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Pulmonary delivery of growth hormone using dry powders and visualization of its local fate in rats

Cynthia Bosquillon, Véronique Préat, Rita Vanbever*

Department of Pharmaceutical Technology, Université catholique de Louvain, School of Pharmacy, Avenue E. Mounier, 73 UCL 73.20, 1200 Brussels, Belgium

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Abstract

A dry powder aerosol formed of human growth hormone (hGH), lactose and dipalmitoylphosphatidylcholine was assessed for systemic delivery of the hormone in rats. The fate of the protein locally in the deep lung was examined post-delivery. The powder was prepared by spray-drying and presented a primary particle diameter of 4.4 μm and a tap density of 0.069 g/cm^3. The mass median aerodynamic diameter was 4.4 μm in the multi-stage liquid impinger at 60 l/min using a Spinhaler™ device. The emitted dose and fine particle fraction were 89% and 58%, respectively. Varying the airflow rate from 30 to 90 l/min had limited impact on aerosolization properties in vitro. No hGH dimers or glycation adducts were produced during formulation of the powder. hGH absorbed into the bloodstream with a time to peak of 23 and 52 min and with an absolute bioavailability of 23% and 8% following intratracheal insufflation of the dry powder and intratracheal spray-instillation of a solution of the hormone, respectively. Confocal imaging of rat lung revealed an intense uptake of fluorescein isothiocyanate (FITC)-hGH by alveolar macrophages as early as 1 h post-delivery. A dry powder aerosol made of selected GRAS excipients improved absorption of hGH from the lung over a simple solution.
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1. Introduction

Recombinant human growth hormone (hGH, 22 kDa) is currently delivered over several years by subcutaneous injection to children with a short stature due to growth hormone deficiency [1]. The welfare of these patients would therefore greatly benefit from a non-invasive delivery method of hGH. The oral route would represent the most convenient alternative; yet, due to enzymatic degradation in the gastrointestinal tract, the bioavailability of oral hGH is lower than 0.01% [2]. The nasal mucosa has also been tested for systemic administration of hGH but, in absence of penetration enhancers, absorption is low (< 1%) as well [3]. In contrast, delivery of hGH to the lung resulted in a bioavailability between 5% and 45% in rats and rabbits, according to the study and method of delivery [4–6]. More recently, a bioavailability on the order of 5–10% was obtained in humans following inhalation of large porous hGH powder particles [7].

Efficient absorption from the lung results from unique anatomical features of the alveoli as well as

* Corresponding author. Tel.: +32-2-764-73-25; fax: +32-2-764-73-98.

E-mail address: vanbever@farg.ucl.ac.be (R. Vanbever).

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from aerosol generation systems that successfully bypass particle filtering in upper airways [8]. The alveoli provide a 100 m^2 surface for absorption, a 0.5 μm diffusion path to the bloodstream and a dense capillary network that allows passage of 5 l of blood per minute. Filtering of particle aerosols in the mouth, trachea and main bronchi occurs by inertial impaction in the case of aerosol particles with an aerodynamic size >5 μm and deposition on the alveolar epithelium has been shown optimal for 1 to 5 μm particles [9].

Dry powder inhalers may be particularly suitable devices for pulmonary administration of proteins like hGH because of their facility of use and the improved drug stability provided by the dry state of the formulation [10]. Dry powder formulations include the drug as well as excipients intended for improving its physico-chemical stability and/or the aerosolization properties of the dry powder. For instance, insulin has been formulated with mannitol, glycine and sodium citrate in dry powder aerosols that are tested in clinical trials [11]. The formation of large porous particles has been shown facilitated by using dipalmitoylphosphatidylcholine (DPPC), a principal component of lung surfactant [12]. Yet, limited experimental work has assessed the impact of formulation physical state and dry powder excipients on drug absorption.

The mechanisms and specific pathways underlying lung protein clearance remain largely unknown as well [13]. However, enhancement of protein absorption from the lung could arise from a deeper understanding of elimination processes encountered by molecules locally in the airway lumen and respiratory tissue. In this regard, we recently reported that alveolar macrophages comprised a major barrier to transport of macromolecules from the lung into the bloodstream, opening up novel approaches to inhibit protein uptake by alveolar macrophages [14].

The aims of this work were (i) to compare the efficiency of inhalation dry powders and aqueous solutions for pulmonary delivery of hGH and highlight a possible impact of dry powder excipients on protein absorption, (ii) to visualize the overall fate of hGH in situ and examine if alveolar macrophages were involved in its local clearance as reported for larger proteins [14]. Dry powders were prepared by spray-drying and formed of hGH, lactose and DPPC. The aerodynamic behavior of the powders was assessed in vitro in a multi-stage liquid impinger and the chemical integrity of the protein was verified after formulation. Systemic absorption of hGH from the lung was measured in rats following intratracheal insufflation of the dry powder and intratracheal instillation of a solution and the fate of FITC-hGH was visualized by confocal laser scanning microscopy post-delivery in rat lung.

