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Sustained release hGH microsphere formulation produced by a novel supercritical fluid technology: *In vivo* studies

F. Jordan [a], A. Naylor [a], C.A. Kelly [b], S.M. Howdle [a,b], A. Lewis [a], L. Illum [a,*]

[a] Critical Pharmaceuticals Ltd, BioCity Nottingham, Pennyfoot Street, Nottingham NG1 1GF, UK
[b] School of Chemistry, University of Nottingham, University Park, Nottingham NG7 2RD, UK

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ABSTRACT

Novel sustained release formulations of hGH prepared by supercritical fluid processing of PLGA/PLA (the CriticalMix™ process) were produced in the form of microparticles for subcutaneous injection. The basis of the process is that PLGA/PLA polymers liquefy when exposed to supercritical CO_2, thereby allowing the hGH to be mixed efficiently into the polymers at an ambient temperature and in the absence of solvents. The CO_2 was removed from the mixture by depressurisation through a nozzle, resulting in the production of microparticles containing the hGH, which were collected in a cyclone. The best microparticle formulations showed an initial *in vitro* burst of around 35% and a sustained release over 14 days. When tested in the rat model, which displays a faster clearance rate of hGH than other animal models, two formulations showed prolonged release over 2–3 days with sustained plasma levels at 1–5 ng/ml whereas the soluble hGH formulation was cleared within 24 h. Two selected sustained release formulations were tested in cynomolgus monkeys and compared to a single injection of soluble hGH. The burst release from the sustained release formulations was similar in magnitude to a daily dose of hGH and serum hGH levels were maintained for a seven day period. It is probable from the data that the sustained release would have continued for up to 14 days if sampling had been continued. The IGF-1 results showed there was no significant difference between the levels obtained for once daily injection of soluble hGH and the two sustained release formulations.

1. Introduction

Human growth hormone (hGH) is a 22 kDa protein, that stimulates growth and cell reproduction in humans and other animals. In healthy individuals it is synthesized and stored in the anterior pituitary gland and secreted into the circulation in a pulsatile pattern. Recombinant hGH is currently used therapeutically to treat a number of conditions including growth hormone deficiency in children with hypopituitary disorders, growth failure due to chronic renal insufficiency, Turners syndrome and growth hormone deficiency in adults. hGH is poorly absorbed orally and therefore is normally administered by daily injections for a period of several years [1,2]. This treatment regimen has a considerable impact upon patients' lives, and can affect patient compliance. A sustained release formulation of hGH to be administered twice a month would therefore provide a significant therapeutic advantage over current treatments. Clinical studies have shown that a continuous infusion of hGH via a pump is as efficacious as daily administration with equivalent growth velocity and IGF-I levels, the principle mediator of hGH effects [3–6]. No significant difference in safety profile between the two modes of delivery was shown [3–6]. A sustained release formulation of hGH can be achieved by encapsulation of the drug into injectable microspheres of biodegradable and biocompatible polymers such as PLGA or PLA. The hGH is slowly released from the microspheres by diffusion and by degradation of the polymer to lactic or glycolic acid. Such a formulation, Nutropin Depot, manufactured using Alkermes' Prolease microsphere system, was approved by the FDA and marketed in 1999 by Genentech for treatment of pediatric growth hormone deficiency. The hGH was stabilized by forming an insoluble complex with zinc and encapsulated into PLGA microspheres using a non-aqueous cryogenic method [7,8]. The product was removed from the market in 2004 due to its non-competitiveness with the daily injection formulation. The reasons for this were mainly the high costs associated with the lengthy production process in which it took two weeks to manufacture a batch [9].

Jostel et al. [10] has described a sustained release hGH microsphere formulation based on encapsulation of hGH in amylopectin microspheres coated with PLGA that gave sustained release in human volunteers over 14 days. A similar hGH formulation based on hydroxyethyl methacrylated dextran microspheres showed sustained release over 7 days in human volunteers [11]. For these different microsphere preparations, processing methods such as encapsulation by emulsification processes was used, which require multiple steps, increasing production time and cost.

DeBenedetti et al. (1993) applied a Rapid Expansion of Supercritical Solvents (RESS) technique to encapsulate drugs into polymers for

* Corresponding author. Tel.: +44 115 8820100; fax: +44 115 9124371.
E-mail address: Lisbeth.illum@illumdavis.com (L. Illum).

