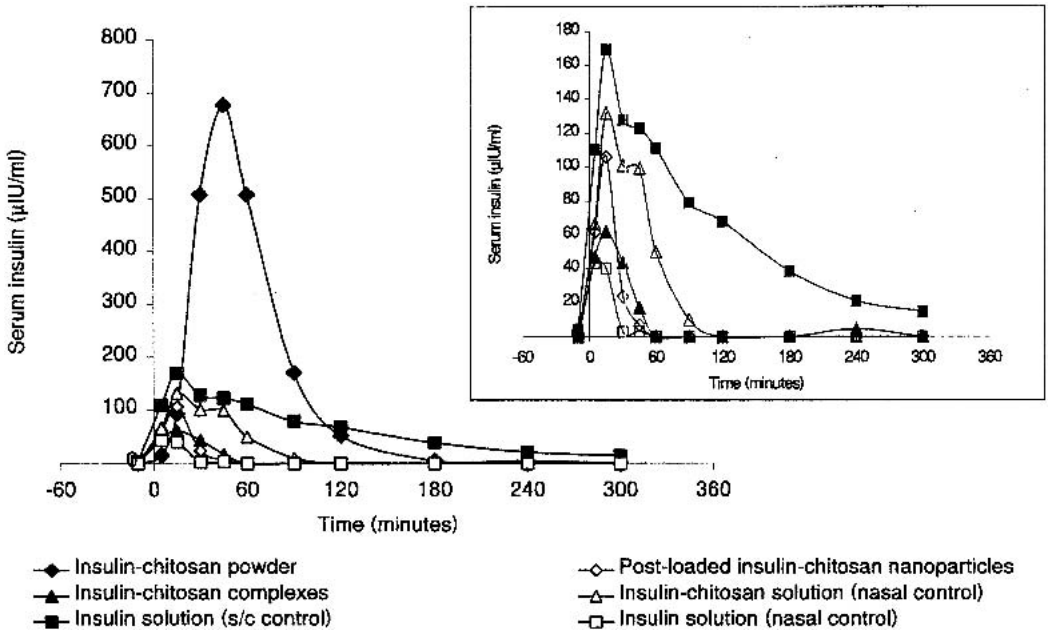

Fig. 2. Serum insulin concentrations following the nasal or subcutaneous administration of various insulin formulations to sheep. The insert figure is enlarged from the main figure containing the serum insulin profiles for all but the insulin-chitosan powder formulation. (n = 6)

Both the post-loaded insulin-chitosan nanoparticles and the insulin-chitosan complexes were significantly less effective ($P < 0.05$) in lowering the blood glucose levels than the insulin-chitosan solution formulation. After nasal dosing of the nanoparticle and complex formulations (100 IU doses) values of C_{max} were in the region of 106 μIU/ml and 67 μIU/ml with corresponding T_{max} of 15 and 12 min., respectively (Table VI). As for the control formulation, elevation in serum insulin levels after dosing these formulations were relatively short lived (<60 min.) which was reflected in the F_{rel} obtained of 1.3% and 1.8%, respectively. These values were significantly ($P < 0.001$) lower than that obtained after dosing the insulin-chitosan solution formulation. There was no significant difference between the pharmacokinetic/pharmacodynamic parameters obtained for the chitosan nanoparticles and complexes compared to the control solution. A substantial improvement (F_{rel} 17%) in the nasal absorption of insulin was observed after dosing the insulin-chitosan powder formulation (average dose 128 IU). Values of C_{max} and T_{max} were around 743 μIU/ml and 40 min., respectively (Table VI) and insulin serum levels were elevated for 120–180 min. Appreciable increase in serum insulin concentrations was observed after 15 min (Fig. 2). F_{rel} was significantly higher ($P < 0.001$) after dosing the chitosan powder formulation than after dosing any of the other nasal formulations. This was also supported by the glucose data with significantly better AOC ($P < 0.05$) for the chitosan powder formulation than for the other nasal formulations. Notably, the nadir in glucose levels for this formulation was comparable to that obtained after subcutaneous administration although the hypoglycaemic response was less prolonged.