2. Materials and methods

2.1. Chemicals

Human growth hormone (isoelectric point, pI, = 5.2) was donated by Eli Lilly (Indianapolis, USA). DPPC was purchased from Lipoid (Lipoid, Ludwigshafen, Germany) and α-lactose monohydrate from Acros Organics (New Jersey, USA). D-Trehalose dihydrate, 96% ethanol, xylazine, sulforhodamine 101, glutaraldehyde and fluorescein isothiocyanate (FITC) isomer were obtained from Sigma (Sigma-Aldrich, Bornem, Belgium). Formaldehyde and disodium hydrogen phosphate dihydrate (Lichropur®) were supplied by VWR International (Leuven, Belgium), cacodylate sodium by Federa (Brussels, Belgium) and Hank's balanced salt solution (without Ca^{2+} and Mg^{2+}) by Life Technologies (Merelbeke, Belgium). Sodium pentobarbital, ketamine (Ketalar®) and fentanyl/droperidol mixture (Thalamonal®) were purchased from Certa (Medeva Pharma, Braine-l'Alleud, Belgium), Warner-Lambert (Zaventem, Belgium) and Janssen-Cilag (Berchem, Belgium), respectively.

2.2. Animals

Male Wistar rats (9 to 12 weeks old; Elevage Janvier, Le Genest St Isle, France) were used for the pharmacokinetic and confocal microscopy studies. Animals had free access to tap water and laboratory diet (pelleted commercial standard diet, n°A04, Usine Alimentation Rationnelle, Epinay-sur-Orge, France) during the experimental period. All experimental protocols in rats were approved by the Ethical Committee for Animal Care and Use of the Faculty of Medicine of the Université catholique de Louvain.

2.3. Fluorescence labeling of hGH with FITC

hGH was labeled with FITC for determination of the actual protein content in the formulation and visualization of hGH fate in pulmonary tissue using confocal laser scanning microscopy (CLSM). Twenty microliters of FITC solution (100 mg/ml in dimethylsulfoxyde, DMSO) were added drop-wise per 1 ml of hGH solution (10 mg/ml in bicarbonate buffer, pH 8.8 0.1 M). The solution was protected from light and incubated under gentle stirring for 3 h at room temperature. A first dialysis (cut-off 10,000 Da; Snakskin Dialysis Tubing; Perbio Science, Erinbodegem-Aalst, Belgium) was performed to eliminate the largest part of unconjugated FITC. The labeled protein was further purified on a Sephadex G-25M column (Amersham Pharmacia Biotech Benelux, Roosendaal, Nederlands). The FITC-hGH solution was finally dialyzed against ultrapure water for salt elimination and lyophilized. Following this procedure, 1 mol hGH was conjugated with 2.7 mol FITC without alteration to protein molecular weight as analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (data not shown) [15].

2.4. Formulation of the dry powders

Dry powders were prepared by spray-drying using hGH, lactose and DPPC in proportion 20/20/60 w/w/w unless otherwise noted [16]. FITC-hGH replaced native hGH for determination of the actual protein content in the powder by spectrofluorimetry ($n=2$; Perkin Elmer Luminescence Spectrometer LS50B, $\lambda_{ex}=495$ nm, $\lambda_{em}=520$ nm). Sulforhodamine 101, a fluorescent dye, was added at a low load (0.2% w/w) to the formulation analyzed in vitro in order to easily quantify the powder fractions deposited on the multi-stage liquid impinger stages (see below). DPPC was dissolved in 96% ethanol; hGH, the sugar and/or sulforhodamine in 0.5 mM phosphate buffer, pH 7.4. The two solutions were then combined to form a 70% ethanolic solution of 0.1% w/v total excipient concentration.

The powders were produced using a LabPlant laboratory-scale spray-dryer (Lab-Plant Limited, Huddersfield, England) at low relative humidity (30–40%). The solutions were pumped into the drying chamber at a flow rate of 10 ml/min and pneumatically atomized through a two fluids external mixing 0.5 mm nozzle using compressed air at 0.5 bar. The inlet temperature was 100 °C and under these conditions, the outlet temperature varied between 50 and 62 °C. The powders were collected and stored in a dessicator at 25% relative humidity and 4 °C until analysis or in vivo assessment. Yields ranged between 10% and 20% [16].

2.5. Particle size, density and morphology

The primary geometric particle diameter (d) was measured by laser diffraction (HELOS, Sympatec, Clausthal-Zellerfelg, Germany). Powder samples were suspended in water in a 50 ml glass cuvette and stirred with a magnetic bar at 1000 rpm. A short period of sonication (30 or 60 s) at a power of 60 W (CUVETTE, Sympatec; 8.5 mm diameter ultrasound tip) was applied before sizing. A R2 lens allowing measurements in the range of 0.25–87.5 μm was used. The particle size analysis was performed by a WINDOX 3.4 software and the mass median particle diameter was taken into account [17].

The powder density (ρ) was determined by tap density measurements, i.e., following 1000 taps which allowed the density to plateau [18].

The primary aerodynamic diameter of the particles, d_{aer}, was calculated based on the following definition: $d_{aer}=(\sqrt{\rho/\rho_1})d$, with $\rho_1=1$ g/cm^3 [19].