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sustained release applications. In this technique both the drug and the polymer need to be soluble in supercritical CO_2 ($scCO_2$), which often has a co-solvent such as acetone added to it. Hence, this technique is only applicable to very low molecular weight polymers (around 1000 Da) and small molecular weight drugs [25].

The PGSS (Particles from Gas Saturated Solutions) production process used here to produce the present hGH microsphere formulations was a simple, one-step process based on a supercritical fluid production technology (CriticalMix™). This technology has significant advantages over conventional production methods, as no organic solvents are required for the process and therefore there is no potential for residual solvents in the final product. Furthermore, the method results in near 100% encapsulation of the protein, with no structural changes to the protein during processing and drug loading as high as 20–30% dependent on the drug [12,13]. When amorphous polyesters, such as PLGA or PLA (even at high molecular weights) are exposed to supercritical carbon dioxide ($scCO_2$) the $scCO_2$ dissolves into the polymer, and acts as a molecular lubricant liquefying the polymer at temperatures significantly below its glass transition temperature. Therefore, PLGA or PLA exposed to $scCO_2$ in a pressure vessel, will liquefy allowing the API, here hGH, in the dry state to be mixed efficiently into the polymer at an ambient temperature and in the absence of solvents. Following mixing, the mixture is depressurised through a nozzle whereby the CO_2 returns to a gaseous state and evaporates solidifying the polymer around the hGH, and resulting in the production of microparticles containing the hGH [12,13].

The present study evaluated the effect of process variables of the supercritical fluid microparticle technology such as polymer composition and addition of different excipients on the *in vitro* release of the entrapped hGH. Promising sustained release hGH formulations were further evaluated *in vivo* in two selected animal models i.e. the rat and the monkey and compared to a daily injection of soluble hGH.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-co-glycolide) (PLGA) with an inherent viscosity of 0.16–0.24 dl/g and a ratio of lactide to glycolide of 50:50 and Poly(D,L-lactide) (PLA) with an inherent viscosity of 0.16–0.24 dl/g were purchased from Boehringer Ingelheim GmbH (Ingelheim, Germany). Solutol HS 15, Poloxamer 407 (Lutrol® F127) and Poloxamer 188 (Lutrol® F68) were obtained from BASF (Ludwigshafen, Germany). hGH was kindly donated by Bioker (Sardinia, Italy). HEPES, acetone and dichloromethane (DCM) were purchased from Fisher Scientific (Leicestershire, UK). Carbon dioxide was purchased from BOC (Surrey, UK).

2.2. Manufacture of hGH microparticle formulations

A number of formulations of hGH loaded PLGA/PLA microparticles were prepared using a CriticalMix™ process described previously [12]. During this process the protein remains in its solid state and therefore requires micronisation before encapsulation. Two methods to micronise the hGH were used — spray drying and zinc precipitation. Spray drying was performed by Upperton Ltd (Nottingham, UK) using a Buchi B-191 spray dryer equipped with a Schlik 0.5 mm Nozzle and an inlet temperature of 85 °C. 0.952 g of hGH was dissolved in 48 ml of 5 mM phosphate buffer containing 20% v/v Tween-20, and zinc added at a 2:1 ratio Zn:hGH. This produced free flowing hGH particles with a mean diameter of approximately 2–5 μm as measured by laser diffraction (Sympatec, Germany). As an alternative to spray drying, hGH was precipitated into nanoparticles by complexation with zinc at a molar ratio of 5:1 zinc:hGH. 10 mM zinc acetate was added dropwise to a 9.1 mg/ml solution of hGH in a quartz cuvette, whilst stirring at 1200 rpm using a magnetic stirrer. The resulting suspension was freeze dried before encapsulation.

