Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmaceutical Sciences 26 (2005) 9–15
EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES
www.elsevier.com/locate/ejps

Intranasal delivery of recombinant human growth hormone (somatropin) in sheep using chitosan-based powder formulations

Yu-Hui Cheng, A. Margaret Dyer, Inderjit Jabbal-Gill, Michael Hinchcliffe, Richard Nankervis, Alan Smith, Peter Watts *

Archimedes Development Ltd., Albert Einstein Centre, Nottingham Science and Technology Park, University Boulevard, Nottingham, NG7 2TN, UK

Received 23 July 2004; received in revised form 14 February 2005; accepted 24 March 2005
Available online 20 June 2005

Abstract

The effectiveness of chitosan in promoting the intranasal bioavailability of recombinant human growth hormone (hGH) has been evaluated. hGH was formulated with chitosan to produce a powder blend (Formulation A) and granules (Formulation B) for intranasal administration. The in vivo pharmacokinetic performance of the formulations was evaluated in a group of six sheep in a randomised crossover study. A subcutaneous injection of hGH solution was administered as a control. The intranasal and subcutaneous doses of hGH were 0.3 and 0.03 mg/kg, respectively. The intranasal formulations appeared to be well tolerated. Mean bioavailabilities of hGH from Formulations A and B were 14 and 15%, respectively relative to subcutaneous injection. It is concluded that chitosan-based intranasal powder formulations may provide a practical means for non-injectable delivery of hGH and, potentially, other therapeutic protein molecules.

Keywords: Nasal; Chitosan; Human growth hormone; Sheep

1. Introduction

The intranasal route offers a number of attractions as a non-invasive means of delivering therapeutic macromolecules. It is convenient to the patient, offers a relatively benign environment for macromolecule stability, is generally well tolerated and, by comparison to the pulmonary route, is less demanding in terms of formulation technology, such as particle size requirements, and complexity of delivery device (Arora et al., 2002; Illum, 2003; Illum and Fisher, 1997).

Synthetic human growth hormone (hGH, somatropin) is manufactured by recombinant DNA technology and is a 191 amino acid polypeptide (MW 22 kDa) with an amino acid sequence and two internal disulphide bridges identical to that of the major component of human pituitary growth hormone (Pearlman and Bewley, 1993). Therapeutically, hGH is used in children to treat growth retardation, for example short stature due to insufficient growth hormone secretion, Turner's syndrome or chronic renal insufficiency. In adults it is used as a treatment for growth hormone deficiency and for management of HIV-related wasting and cachexia (Sweetman, 2002)

There are a number of reports on the intranasal administration of hGH. The effect of sodium tauro-24,25-dihydrofusidate (STDHF) on the nasal absorption of hGH in the rat, rabbit and sheep has been evaluated (Baldwin et al., 1990). Compared to a simple aqueous solution of hGH, the addition of STDHF produced a 11-fold increase in intranasal hGH bioavailability (area under curve) in rats and rabbits and a 21-fold increase in sheep. STDHF-based hGH solutions were also administered to growth hormone-deficient patients (Hedin et al., 1993). The bioavailability relative to subcutaneous injection (s.c.) was in the range 1.6–3.0%.

In a similar study, formulations containing hGH and L-α-phosphatidylcholine (LPC) were administered to rats (up to 17.5% bioavailability achieved), rabbits (72.8%) and sheep (up to 16%) (Fisher et al., 1991). Bioavailabilities in the

* Corresponding author. Tel.: +44 115 9078700; fax: +44 115 9078701.
E-mail address: peterwatts@archimedespharma.com (P. Watts).

0928-0987/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejps.2005.03.014

absence of LPC were 2.3, 1.4 and 0.2% in the rat, rabbit and sheep, respectively.

In sheep the intranasal absorption of hGH administered as an aqueous solution and as lyophilised powders containing starch microspheres and starch microspheres mixed with LPC has been reported (Illum et al., 1990). Relative to s.c. injection, the nasal formulations produced bioavailabilities of 0.1, 2.7 and 14.4%, respectively. The possibility of LPC causing damage to cell membranes was noted. An intranasal hGH formulation containing α-cyclodextrin and didecanoyl-L-α-phosphatidylcholine provided a bioavailability of 20% in rabbits, but was associated with severe damage to the nasal epithelial membrane (Agerholm et al., 1994). Formulations containing hGH and didecanoyl-L-α-phosphatidylcholine have been intranasally administered to growth hormone-deficient patients (Laursen et al., 1996). Depending on dose, the bioavailability ranged from 3.8 to 8.9%.