2.6. Aerosolization properties of the powders in vitro

The pulmonary deposition of the dry powders was estimated in vitro in a multi-stage liquid impinger equipped with a USP induction port (Copley Scientific, Nottingham, UK) under low relative humidity (30–40%). Twenty milliliters of water were poured into each of the four stages of the impinger to wet the collection surfaces. A hard gelatin capsule (size 2, Capsugel), previously stored in a dessicator for at least 2 days, was half-filled with the powder and placed in a Spinhaler™ inhaler (Fisons, Bedford, MA). The capsule was then pierced and the liberated powder drawn through the impactor operated at 30, 60 or 90 l/min for 8, 4 or 2.7 s, respectively. This allowed the aspiration of 4 l of air through the apparatus as recommended by pharmacopoeias [20]. The powder

deposited on the four impinger levels was recovered by agitating the apparatus, removing the initial water and rinsing with additional fractions of water and ethanol up to reaching a total of 250 ml of a 60% ethanolic solution. The powder deposited in the throat and on the back filter was also collected. After dissolution of the particles, the fluorescence of each solution due to sulforhodamine was determined by spectrofluorimetry ($\lambda_{ex} = 586$ nm, $\lambda_{em} = 602$ nm). Measurements were performed in triplicate [21].

The emitted dose was determined as the percent of total powder mass exiting the capsule. The cumulative mass of powder less than the stated size of each stage of the impactor was calculated and plotted on a log probability scale, as percent of total mass recovered in the impactor against the effective cut-off diameter. The cut-off diameter of each individual stage (D) was determined as $D = D_{60}\sqrt{(60/Q)}$ where D_{60} is the cut-off diameter at a flowrate of 60 l/min, i.e., 13.0, 6.8, 3.1 and 1.7 μm for stages 1 to 4, respectively, and Q is the flowrate employed in the test [20]. The experimental mass median aerodynamic diameter (MMAD) of the particles was defined from this graph as the particle size at which the line crosses the 50% mark and the geometric standard deviation (GSD) as $\mathrm{GSD} = \sqrt{(\mathrm{Size}X/\mathrm{Size}Y)}$, where size X is the particle size for which the line crosses the 84% mark and size Y the 16% mark. The fine particle fraction was calculated by interpolation from the same plot as the fraction of powder emitted from the inhaler with an aerodynamic diameter ≤ 5 μm [20].

2.7. Protein aggregation and glycation

The amount of soluble hGH aggregates in the dry powders was evaluated by size-exclusion chromatography [22]. The powder was suspended in 5 mM phosphate buffer, pH = 7.4 for 1 h, which allowed the release of 90% of the amount of hGH incorporated in the formulation (as estimated by a Bradford assay). After centrifugation of the powder suspension ($2700 \times g$ at room temperature for 10 min), 50 μl of the supernatant was loaded onto a Va300/7.7 Nucleosil 125-5 GFC column (Macherey-Nagel, Düren, Germany) and hGH detected at 210 nm (Bio-Tek Instruments, Milano, Italy). The mobile phase consisted of 50 mM di-sodium hydrogen phosphate (Lichropur®) and sodium chloride 0.15 N, pH = 7.4, pumped at a flow rate of 1 ml/min. Under those chromatographic conditions, the retention time of hGH was 7 min. The limit of quantification was 5 μg/ml and the inter-assay relative standard deviation was 3.7%. The percentage of aggregates was evaluated by comparing the relative peak heights of hGH and dimer.

The formation of lactose-hGH adducts during formulation and storage was assessed by isoelectric focusing. A certain amount of powder made of 60% DPPC, 30% lactose or trehalose and 10% hGH w/w/w was suspended in Tris–EDTA buffer, pH = 8 for 1 h in order to reach a protein concentration of 0.5 mg/ml in the supernatant. After centrifugation ($2700 \times g$ at room temperature for 10 min), 20 μl of each sample were applied on Ampholine® PAGE plate gels with a pH gradient ranging from 3.5 to 9.5 (Amersham Pharmacia Biotech, Piscataway, USA) and focusing was carried out in a Multiphor II Electrophoresis System (Amersham Pharmacia Biotech) under the following conditions: 1500 V, 25 mA and 15 W. hGH was visualized by Coomassie blue staining (detection limits: 0.1–0.5 μg protein, Amersham Pharmacia Biotech) and its pI determined using protein standards in the 2.5–6.5 pI interval (Amersham Pharmacia Biotech).

2.8. Pharmacokinetic studies

Catheters (polyurethane tubings, 3 French 0.6 mm ID × 0.9 mm OD, Access Technologies, Skokie, IL, USA) for blood sampling and intravenous injection were implanted in the jugular veins of rats (9–10 weeks old, 340–400 g) 24 h before the pharmacokinetic study. The animals were anesthetized with Thalamonal® and then received a hGH dose by intratracheal insufflation of a dry powder, intravenous (IV) or subcutaneous (SC) injection, or intratracheal instillation of a solution. All hGH solutions were prepared with phosphate buffer, 5 mM, pH = 7.4, and the volume delivered by injection was adjusted to the rat weight to keep the dose per kg constant.

Approximately 1 mg of powder, corresponding to 200 μg of hGH, was delivered directly into the trachea through the mouth using a powder insufflator (Model DP-3; Penn-Century, Philadelphia, PA). Administration of the powder was performed by insufflation of 3 ml of air contained in a syringe connected to the device. The insufflator was weighed before and after

powder filling as well as after administration to determine the actual dose insufflated per rat [21].