The $scCO_2$ microparticle manufacturing process used was described in detail by Whitaker et al. [12]. The process parameters were kept constant for all microparticle batches but the excipients and polymer ratios were altered to determine their effect on release kinetics, particle size distribution and morphology. The selection of polymers, the ratio of these and the processing aids were chosen based on a range of preliminary *in vitro* release studies and particle size measurements. It was found that neither the use of PLGA alone nor PLA gave the desired properties for obtaining a sufficiently small particle size, low burst and sustained release characteristics. Briefly, micronised hGH (10% w/w of the formulation), suitable quantities of PLGA and PLA in different ratios (80% w/w of the formulation) and GRAS excipients consisting of poloxamer 188, poloxamer 407 or Solutol HS15 (alone or in combination to make up the remaining 10% w/w of the formulation) were loaded into a pressure vessel which was sealed. CO_2 was introduced into the pressure vessel and the temperature and pressure were increased to above 32 °C and 76 bar, respectively. The $scCO_2$ dissolved into the polymers which became liquefied. The liquefied polymer, micronised hGH and excipients were then mixed in the pressure vessel using a stirrer at 150 rpm to produce a homogenous mixture. Microparticles were formed upon atomisation and depressurisation of the mixture through a nozzle into a lower pressure environment. All batches were made on a laboratory scale apparatus with a 2 g batch size and in triplicate. A total of five formulations were prepared and details of the compositions are given in Table 1.

The microparticles were characterised in terms of encapsulation efficiency, hGH loading, morphology and particle size distribution. To determine the encapsulation efficiency and loading of the formulations, hGH was extracted from a known mass of microparticles by adding 1 ml of a 2:1 mixture of DCM: acetone to dissolve the polymer component. The tubes were centrifuged at $4355 \times g$ to pellet the protein and the supernatant discarded. The pellet was resuspended in 2:1 DCM: acetone and the extraction procedure repeated a further two times. After the final wash and centrifugation the supernatant was discarded and the pellet collected. The pellet was dried to remove any residual solvent, dissolved in 0.063 M phosphate buffer and assayed for hGH content by size exclusion high performance liquid chromatography (SEC-HPLC) following the method described in the European Pharmacopoeia (E.P.). Determination of drug in several samples of microparticles showed the homogeneity of the drug distribution in the batch. The encapsulation efficiency of the hGH microparticles was determined by calculating the percentage of the measured drug loading in relation to the theoretical drug loading.

Scanning electron microscopy (SEM) of the microparticles was carried out using a JEOL JSM-6060LV (Tokyo, Japan). The particles were coated with gold in an argon atmosphere using a Balzers Sputter Coater (AG, Liechtenstein) for 4 min before analysis using the SEM.

hGH was assayed and soluble aggregate formation quantitated using SEC-HPLC as described in the European Pharmacopoeia. Briefly, an Agilent 1100 HPLC system was fitted with a TSKgel G2000SWXL column (TOSOH Bioscience, Japan), and the column was equilibrated with a mobile phaseof 97%v/v 0.063 M phosphate buffer pH 7 and 3% v/v 2-propanol at a flow rate of 0.6 ml/min before injection of 20 μl of

Table 1
Composition of the hGH microparticle formulations.

	PLGA:PLA ratio	hGH (% w/w)	Other excipients (% w/w)
Formulation A	90:10	10% Spray dried hGH	10% Poloxamer 407
Formulation B	85:15	10% Spray dried hGH	10% Poloxamer 188
Formulation C	87.5:12.5	10% Spray dried hGH	9.5% Poloxamer 188 0.5% Solutol HS15
Formulation D	90:10	10% Spray dried hGH	9.5% Poloxamer 188 0.5% Solutol HS15
Formulation E	85:15	10% Zn:hGH precipitate	10% Poloxamer 407

the sample to be analysed. Protein detection was measured by optical absorption at 214 nm, and concentration measured by comparison of the peak area to a standard.

A Sympatec HELOS laser diffractor was used for analysis of particle size and size distribution of the polymer microparticles. Approximately 5 mg of the sample was suspended in deionised water with 10 drops of 1% v/v Tween 20. Volume mean diameter (Vmd) was the diameter at the 50% point of the entire volume distribution. The distribution was defined as d10, d50 and d90 which relate to the respective diameters at 10, 50 and 90% cumulative volume.

2.3. In vitro drug release

Triplicate 10 mg samples of microparticles were weighed into Eppendorf tubes and 1 mL of a release buffer containing 10 mM HEPES, 100 mM NaCl, 0.02%v/v Tween 20 and 0.05% sodium azide, was added to each vial. The eppendorf tubes were then placed on an Intellimix rotator mixer set to rotate at 10 rpm. Samples were taken at 1 h, 4 h, 24 h, and 2, 3, 4, 7, 10 and 14 days after addition of the release buffer. At each time point the tubes were removed from the mixer and centrifuged at 4355 $\times g$ for 3 min. Aliquots were taken after centrifugation and fresh HEPES release buffer was replaced. The hGH content in the HEPES was analysed by SEC-HPLC using the E.P. method and the cumulative release determined. All studies were carried out in triplicate.