It is therefore clear that there may be opportunities to deliver efficacious amounts of hGH into the systemic circulation via the intranasal route, but this will require the use of additives to enhance bioavailability. For long term use in a human medicine, any such additive(s) will need to be demonstrated to be both effective and safe.

A number of studies have reported the use of chitosan as an excipient for improving intranasal absorption of polar small molecules and peptides (Dyer et al., 2002; Illum et al., 1999, 2000, 2002; Illum, 2003; Roon et al., 1999; Sinswat and Tengamnuay, 2003) and for enhancing the efficacy of intranasal vaccines (Illum et al., 2001; Mills et al., 2003). Chitosan is a polysaccharide comprising copolymers of glucosamine and *N*-acetylglucosamine and is derived by the partial deacetylation of chitin, a material found in abundance in shells of Crustacea such as lobsters, prawns and crabs. Being positively charged, chitosan can bind strongly to negatively charged materials such as epithelial cell surfaces and mucus. Chitosan has been shown to behave as a bioadhesive polymer, increasing significantly the half-time of nasal clearance (Soane et al., 1999). In addition to improved adhesion between the formulation and the nasal tissue, there are also reports that chitosan can increase the permeability of cell monolayers and may have transient effects, in vitro, on the integrity of tight junctions (Artursson et al., 1994; Dodane et al., 1999; Ranaldi et al., 2002; Smith et al., 2004), although it is not clear how relevant this phenomenon is to the ability of chitosan to improve drug absorption in vivo.

From a pharmaceutical regulatory viewpoint the use of chitosan as an additive to improve intranasal drug absorption is attractive since it has pharmacopoeial recognition (European Pharmacopoeia, 2002) and is available commercially as a high purity material made in compliance with Good Manufacturing Practice (Illum, 2002). A considerable body of human clinical data is also being generated on chitosan-based intranasal formulations: In clinical trials to date, in excess of 700 human subjects have received a total of more than 2000 intranasal doses (unpublished data).

However, there are no reports on whether chitosan has any utility in improving the intranasal absorption of high molecular weight (>10 kDa) therapeutic proteins. This paper presents work to explore the feasibility of delivering hGH intranasally using chitosan-based delivery systems. The sheep was used as the animal model. Both chitosan powder blend and granule formulations were prepared and characterised. The latter was developed with a view to exploring whether this particular presentation would provide for improved powder flow and/or blend homogeneity compared with a powder blend formulation.

2. Materials and methods

2.1. Materials

Recombinant human growth hormone (frozen bulk solution containing 8.8 mg/ml protein) was supplied by Biochemie, Kundl, Austria. Chitosan glutamate (Protasan UP G213) was purchased from NovaMatrix, Drammen, Norway and polyvinylpyrrolidone (PVP) (Kollidon® 30) from BASF, Ludwigshafen, Germany. Dichloromethane, disodium hydrogen orthophosphate dihydrate, sodium dihydrogen orthophosphate dihydrate, hydrochloric acid and sodium hydroxide were purchased from Fisher Scientific, Loughborough, UK. Deionised water was used throughout (Prima and Maxima water purification units, ELGA Lab Water, High Wycombe, UK).

2.2. Formulation preparation

Defrosted hGH solution (170 ml) was transferred into a glass beaker, frozen using liquid nitrogen and lyophilised for 48 h (ThermoSavant ModulyoD freeze dryer, Thermo Life Sciences, Basingstoke, UK). The resultant powder was passed through a sieve (0.85 mm aperture size) before use.

To prepare the powder blend (Formulation A), 648 mg of freeze-dried hGH and 408 mg of chitosan glutamate were gently mixed in a glass mortar using a pestle. This powder was transferred to a glass vial and mixed using a Turbula T2 mixer (Willy Bachofen, Bubendorf, Switzerland) for 30 min. The final powder was stored refrigerated in a glass vial.

To prepare granules (Formulation B), 15 mg of PVP was dissolved in 2–3 ml of dichloromethane in a glass beaker. To the PVP solution, were added 864 mg of freeze-dried hGH and 529 mg of chitosan glutamate and these were mixed using a spatula to form a homogeneous mass. The majority of the dichloromethane was allowed to evaporate in a fume hood and then the wet mixture was passed through a 0.25 mm aperture size sieve and oven dried at 40 °C to constant weight. The dried granules were gently milled in a mortar and passed through a 0.15 mm aperture size sieve. The sieved material was stored desiccated in a refrigerator in a glass vial.