One hundred and 200 μg/kg hGH (corresponding to approximately 35 or 70 μg/rat) were injected intravenously and subcutaneously, respectively. Two hundred micrograms of hGH (per rat) were administered intratracheally via the mouth using a spray-instillator that delivers a fixed volume of 100 μl (Microsprayer®, PennCentury, Philadelphia, USA). hGH was released from the dry powder formulation by suspending the dry powder in buffer for 1 h. After centrifugation ($2700 \times g$ at room temperature for 10 min), hGH concentration in the supernatant was determined by a Bradford assay and the solution was then injected subcutaneously to rats at a hGH dose of 200 μg/kg.

A total of 12 blood samples (200 μl) were collected from the jugular vein into lithium-heparinized tubes (Microtainer brand tubes lithium heparin, Becton Dickinson, Aalst, Belgium) over 4 h (IV injection), 5 h (powder insufflation) or 7 h (SC injection and spray-instillation) and replaced by an equivalent volume of sodium chloride 0.9% w/v to compensate for the decrease in blood volume. Plasma was separated by centrifugation (15 min at $3000 \times g$ and 4 °C) and stored at − 20 °C until analysis. The hGH plasma concentrations were measured using an ImmunoRadiometric assay (IRMA, DiaSorin, Saluggia, Italy). In this assay, one monoclonal antibody attached to a plastic tube binds hGH and a second one labeled with ^{125}I recognizes a distinct hGH epitope. hGH fragments or molecules that have lost their tertiary structure are not detected and normal rat plasma does not cross-react in the assay. The dosage range was 0.5–50 ng/ml and the inter-assay relative standard deviation was 5.2%. Plasma samples were assayed at proper dilutions, in duplicate, and hGH concentrations were determined by interpolation from the calibration curve.

Areas under the plasma concentration–time curve (AUC) were calculated using the linear trapezoidal rule from time 0 to t (last blood sampling time) and extrapolation to infinity (plasma concentration at time t divided by the elimination rate constant k). The maximal plasma concentration (C_{max}) and the time to peak (t_{max}) were obtained from the individual concentration–time curves. Bioavailability parameters (absolute and relative bioavailability, F_{abs} and F_{rel}) were calculated as $[(\mathrm{AUC} \times \mathrm{dose}_{ref})/(\mathrm{AUC}_{ref} \times \mathrm{dose})] \times 100$ and the plasma elimination half-life ($t_{1/2}$) as $\ln 2/k$. The elimination rate constant (k) was estimated by linear regression of the last time points of the log concentration vs. time curve. The clearance (CL) was calculated as $[\mathrm{dose} \times F_{abs}]/\mathrm{AUC}$ and the volume of distribution (V_d) as CL/k. The last two parameters were normalized to rat weight. Mean residence times (MRT) were calculated as the ratio between areas under the first-moment vs. time curve (AUMC) and AUC. Mean absorption times (MAT) were calculated by subtracting MRT after IV injection from that after pulmonary delivery or SC injection.

2.9. Confocal microscopy studies

Rats (10–12 weeks old, 400–480 g) were anesthetized by an intraperitoneal injection of ketamine/xylazine (90/10 mg/kg) and 500 μg of FITC-hGH (dissolved in 100 μl buffer) were delivered intratracheally using the spray-instillator. Immediately, 1 and 4 h after delivery, the abdominal cavity was incised and a 16-gauge catheter (Ohmeda, Helsingborg, Sweden) connected to a double reservoir was inserted in the posterior vena cava. The two reservoirs contained respectively solution (1): 0.1% sulforhodamine in phosphate-buffered saline (138 mM NaCl, 8.1 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 2.7 mM KCl, pH 7.4) and solution (2): 0.1% sulforhodamine, 0.6% formaldhehyde, 0.9% glutaraldehyde in cacodylate buffer, 75 mM, pH 7.4. Both carotid arteries were severed and the lung was perfused with solution (1) at 10 ml/min for 5 min and then fixed by perfusing solution (2) at 5 ml/min for an additional 5 min [15]. The thoracic cavity was opened and the lung was removed for analysis by CLSM. The total time for intratracheal delivery and perfusion was approximately 20 min. Each experimental condition was repeated at least three times.

Slices (± 2 mm) of the pulmonary lobes were directly placed in a sample holder and covered with a coverslip glass. The microscope used was a Bio-Rad MRC 1024 confocal unit equipped with an argon–krypton laser and mounted on a Zeis Axiovert 135 M inverted microscope. Laser excitation wavelengths of 488 nm and 568 nm were used individually to scan lung tissue, and fluorescent emissions from FITC-hGH (emission $\lambda = 515–545$ nm) and sulforhodamine (emission $\lambda = 589–621$ nm) were collected using separate channels. Images were

acquired with a Zeiss Plan-Neofluor 40 x oil immersion lens. Grayscale images (obtained from each scan) were pseudo-colored green (FITC-hGH) and red (sulforhodamine) and then overlaid (BioRad Lasersharp 2000 software, Nazareth, Belgium) to form a multicolored image.

To assess the autofluorescence of the pulmonary tissue, samples were examined after rinsing and fixation of the tissue. The autofluorescence of the rat lung in the green channel was found to be very low with the confocal settings used in this study, except in peripheral regions where fiber networks and cells exhibited high autofluorescence (data not shown). Confocal images were therefore collected at least 10 μm below the lung surface [15].