It was previously shown for other drugs such as goserelin, that a chitosan powder formulation was better at enhancing the nasal absorption of the drug than the corresponding chitosan solution formulation with bioavailabilities of 25.6% and 11.8%, respectively (10). The improved effect of the chitosan powder as compared to the chitosan solution can be readily explained by the longer residence time of the powder ($T_{1/2}$ = 115 min) in the sheep nasal cavity, as compared to the chitosan solution ($T_{1/2}$ = 45 min) (25). This longer residence time will promote increased transport of the insulin across the nasal mucosa. Furthermore, the absorption of water from the mucosa by chitosan powder may have an additional effect on the opening of the tight junctions.

The results obtained in the present study on the effect of the chitosan nanoparticles on nasal absorption of insulin compared to the chitosan solution formulation are not in agreement with the results from Fernandez-Urrusuno *et al.* (19,20). These workers reported the superior efficacy of insulin-loaded nanoparticles as compared to chitosan solutions in terms of their ability to enhance insulin absorption via the nasal route. As can be seen from Table VII the characteristics of the nanoparticles used in the two studies are quite similar with the major difference being the dose of insulin, the nanoparticle size range, and the animal models used. The difference in particle size of about 300–400 nm should not be critical. As discussed in the Fernandez-Urrusuno *et al.* publications, the effect of the nanoparticle system is most likely due to an effect of the positively charged chitosan particle on the epithelial membrane. This is mainly in terms of bioadhesion and possibly transient opening of the tight junctions and is not likely due to the particles being transferred across the nasal membrane. In support of this, it was shown by our own group that even polystyrene particles (100 nm in diameter) coated with chitosan were only taken across the nasal membrane to a very low degree (<3% over 3 h) (26).

The chitosan used in the present studies was chitosan glutamate with a mean molecular weight of 205 kDa and a degree of deacetylation of about 83%. The chitosan used in the Fernandez-Urrusuno *et al.* studies was chitosan HCl with a mean molecular weight of 130 kDa and a degree of deacetylation of >70%. It has been shown previously that the degree

Table VII. A Comparison between Characteristics and Doses of Nanoparticles Used in the Study by Fernandez-Urrusuno et al. [19,20] and the Nanoparticles Used in the Present Study

Formulation (Reference)	Insulin dose (IU/kg)	Chitosan dose (mg/kg)	Mean particle size (nm)	CHI/TPP ratio	Zeta potential (mV)
Rabbits	5	0.16	300	6/1	25–54
(23,24)		0.35	400		
Rats	2	0.5	473	6/1	25.0
INS CHI NP[a]			(423–501)		(23.7–25.7)
Rats	2	0.5	752	6/1	37.2
INS CHI NP PL[b]			(671–877)		(35.5–38.3)
Sheep	2	0.1	717	6/1	43.7
INS CHI NP PL[b]			(660–799)		(43.8–44.0)

[a] INS CHI NP: Insulin chitosan nanoparticles loaded with insulin during production.
[b] INS CHI NP PL: Insulin chitosan nanoparticles post-loaded with insulin.

of deacetylation is critical for the absorption promoting effect and that the lower the degree of deacetylation the less the absorption promotion (27,28). Similarly, it was shown that the molecular weight had some importance in that a molecular weight of at least 100 kDa was needed to obtain the optimal effect (29). Hence, it could be expected that the chitosan used in the Fernandez-Urrusuno *et al.* studies might have had a lower effect on absorption promotion, at least as a solution formulation.

It should be noted that the insulin absorption data in terms of blood glucose profiles and plasma insulin levels seen in the rat and the sheep studies are very similar to results from studies in rats and sheep previously published by our group (5,11,23). Chitosan solution has also been shown to have a very good absorption promoting effect on other drugs such as morphine (F = 60%) and goserelin (F = 12%) (9,10).

It can be concluded from these studies, that both chitosan solution and chitosan powder formulations are superior in terms of their nasal absorption promoting ability compared with chitosan nanoparticulate systems such as those prepared by ionic gelation of chitosan and TPP (19,20) and those described in the present paper prepared by complexation between chitosan and insulin. It can further be concluded that for nasal absorption of insulin, the chitosan powder formulation was superior to the chitosan solution formulation.

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