Phosphate buffer solution was prepared by dissolving 56 mg of disodium hydrogen orthophosphate dihydrate and 29 mg of sodium dihydrogen orthophosphate dihydrate in

Table 1
Theoretical composition of hGH formulations administered to sheep

Formulation	Composition
Intranasal hGH/chitosan glutamate powder blend (Formulation A)	50% (w/w) hGH, 11.4% (w/w) buffer salts, 38.6% (w/w) chitosan glutamate
Intranasal hGH/chitosan glutamate granules (Formulation B)	50% (w/w) hGH, 11.4% (w/w) buffer salts, 37.5% (w/w) chitosan glutamate, 1.1% (w/w) PVP
Subcutaneous injection	0.57 mg/ml hGH in pH 7 phosphate buffer

45 ml of water, adjusting to pH 7 using 1 M sodium hydroxide solution and then making up to 50 ml with water. An hGH s.c. injection (0.57 mg/ml) was prepared by diluting 1.62 ml of hGH bulk solution to 25 ml with the phosphate buffer and then passing through a sterile 0.2 μm syringe filter (Acrodisc®, Pall, Portsmouth, UK) into sterile glass injection vials. The injection vials were sealed and stored refrigerated until required.

The composition of the hGH formulations is summarised in Table 1.

2.3. Analysis of hGH by circular dichroism (CD)

A sample of lyophilised hGH was reconstituted in water to a concentration of 8.8 mg/ml. This solution and a sample of defrosted hGH bulk solution were further diluted to a concentration of 88 μg/ml and CD spectra recorded (analysis performed by Department of Chemistry, University of Nottingham, UK).

2.4. Characterisation of formulations

The appearance of the powder formulations was noted and a qualitative visual assessment of flow properties performed. The morphology of the formulations was observed by light microscope.

The hGH formulations were analysed for both total protein and hGH content. To measure total protein, 10 mg samples of each formulation were weighed into 10 ml glass vials in triplicate. Each sample was dissolved in 5 ml-acidified water (pH 3 with hydrochloric acid) followed by two washes of the vial, transferring all the aliquots into a 10 ml volumetric flask and making up the volume with acidified water. The protein concentration of each sample was then determined utilising a bicinchoninic acid (BCA) method (Pierce and Warriner, Chester, UK) using a standard curve of hGH in acidified water. The homogeneity of each formulation was assessed by calculating the variability of protein content in the triplicate samples. The hGH content of the powders was measured by SEC-HPLC using an Agilent system. For analysis, 10 mg samples of each powder formulation were dissolved in triplicate in mobile phase (20 mM phosphate buffer, pH 7.2) and diluted to provide a concentration of approximately 400 μg/ml hGH. The reconstituted samples (20 μl) were injected onto a BioSep SEC2000 column (300 mm × 7.8 mm) (Phenomenex, Macclesfield, UK) at room temperature. Analyses were performed under isocratic conditions at a flow rate of 0.3 ml/min and detection was by UV at 214 nm. The concentration of hGH in each sample was calculated by reference to a calibration curve of hGH in mobile phase.

2.5. In vivo testing

The pharmacokinetic evaluation of the hGH formulations in sheep was performed under a United Kingdom Home Office Project Licence and had been approved by the Ethical Review Committee at the University of Nottingham.

Six Leicester Mule and Texel crossbred sheep weighing 56 ± 6 kg (mean ± S.D.) were housed in a controlled environment at the School of Biosciences, University of Nottingham. The study was performed as a partially randomised crossover with a minimum of 2 days washout between successive study days. On study days 1 and 2 the two intranasal hGH powders (Formulations A and B) were administered according to a randomised design. On study day 3 all animals received a s.c. injection of hGH. Food was withdrawn for a period of about 5 h on each study leg starting approximately 1 h before dosing. Throughout, the animals had free access to drinking water. To assist in handling during nasal dosing, animals were sedated for around 3 min by administering 2.25 mg/kg ketamine hydrochloride (Ketaset® injection, Fort Dodge Animal Health, Southampton, UK) by venepuncture of a jugular vein.