The uptake of FITC-hGH by alveolar macrophages was confirmed by analyzing the cellular components of a bronchoalveolar lavage (BAL) under the confocal microscope [15]. Rats received a lethal dose of pentobarbital and 10 ml of Hank's balanced salt solution was slowly instilled into the trachea. After 30 s, the liquid was withdrawn from the lung. The lavage procedure was repeated with four additional 10 ml aliquots until a total volume of 50 ml was instilled. The BAL was centrifuged at $700 \times g$ at 4 °C during 10 min. The supernatant was removed and the cells were resuspended in 1 ml of Hank's balanced salt solution. A few droplets of the cell suspension were mounted on a slide and covered with a coverslip glass for analysis by CLSM.

2.10. Statistics

The data were validated by the Dixon test. All results are expressed as mean ± standard errors of the mean (S.E.M.). One-way analysis of variance (ANOVA) test and Tukey test were performed to demonstrate statistical differences ($p < 0.05$), using the software Sigma-stat for Windows (SPSS, San Rafael, CA, USA).

3. Results

3.1. Powder preparation and characterization

A powder made of 20% hGH, 20% lactose and 60% DPPC was prepared by spray-drying according to formulation parameters previously optimized in terms of aerodynamic behavior in cascade impactors in vitro [16]. Dry powders from different spray-drying runs presented a primary particle size between 4.2 and 4.6 μm and a tap density between 0.05 and 0.08 g/cm^3. Theoretical estimates of primary particle aerodynamic diameters ranged between 1.0 and 1.3 μm, indicating that the powder was suitable for deposition in the deep lung.

The influence of increasing the airflow from 30 to 90 l/min on the aerosolization properties of the dry powder was assessed in the multi-stage liquid impinger. The emitted dose reached values between 85% and 95% and was independent of the airflow rate (Fig. 1A; Anova, p>0.05). The fine particle fraction was 41% at 30 l/min, increased to 58% at 60 l/min (Tukey test, $p < 0.05$) and then plateaued at 90 l/min (Fig. 1A; Tukey test, p>0.05). The mass median aerodynamic diameter (MMAD) decreased when increasing the airflow from 30 to 60 l/min (Tukey test, $p < 0.05$) and then plateaued at 90 l/min (Fig. 1B; Tukey test, p>0.05). The geometric standard deviation varied between 1.4 and 1.7, independently of the airflow (Anova, p>0.05).

The actual hGH content in the dry powder was assessed by spectrofluorimetry on dry powders prepared with FITC-hGH instead of native hGH. It was 86 ± 3% of the theoretical content. This value was taken into account to determine the hGH dose delivered to rats in the pharmacokinetic study (see below).

3.2. Chemical integrity of hGH after formulation

Since hGH is prone to aggregation at air–liquid interfaces, the proportion of soluble hGH dimers after powder production was evaluated by size-exclusion chromatography [23]. The dimers content was < 1% in native hGH as well as in hGH powder, indicating that incorporation of hGH in the dry powder formed of lactose and DPPC did not induce dimerization. In contrast, 3.9% of hGH dimers was measured following spray-drying in absence of DPPC (data not shown).

Lactose is a reducing sugar and a Maillard reaction of this excipient with the primary amine of lysine residues of hGH could occur during dry powder production and/or storage [24]. A potential

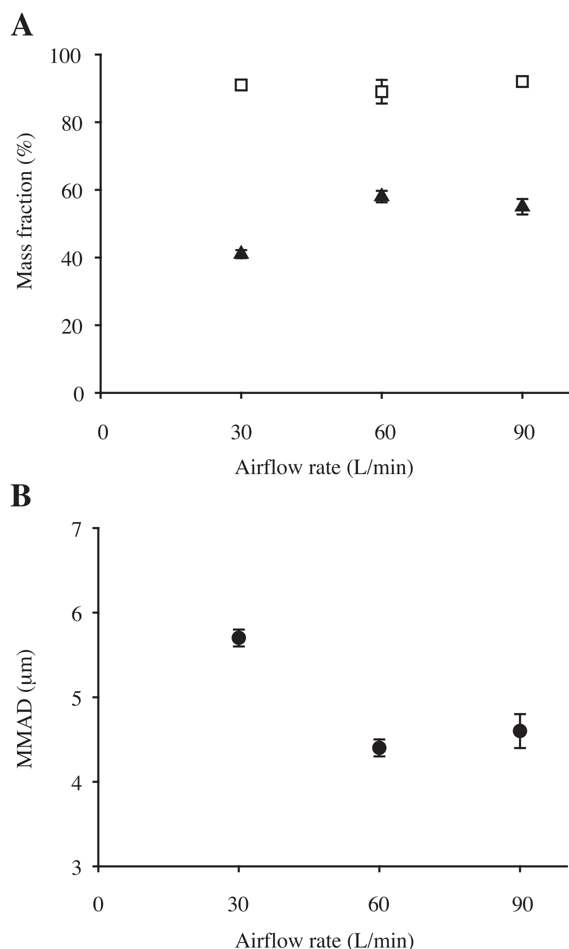

Fig. 1. Influence of the airflow rate on the fine particle fraction (A; triangle), emitted dose (A; square) and mass median aerodynamic diameter (MMAD; B) of a powder made of 20% hGH, 20% lactose and 60% DPPC. Error bars are standard errors of the mean.

acidic shift in hGH pI was analyzed by isoelectric focussing. A dry powder containing trehalose, a non-reducing sugar, instead of lactose was used as a negative control. No modification to the protein pI was observed after formulation of both types of powders and up to 5 months of storage at 4 °C for the powder containing lactose (Fig. 2).