2.4. Evaluation of hGH stability and integrity

In order to evaluate the effect of the scCO2 production process on the encapsulated hGH, the hGH extracted from the microspheres were analysed using SEC-HPLC, reverse phase HPLC and circular dichroism. Controls were spray dried hGH which had not been through the production process. hGH was extracted from the microparticles using the same procedure used for measuring the loading and encapsulation efficiency. SEC-HPLC was carried out on the samples following the E.P method as described above. For reverse phase HPLC (RP-HPLC) the extracted hGH was dissolved in 0.025 M phosphate buffer to a maximum concentration of 2 mg/ml. A butylsilyl silica gel column, 0.25 m long and 4.6 mm in internal diameter (Grace-Vydac, 214TP54 column) was equilibrated to 45 °C and washed with 50%v/v acetonitrile/0.1% trifluoroacetic acid until the baseline was stable. The column was then equilibrated with the mobile phase consisting of 71%v/v 0.05 M tris–hydrochloride buffer solution pH 7.5 and 29%v/v 1-propanol at a flow rate of 0.5 ml/min. 20 µl of each sample was injected onto the column and UV absorption was measured at 220 nm. The amount of hGH oxidation and degradation was then quantitated as described in the European Pharmacopeia.

Circular dichroism analysis was performed on an Applied Photophysics Pi-Star-180 Spectrophotometer. hGH was extracted from formulations A and B as described above, dissolved in 300 µl 20 mM sodium phosphate buffer (pH 6.8) and diluted to give a concentration of 10 µmol dm^{-3}. Spectra were obtained from 300 µl of sample in a 1 mm pathlength cuvette. A blank spectrum was also recorded and subtracted from the hGH spectrum. The secondary structure content was investigated by recording the spectra from 250 nm to 200 nm in 1 nm increments. The molar ellipticity was calculated (Eq. (1)) and plotted in order to remove any concentration effects.

$$[\Theta] = \frac{\Theta}{10ncl} \quad (1)$$

Eq. (1) Calculation of the molar ellipticity from CD: [Θ] = Molar ellipticity (deg cm^2 $dmol^{-1}$); Θ = Ellipticity (deg); n = Number of amino acid residues in the protein; c = Concentration of hGH (mol dm^{-3}) as determined by HPLC SEC; and l = Path length (cm)

2.5. Evaluation of hGH activity using a cell based bioassay

The activity of the hGH released from the microparticles and prior to processing was evaluated using a cell proliferation assay on a NB2-11 cell line (Kit Biotrak ELISA system, BR-dU, GE Healthcare, UK). Briefly, cells were cultured in the presence of the hGH released from the microparticles *in vitro* at 37 °C for 1–5 days in a 96-well microplate. 5-bromo-2-deoxyuridine (BrdU) was then added to the cells and reincubated for 24 h. During this period the pyrimidine analogue BrdU is incorporated in the place of thymidine into the DNA of the proliferating cells. The cell culture was removed and fixative added to denature the DNA followed by the addition of peroxidase-labelled anti-BrdU. The immune complexes were detected by the subsequent substrate reaction and the plate was read at 450 nm on a microplate spectrophotometer. Both the test samples and the standards were applied to the cells at the same time but in different wells of the tissue culture plate. The cells were of the same population and therefore the same passage. The effect of increasing culturing time is therefore controlled.

2.6. In vivo studies in rats

Male Sprague Dawley rats (Charles River) (mean weight of 300 g) were divided into five groups of four rats. The five different microparticle formulations were suspended in an aqueous injection vehicle consisting of 0.5% w/v carboxymethylcellulose, 5.0% w/v mannitol and 0.1% v/v Tween 80 and injected subcutaneously at a dose of 5 mg/kg hGH. Two additional groups of four rats received a subcutaneous or an intravenous dose of hGH solution to allow the bioavailability of the microparticle formulations to be determined. Blood samples were taken prior to dosing and for up to 98 or 168 h after administration. All animal experimentation was carried out in compliance with current UK legislation on animal experimentation (Animals (Scientific Procedures) Act 1986) and within the requirements of Commission Directive 86/609/EEC concerning the protection of animals used for experimental and other scientific purposes. Serum samples were measured for hGH by ELISA using a DSL-10-1900 Active (R) Human Growth Hormone ELISA kit (Diagnostic System Laboratories Inc, Texas USA).