The intranasal hGH dose was 17 mg (approximately 0.3 mg/kg), administered as two equal portions into each nostril. Each portion of dose was loaded (with 3 ± 0.5 mg overfill) into an individual 5 mm siliconised tracheal tube (SIMS Portex, Hythe, UK), which was inserted 6–7 cm into the nasal cavity and the contents emitted using a oneway bellows. The exact amount of powder administered to each sheep was determined by weighing each tube before and after dose administration. The s.c. dose was 1.7 mg of hGH (approximately 0.03 mg/kg) injected into a shaved area of the flank.

Blood samples (4 ml) were collected by venepuncture of a cephalic vein under local anaesthesia prior to intranasal hGH administration and at 20, 40, 60, 80, 100, 120, 180, 240, 300, 360 and 540 min post-administration. Following s.c. administration, the blood sampling schedule differed only in that additional samples were collected at 90, 720 and 1440 min and the 80, 100 and 300 min samples were omitted. After collection, each blood sample was dispensed into a serum tube and the sample mixed at room temperature for 45–60 min prior to separation of serum by centrifugation. The separated serum was stored frozen at −80 °C.

2.6. Bioassay

Serum samples were analysed by Nottingham City Hospital NHS Trust, Nottingham, UK using an IMMULITE® 2000 Growth Hormone (hGH) kit (Euro/DPC, Gwynned, Wales). The method is a chemiluminescent immunometric assay using hGH-specific antibodies, murine monoclonal anti-hGH antibody coated beads and alkaline phosphatase-conjugated rabbit polyclonal anti-hGH antibody, employing the IMMULITE® 2000 analyser. The hGH concentration of the samples was determined against a standard curve of hGH in sheep serum. The variability of the assay was evaluated using the data obtained for hGH quality control samples prepared in sheep serum and the standard curve. The validation parameters of precision (CV of <15%) and accuracy (recovery of ±20%) were acceptable and the lower limit of quantitation (LOQ) was 0.56 ng/ml.

2.7. Pharmacokinetic analysis

Pharmacokinetic (non-compartmental) analysis on the serum hGH data was performed using WinNonlin Version 1.1 (Scientific Consulting, North Carolina, USA). The principal pharmacokinetic parameters used were maximum (peak) serum hGH concentration (C_{max}), time of maximum concentration (T_{max}) and area under the serum hGH concentration-time curve extrapolated to infinity (AUC_{inf}).

For each animal, the bioavailability of intranasally (i.n.) administered hGH relative to s.c. injection (F_{rel}) was calculated as follows

$$F_{rel}\,(\%) = \frac{AUC_{inf(i.n.)} \times \text{mean dose}_{(s.c.)}}{\text{mean } AUC_{inf(s.c.)} \times \text{dose}_{(i.n.)}} \times 100$$

2.8. Statistical analysis

Data are presented as mean values ± S.D.

The principal pharmacokinetic measures obtained for the intranasal dose groups were compared statistically using GraphPad™ Instat® Version 3.01 (GraphPad, San Diego, USA). A paired *t*-test was performed on dose-normalised log-transformed values of C_{max} and AUC_{inf}. Values of T_{max} were analysed using a Wilcoxon matched-pairs signed-ranks test (non-parametric alternative to paired *t*-test). Throughout, the significance level was set at 5% (i.e. $\alpha = 0.05$).

3. Results and discussion

CD spectra of the reconstituted hGH and diluted bulk solution are provided in Fig. 1. The spectra were essentially identical, with minima at 221 and 210 nm and a maximum at 191 nm, indicative of folded protein with a high α-helical content. This result indicated that the secondary and tertiary structure of hGH was not adversely affected by lyophilisation.

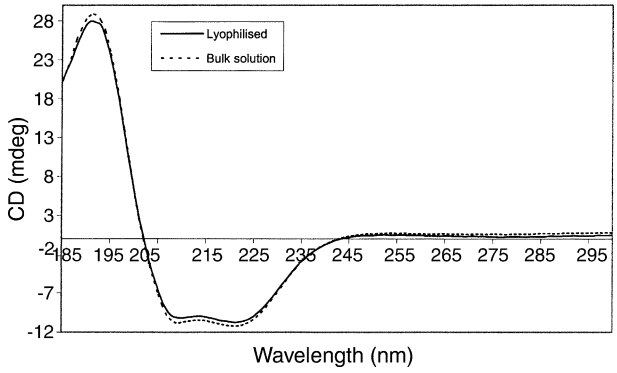

Fig. 1. CD spectra of hGH bulk solution and reconstituted lyophilised hGH, both diluted to an hGH concentration of 88 μg/ml.