3.3. Pharmacokinetic study

In order to evaluate the potential of the dry powder for systemic delivery of hGH, the powder was insufflated in rat lung and the pharmacokinetic parameters

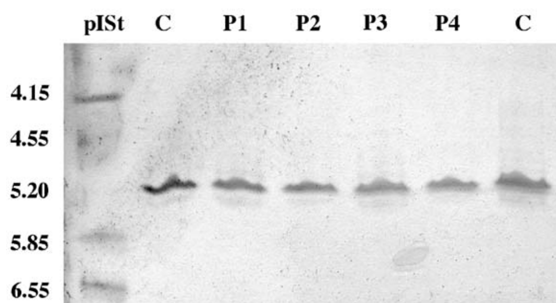

Fig. 2. hGH glycation assayed by isoelectric focussing. pISt = isoelectric point standards, C = hGH dissolved in buffer, P = hGH released from powders made of hGH/lactose/DPPC 10/30/60% w/w/w stored at 4 °C for 5 months (P1); 4 months (P2) or 2 months (P3) and from a powder containing trehalose (P4).

were compared with those obtained after intratracheal spray-instillation, IV and SC injections of a hGH solution.

Dry powder inhalation resulted in high absorption of hGH: the absolute bioavailability reached 23% and the bioavailability relative to SC 56% (Fig. 3, Table 1). In contrast, intratracheal instillation of a hGH solution showed threefold lower systemic absorption (Table 1). Additionally, absorption from the lung occurred over a shorter period and plasma hGH concentrations peaked sooner after powder insufflation than after spray-instillation (Fig. 3, Table 1; Tukey test, $p < 0.05$). This

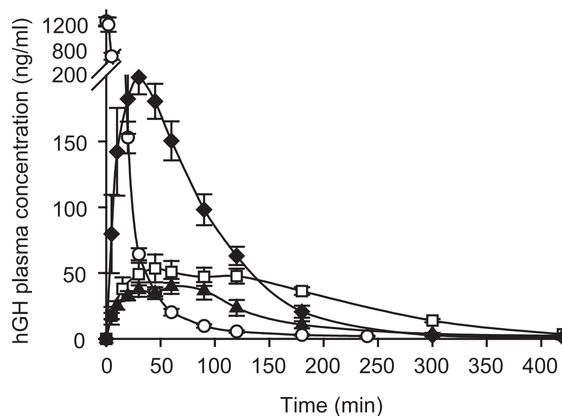

Fig. 3. hGH plasma concentration–time curves following: intravenous injection of 35 μg hGH (circles; $n = 5$), subcutaneous injection of 74 μg hGH (squares; $n = 6$), intratracheal spray-instillation of 200 μg hGH (triangles; $n = 8$) and intratracheal insufflation of a powder containing 209 μg hGH (diamonds; $n = 8$). Error bars are S.E.M.

Table 1
Pharmacokinetic parameters

	IV[a]	SC[b]	Spray-instillation	Powder insufflation
Number of rats	5	6	8	8
Rat weight (g)	354 ± 4	369 ± 12	363 ± 4	378 ± 5
dose/rat (μg)	35.4 ± 0.4	74 ± 2	200	209 ± 5
C_0 or C_{max} (ng/ml)[c]	1280 ± 63	55 ± 9	46 ± 6	234 ± 17
t_{max} (min)[d]		64 ± 7	51 ± 7	23 ± 3*
AUC (ng min/ml)[e]	14578 ± 570	12144 ± 1205	6320 ± 1026	19520 ± 1436
CL (ml/min/kg)[f]	6.9 ± 0.3	6.9 ± 0.6	8.1 ± 1.1	6.8 ± 0.6
Vd (ml/kg)[g]	646 ± 71	761 ± 106	873 ± 148	349 ± 32*
$t_{1/2}$ (min)[h]	65 ± 7	75 ± 7	73 ± 6	36 ± 3*
MRT (min)[i]	16 ± 4	124 ± 10	93 ± 11	60 ± 6
MAT (min)[j]		108 ± 14	77 ± 15	44 ± 10
F_{abs} (%)[k]		42 ± 5	8 ± 1	23 ± 2
F_{rel} to SC (%)[l]			18 ± 4	56 ± 9

Data are presented as mean ± S.E.M.

[a] Intravenous injection. [b] Subcutaneous injection of a hGH solution. [c] Maximal plasma concentration. [d] Time to peak. [e] Area under the plasma concentration–time curve. [f] Clearance. [g] Volume of distribution. [h] Elimination half-life. [i] Mean residence time. [j] Mean absorption time. [k] Absolute bioavailability. [l] Bioavailability relative to SC.

*Indicates significant decrease in the pharmacokinetic parameter as compared to the other routes of administration (Tukey test, $p < 0.05$).

suggests a permeation enhancer effect of the dry powder excipients. The elimination half-life ($t_{1/2}$) and volume of distribution (V_d) were smaller following powder insufflation than following spray-instillation, IV or SC injections (Table 1; Tukey test, $p < 0.05$). In order to test whether smaller $t_{1/2}$ and V_d values resulted from the method of powder administration to rat lungs or from an alteration to the protein after incorporation in the dry powder, the protein released from the powder was injected subcutaneously. In this case, $t_{1/2}$ and V_d were similar to the values obtained following SC injection of native hGH (Anova, $p > 0.05$; data not shown), suggesting that alterations to pharmacokinetic parameters were due to physiological caused by perturbations by intratracheal insufflation of the powder.