Formulation A was a relatively free-flowing white powder and Formulation B was a free-flowing white powder. Photomicrographs of the two formulations are presented in Fig. 2 and their difference in appearance is consistent with the methods of manufacture. The lyophilised hGH used in the preparation of both the powder and granule formulations was passed though a 0.85 mm sieve prior to processing. For the powder formulation, chitosan was triturated directly with the sieved hGH using a pestle and mortar and no further particle size reduction was performed. For the granule formulation, granules were passed through a 0.15 mm sieve following drying. The difference in particle size between the two formulations may be explained by the ease of passing granu-

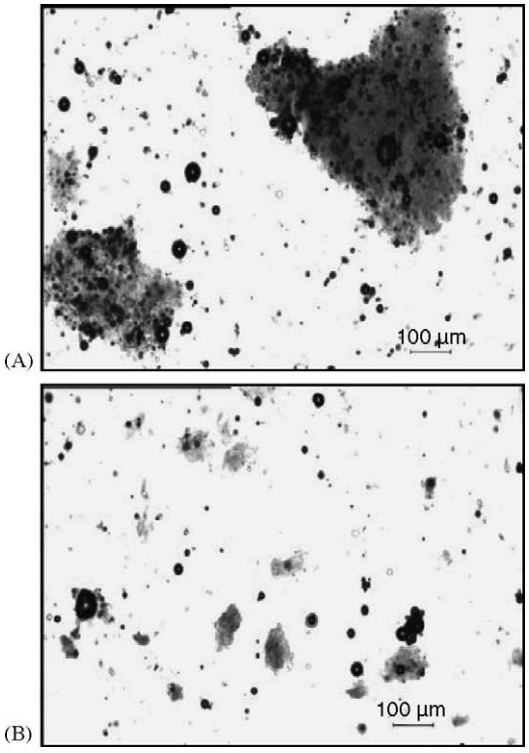

Fig. 2. Light micrographs of hGH intranasal formulations: (A) powder blend; (B) granules.

lated material through a relatively fine mesh sieve (0.15 mm) compared with lyophilised hGH (0.85 mm), the latter of, which is characteristically light, fluffy and electrostatic. Because of the significant difference in particle size between lyophilised hGH and chitosan glutamate powder, mean particle size data was considered inaccurate and thus was not generated.

The mean protein contents of Formulations A and B (theoretical values = 50% (w/w) hGH) were 50.8 ± 2.4% (w/w) and 53.3 ± 1.1% (w/w). Protein content uniformity of both formulations was satisfactory, with a coefficient of variance (CV) of less than 10%. The protein content of the subcutaneous injection (theoretical hGH content = 0.57 mg/ml) was 0.60 mg/ml. The hGH content (SEC-HPLC) of Formulation A and B was 41.1 ± 1.8% (w/w) and 44.4 ± 1.2% (w/w), respectively. The shortfall between assayed hGH content and expected content is attributed to incomplete recovery of hGH in the presence of chitosan. The hGH content of the s.c. injection was 0.56 mg/ml ($n = 2$).

The mean dose of hGH administered to sheep for Formulations A and B was 20 ± 1 and 19 ± 1 mg, respectively, as calculated by weight difference of the tracheal tubes before and after dosing (see Table 2). There were no adverse systemic events evident in the sheep following the intranasal or s.c. administrations of hGH and, based on observations of sneezing/snorting and nasal mucus discharge, both intranasal formulations appeared to be well tolerated.

The pharmacokinetic data from the administration of hGH formulations to sheep are presented in Table 2 and serum concentration–time profiles in Fig. 3. The mean serum hGH concentration–time profile was similar for the two intranasal formulations, characterised by an early hGH peak, indicative of relatively rapid absorption and distribution of the drug, followed by slower decline due to elimination (Fig. 2). The (terminal) elimination half-life of hGH was around 70 min for both formulations. In contrast, the elevation and especially the subsequent decline in serum hGH was much more gradual following s.c. administration, attributable to slow absorption from the injection site; the elimination half-life was around 240 min (Fig. 2). A similar pattern of subcutaneous absorption and clearance for s.c. hGH is reported in humans (Dollery, 1999).

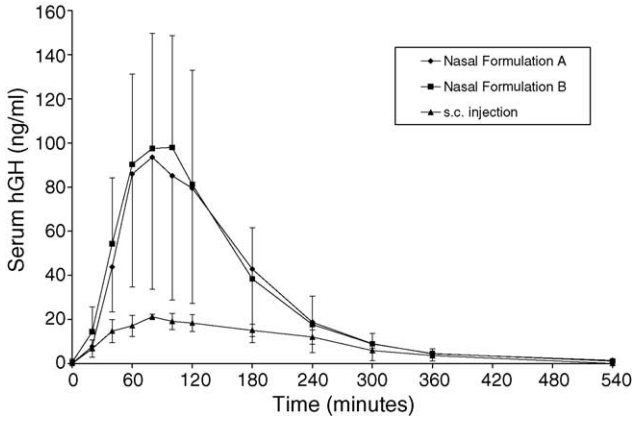

Fig. 3. Serum hGH concentrations following intranasal administration of chitosan-based formulations and subcutaneous administration of hGH solution (mean ± S.D., $n = 6$).

The principal PK data also confirm that the two intranasal formulations were highly comparable in spite of their different physical characteristics, such as particle size distribution; mean peak serum concentrations (C_{max}) were 98 and 106 ng/ml for Formulations A and B, respectively reached at (T_{max}) 90 and 73 min, respectively. Mean bioavailabilities (adjusted for dose), relative to s.c. injection, were 14–15%. There were no significant differences ($p > 0.05$) between the PK measures obtained for the two intranasal formulations (Table 2). Following s.c. dosing, the mean peak serum concentration of hGH was around 22 ng/ml at 110 min.

The variability in the pharmacokinetic parameters was higher for the intranasal doses than s.c. injection. One of the factors that could have increased variability in the nasal dose groups was the method of dosing. It is possible that the tube and bellows system used to dose the sheep may not provide an optimum distribution of powder in the nasal cavity, which could consequently compromise the consistency of hGH absorption. Although spray devices that will provide a more uniform deposition of powder are commercially available, these are principally designed for use in humans and will not work satisfactorily in sheep, due to their much longer nostrils.

The similar pharmacokinetic performance of the powder and granule formulations suggested that, despite their

Table 2
Summary of pharmacokinetic (PK) measures obtained after intranasal or s.c. administration of various hGH formulations to sheep[a]

Formulation	hGH dose (mg)	C_{max}[b] (ng/ml)	T_{max}[c] (min)	AUC_{inf}[d] (ng min/ml)	F_{rel}[e] (%)
Intranasal hGH/chitosan glutamate (A)	20 ± 1	98 ± 58	90 ± 17	14543 ± 9317	14 ± 9
Intranasal hGH/chitosan glutamate granules (B)	19 ± 1	106 ± 47	73 ± 16	14995 ± 7629	15 ± 8
s.c. hGH injection	1.7 ± 0	22 ± 1	110 ± 41	9264 ± 1480	100[f]
Statistical testing (nasal data only)	NT	$p = 0.619$	$p = 0.063$	$p = 0.708$	NT

NT: not tested.
[a] Data presented as the mean ± S.D. ($n = 6$).
[b] Maximum or peak serum hGH concentration.
[c] Time of maximum concentration.
[d] Area under the serum hGH concentration–time curve extrapolated to infinity.
[e] Bioavailability of intranasal hGH relative to s.c. injection.
[f] Calculation of S.D. is not applicable; the mean AUC_{inf} following s.c. injection was used during calculation of F_{rel}.

different physical properties, in particular particle size distributions, the rate at which hGH was liberated from the formulations and became available for systemic absorption was comparable.

The bioavailabilities achieved with the intranasal chitosan formulations in this study were comparable to the values reported previously for LPC (16.0%) (Fisher et al., 1991) and for starch microspheres/LPC (14.4%) (Illum et al., 1990). In contrast, these published bioavailabilities were associated with C_{max} values of 51.6 ng/ml for LPC and 55.4 ng/ml for starch microspheres/LPC, whereas in the current study C_{max} values were around two-fold higher for a comparable dose of hGH, which presumably reflected differences between the formulations in the rate of protein absorption from the nasal cavity into the systemic circulation.