3.4. Fate of FITC-hGH in the lung

The fate of FITC-hGH locally in the alveolar region was visualized by confocal laser scanning microscopy after intratracheal spray-instillation of a solution of the protein in rats. Twenty minutes after delivery, FITC-hGH appeared homogeneously spread on alveolar surfaces (Fig. 4a). The red fluorescence from tissue labeling with sulforhodamine was not superimposed on the green fluorescence due to FITC-hGH, indicating that the protein did not diffuse significantly in the alveolar tissue (Fig. 4b). One hour later, the intensity of the green fluorescence had decreased but FITC-hGH remained clearly visible in the alveolar spaces (Fig. 4c). No penetration in the tissue could be observed at 1 h as well, but particular cells in the air spaces, likely alveolar macrophages, displayed intense green fluorescence, indicating that significant protein uptake by these cells had occurred (Fig. 4c). Four hours after administration, the majority of FITC-hGH had been cleared from the alveoli and the protein remained essentially apparent in alveolar macrophages (data not shown). To confirm the uptake of FITC-hGH by alveolar macrophages, the cellular components of a broncho-alveolar lavage collected 4 h after delivery were visualized under the confocal microscope. A large amount of cells exhibited intense punctuated fluorescence in their cytoplasm, attesting that FITC-hGH had been taken up by alveolar macrophages (Fig. 4d).

4. Discussion

A dry powder aerosol prepared with hGH, lactose and DPPC delivered more effectively the hormone to the systemic circulation than intratracheal instillation of a solution in rats (Fig. 3, Table 1). The powder particles presented particularly low densities and

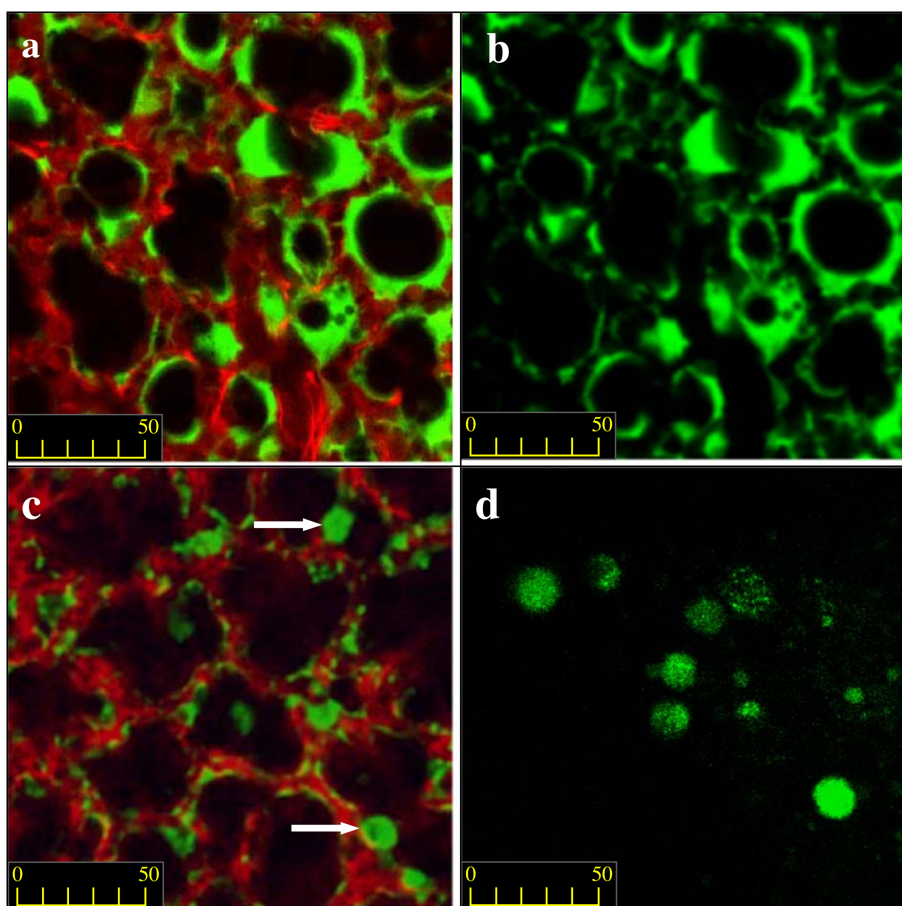

Fig. 4. Confocal imaging of rat alveoli (a) 20 min and (c) 1 h after intratracheal delivery of a solution of FITC-hGH (green). The tissue is labeled in red with sulforhodamine. (b) Same picture as (a) without the red channel. (d) Alveolar macrophages recovered by bronchoalveolar lavage 4 h after intratracheal delivery of FITC-hGH. Arrows show hGH uptake by alveolar macrophages. Scale bar is 50 μm.

aerosolized efficiently from a conventional inhaler with limited dependence on airflow rate (Fig. 1). No hGH byproducts as dimers or glycation adducts were produced during formulation of the powder (Fig. 2). Confocal imaging of rat lung following intratracheal instillation of FITC-hGH suggests that alveolar macrophages might play a role in alveolar clearance of the protein (Fig. 4).