Regarding the potential utility of intranasal chitosan/hGH formulations in humans, existing subcutaneous doses are typically in the range of 6–12 μg/kg in adults with growth hormone deficiency (Sweetman, 2002). These values would equate to a total hGH dose of approximately 0.4–0.8 mg in a 70 kg adult. The sheep is reported to be a good predictive model for intranasal drug absorption in humans (Illum et al., 2003). Hence, working on the assumption that bioavailability data comparable to sheep could be achieved in humans, 0.4–0.8 mg of hGH would be delivered into the systemic circulation by intranasal administration of around 5–10 mg of chitosan-based powder formulation. It is a practical proposition to dose such quantities of powder intranasally to a human. Endogenous growth hormone is secreted in pulses approximately every 3 h and shows diurnal variation with more produced at night than during the day (Toogood et al., 1997). The apparent pulsatile pattern of hGH delivery following intranasal administration of the powders mimics more closely the endogenous pattern of growth hormone secretion than the s.c. injection, which could have therapeutic advantages. This suggestion is supported by a study which showed that growth hormone was more effective in promoting growth in hypophysectomised rats when given as intravenous pulsate doses than when given either as an intravenous infusion or as a s.c. dose (Clark et al., 1985).

4. Conclusions

This study has demonstrated that the ability of the cationic polysaccharide, chitosan to improve the bioavailability of intranasally administered polar drugs and peptides can be extended to a higher molecular weight protein. If comparable bioavailability can be achieved in humans, it should be possible to deliver therapeutically relevant amounts of hGH by the intranasal route and thus offer an effective alternative to the existing injection dosing regimen.

The in vivo performance of the powder blend and granule formulations was comparable, although it is possible that the latter presentation could offer some advantages in terms of handling and flow properties.

Furthermore, there are many other therapeutic proteins of a similar size to hGH, such as interferons, interleukins and colony stimulating factors; chitosan may be an enabling technology for intranasal delivery of these molecules.

References

Agerholm, C., Bastholm, L., Johansen, P.B., Nielsen, M.H., Elling, F., 1994. Epithelial transport and bioavailability of intranasally administered human growth hormone formulated with the absorption enhancers didecanoyl-L-α-phosphatidylcholine and α-cyclodextrin in rabbits. J. Pharm. Sci. 83, 1706–1711.

Arora, P., Sharma, S., Garg, S., 2002. Permeability issues in nasal drug delivery. Drug Discov. Today 7, 967–973.

Artursson, P., Lindmark, T., Davis, S.S., Illum, L., 1994. Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). Pharm. Res. 11, 1358–1361.

Baldwin, P.A., Klingbeil, C.K., Grimm, C.J., Longenecker, J.P., 1990. The effect of sodium tauro-24,25-dihydrofusidate on the nasal absorption of human growth hormone in three animal models. Pharm. Res. 7, 547–552.

Clark, R.G., Jansson, J.-O., Isaksson, O., Robinson, I.C.A.F., 1985. Intravenous growth hormone: growth responses to patterned infusions in hypophysectomised rats. J. Endocrinol. 104, 53–61.

Dodane, V., Khan, M.A., Merwin, J.R., 1999. Effect of chitosan on epithelial permeability and structure. Int. J. Pharm. 182, 21–32.

Dollery, C. (Ed.), 1999. Therapeutic Drugs, Churchill Livingstone, Edinburgh, p. S73.

Dyer, A.M., Hinchcliffe, M., Watts, P., Castile, J., Jabbal-Gill, I., Nankervis, R., Smith, A., Illum, L., 2002. Nasal delivery of insulin using novel chitosan based formulations: a comparative study in two animal models between simple chitosan formulations and chitosan nanoparticles. Pharm. Res. 19, 998–1008.

European Pharmacopoeia, 2002. 4th ed., Council of Europe, Strasbourg, pp. 875–876.

Fisher, A.N., Farraj, N.F., O'Hagan, D.T., Jabbal-Gill, I., Johansen, B.R., Davis, S.S., Illum, L., 1991. Effect of L-α-phosphatidylcholine on the nasal absorption of human growth hormone in three animal species. Int. J. Pharm. 74, 147–156.

Hedin, L., Olsson, B., Diczfalusy, M., Flyg, C., Petersson, A.S., Rosberg, S., Albertsson-Wikland, K., 1993. Intranasal administration of human growth hormone (hGH) in combination with a membrane permeation enhancer in patients with GH deficiency: a pharmacokinetic study. J. Clin. Endocrinol. Metab. 76, 962–967.

Illum, L., Farraj, N.F., Davis, S.S., Johansen, B.R., O'Hagan, D.T., 1990. Investigation of the nasal absorption of biosynthetic human growth hormone in sheep—use of a bioadhesive microsphere delivery system. Int. J. Pharm. 63, 207–211.