Absorption of hGH from rat lung was faster and threefold higher after intratracheal insufflation of the dry powder than after intratracheal spray-instillation of a solution of the hormone (Fig. 3, Table 1). We measured in a parallel study regional deposition of formulations in rat lung and found that a powder of similar composition and aerosolization properties resulted in 60% of powder mass deposited in the trachea (and main bronchi) and 40% in lung lobes [25]. Values of 6% and 94% were respectively obtained after spray-instillation, suggesting that enhanced absorption of hGH from the powder as compared to the liquid did not originate from a deeper deposition within the respiratory tract. Our hypothesis is rather that the dry powder excipients and more particularly DPPC might have membrane permeation enhancer properties. Exogenous DPPC might alter the composition and/or organization of the surfactant mix lining the airway and/or alveolar epithelium and increase its permeability by mecha-

nisms similar to those outlined for medium chain fatty acids and phospholipids [25–27]. Administration of DPPC has previously been reported to increase pulmonary absorption of peptides as insulin (5.8 kDa) and parathyroid hormone (4.1 kDa) in rats [25,28,29]. Our data indicate that DPPC could enhance absorption of large proteins from the lung as well. It might be relevant to verify if DPPC contained in large porous hGH particles was involved in the substantial bioavailability reported for the hormone following inhalation in humans [7]. DPPC might be a particularly advantageous permeation enhancer because it is endogenous to the lung and does not induce acute toxicities as conventional chemical enhancers do [8].

Alveolar macrophages took up FITC-hGH that deposited in the deep lung and significant accumulation in cell cytoplasm became apparent 1 h post-delivery (Fig. 4). Lombry et al. [14] demonstrated that alveolar macrophages presented a significant hindrance to the transport of human chorionic gonadotropin (40 kDa) and immunoglobulin G (150 kDa) from airway lumen to the bloodstream and depletion of alveolar macrophages by liposome-encapsulated dichloromethylene diphosphonate increased bioavailability of both proteins by several fold in rats. Because alveolar macrophages captured molecules in soluble form and with hours persistence in the alveoli, uptake was supposed to predominantly involve adsorptive and/or fluid-phase endocytosis [14,15]. Alveolar macrophages probably impeded pulmonary absorption of the lower molecular weight hGH as well, especially when delivered by spray-instillation. The protein remained in the air spaces for 77 min following delivery of the solution, a duration that is beyond the time needed to observe a significant macrophages uptake of FITC-hGH by confocal microscopy (Table 1, Fig. 4). In contrast, despite the additional step of particle dissolution in the epithelial lining fluid, hGH delivered as a powder remained for only 44 min in the air spaces, suggesting that accelerated absorption from the powder allowed to partly bypass uptake by alveolar macrophages (and other elimination processes as enzymatic cleavage), thereby increasing bioavailability (Table 1).

The dry powder formulated showed good aerodynamic behavior in vitro despite the use of a Spinhaler™ device, a first-generation low efficiency inhaler (Fig. 1). The dry powder did not behave as isolated particles but as particle aggregates since the MMAD was approximately threefold larger than the theoretical estimate of primary aerodynamic diameter. However, most aggregates were respirable (< 5 μm) as a result of low powder density (0.07 vs. 1 g/cm^3 for conventional powders) and thereby small primary aerodynamic diameter [21]. The fine particle fraction increased with airflow rate from 30 to 60 l/min but not further from 60 to 90 l/min (Fig. 1). It is to be noted that the Spinhaler™ device is a low resistance inhaler used by patients at high airflow rates and variation in deposition from 30 to 60 l/min is of little relevance in the clinic [30].

Although approximately 25% of the protein load has been shown to aggregate during spray-drying [23], we did not detect any human growth hormone aggregates following atomization with DPPC and lactose. The formation of aggregates is induced by surface denaturation at the air–liquid interface of droplets in spray and DPPC may act as polysorbate-20 surfactant, compete with protein molecules to occupy the interface and leave a smaller interface for protein to unfold and aggregate [23]. Removing DPPC from the liquid feed increased the formation of aggregates to 3.9%, in line with this hypothesis. Formulation of proteins with lactose has also been reported to minimize aggregation [24]. Although hGH can undergo oxidation of methionine and deamidation of asparagine or glutamine [31], aggregation is the most critical denaturation mechanism because aggregates are immunogenic and have reduced bioactivity [22].

5. Conclusions

Systemic delivery of hGH by inhalation was assessed in rats using a dry powder aerosol of particular composition. The dry powder presented good aerodynamic behavior in vitro due to low bulk powder tap density and showed limited variation in deposition with airflow rate in a multi-stage liquid impinger. Absorption of hGH from rat lung was faster and higher following intratracheal delivery of the dry powder as compared to the solution, likely due to membrane permeation enhancer properties of DPPC incorporated in the dry powder. DPPC presented the additional advantage of protecting hGH against denaturation during spray-drying. Significant uptake of

FITC-hGH by alveolar macrophages was visible in the air spaces 1 h post-delivery, suggesting that these particular cells might play a role in alveolar clearance of the hormone.

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