Illum, L., Fisher, A.N., 1997. Intranasal delivery of peptides and proteins. In: Adjei, A.L., Gupta, P. (Eds.), Inhalation Delivery of Therapeutic Peptides and Proteins. Marcel Dekker, New York, Chapter 5.

Illum, L., Farraj, N.F., Davis, S.S., 1999. Chitosan as a novel nasal delivery system for peptide drugs. Pharm. Res. 11, 1186–1189.

Illum, L., Watts, P., Fisher, A.N., Jabbal-Gill, I., Davis, S.S., 2000. Novel chitosan-based delivery systems for nasal administration of goserelin. STP Pharma 10, 89–94.

Illum, L., Jabbal-Gill, I., Hinchcliffe, M., Fisher, A.N., Davis, S.S., 2001. Chitosan as a novel nasal delivery system for vaccines. Adv. Drug Del. Rev. 51, 81–96.

Illum, L., Watts, P., Fisher, A.N., Hinchcliffe, M., Norbury, H., Jabbal-Gill, L., Nankervis, R., Davis, S.S., 2002. Intranasal delivery of morphine. J. Pharmacol. Exp. Ther. 301, 391–400.

Illum, L., 2002. Nasal drug delivery: New developments and strategies. Drug Dis. Today 7, 1184–1189.

Illum, L., Hinchcliffe, M., Davis, S.S., 2003. The effect of blood sampling site and physicochemical characteristics of drugs on bioavailability after nasal administration in the sheep model. Pharm. Res. 20, 1474–1484.

Illum, L., 2003. Nasal drug delivery: possibilities, problems and solutions. J. Control. Rel. 87, 187–198.

Laursen, T., Grandjean, B., Jorgensen, J.O., Christiansen, J.S., 1996. Bioavailability and bioactivity of three different doses of nasal growth hormone (GH) administered to GH-deficient patients: comparison with intravenous and subcutaneous administration. Eur. J. Endocrinol. 135, 309–315.

Mills, K.H., Cosgrove, C., McNeela, E.A., Sexton, A., Giemza, R., Jabbal-Gill, I., Church, A., Lin, W., Illum, L., Podda, A., Rappouli, R., Pizza, M., Griffin, G.E., Lewis, D.J., 2003. Protective levels of diphtheria-neutralising antibody induced in healthy volunteers by unilateral priming-boosting intranasal immunization associated with restricted ipsilateral mucosal secretory immunoglobulin A. Infect. Immun. 71, 726–732.

Pearlman, R., Bewley, T.A., 1993. Stability and characterization of human growth hormone. In: Swang, Y.J., Pearlman, R. (Eds.), Stability and Characterization of Protein and Peptide Drugs: Case Histories. Plenum, New York, pp. 1–58.

Ranaldi, G., Marigliano, I., Vespignani, Perozzi, G., Sambuy, Y., 2002. The effect of chitosan and other polycations on tight junction permeability in the human intestinal Caco-2 cell line. J. Nutr. Biochem. 13, 157–167.

Roon, K.I., Soons, P.A., Uitendaal, M.P., de Beukelaar, F., Ferrari, M.D., 1999. Pharmacokinetic profile of alniditan nasal spray during and outside migraine attacks. Br. J. Clin. Pharmacol. 47, 285–290.

Sinswat, P., Tengamnuay, P., 2003. Enhancing effect of chitosan on nasal absorption of salmon calcitonin in rats: comparison with hydroxypropyl- and dimethyl-β-cyclodextrin. Int. J. Pharm. 257, 15–22.

Smith, J., Wood, E., Dornish, M., 2004. Effect of chitosan on epithelial cell tight junctions. Pharm. Res. 21, 43–49.

Soane, R.J., Frier, M., Perkins, A.C., Jones, N.S., Davis, S.S., Illum, L., 1999. Evaluation of the clearance characteristics of bioadhesive systems in humans. Int. J. Pharm. 178, 55–65.

Sweetman, S.C. (Ed.), 2002. Martindale, 33rd ed., Pharmaceutical Press, London, pp. 1286–1288.

Toogood, A.A., Naas, R.M., Pezzoli, S.S., O'Neill, P.A., Thorner, M.O., Shalet, S.M., 1997. Preservation of growth hormone pulsatility despite pituitary pathology, surgery and irradiation. J. Clin. Endocrinol. Metab. 82, 2215–2221.