Non-compartmental pharmacokinetic analysis was performed on the hGH concentrations versus the time profile using WinNonlin. Area under the curve (AUC) values were calculated using the linear trapezoidal method and extrapolated to infinite time by dividing the last measurable serum concentration by the terminal elimination rate constant. Elimination rate constants (K_{el}) were calculated by linear regression analysis of the log/linear portion of the soluble individual serum concentration–time curves. C_{max} values were taken to be the highest measured serum concentrations and T_{max} values were the time points of highest serum concentrations.

Local tolerability of microparticle formulations at the injection site was determined seven days after administration. The skin including subcutaneous tissue surrounding the injection site was excised and fixed in 10% v/v neutral buffered formalin. Samples were embedded in paraffin wax, and 5 µm sections taken from the entire injection site, and the sections stained with haematoxylin and eosin prior to microscopic examination.

2.7. In vivo studies in primates

Two groups of 4 male Cynomolgus primates (*Macaca faccicularis)* were used to test the two formulations which were selected on the basis of the results from the *in vivo* studies in rats (formulations A and B). The studies were carried out at the Laboratory for Pharmacology and Toxicology (Hamburg, Germany). The primates were kept singly with room temperatures of 23 °C ± 3 °C and relative humidity of 60% ± 20% with a 12 h light/dark cycle. At the start of the treatment the

animals were between 48 to 72 months old and between 4.2 and 6.4 kg. The doses were administered by subcutaneous administration. In the first phase of the study, primates in group 1 received a daily injection of soluble hGH for seven consecutive days at a dose of 0.21 mg/kg/day which equated to a weekly dose of 1.5 mg/kg. Primates in group 2 received a daily injection of vehicle only. Blood samples were collected at regular intervals for 24 h and then every day (prior to the next injection) for up to 7 days. After a wash out period of 8 days group 1 and 2 were administered microparticle formulations A and B, respectively as a single dose of 1.5 mg/kg hGH on day 0. Blood samples were collected up to a week after administration and the serum frozen at −20 °C for analysis.

Serum concentrations of hGH and insulin-like growth factor-I (IGF-I) in the primates were determined using commercially available ELISA kits (BioSource Europe S.A. Belgium) and a Tecan Sunrise microplate reader (Crailsheim, Germany). Non-compartmental pharmacokinetic analysis of the data was performed using TopFit 2.11. The following pharmacokinetic parameters were determined: area under the curve from time zero to the last day of sampling ($AUC_{0-\infty}$), maximum blood concentration (C_{max}), time to the maximum concentration (T_{max}).

2.8. Statistical analysis

Statistical analysis was carried out using GenStat version 11. Analyses were made using ANOVA and the Least Significant Difference (LSD) at the 5% confidence level was calculated for all data. Any differences in results were considered significant if the p-value was less then <0.05.

3. Results and discussion

3.1. Manufacture and characterisation of the microparticles

The hGH microparticle formulations evaluated in the study differed in the composition of the polymers used (ratio of PLGA:PLA) and in the type and composition of excipients added. All of the formulations were successfully manufactured using the CriticalMix™ process and characterised in terms of particle size distribution, morphology, hGH encapsulation efficiency and hGH loading. The particles appeared rounded with few pores and of an apparent size around 93 µm, although some larger particles were present (Fig. 1).

Whilst there appears to be some heterogeneity in the morphology of the particles it is not likely that this is due to phase separation of the polymers in the formulations. When exposed to scCO2 the polymers and excipients are liquefied and miscible and hence easily blended together by a stirrer. Upon atomisation, particle solidification is extremely rapid, due in part to the adiabatic cooling as the CO_2 expands. The temperature in the spray has been shown to drop from the processing temperature of 40 °C to minus 60 °C in less than a millisecond and high speed video show solidification to occur in <1 ms (data not shown). Hence, the excipients and polymers would not have sufficient time to phase separate. This has been confirmed by DSC analysis on the particles, which showed only one glass transition temperature for the polymer matrix (data not shown). The reason for the presence of different morphologies is most likely due to enhanced atomisation of the liquefied polymer droplets caused by the expansion of high density CO_2.

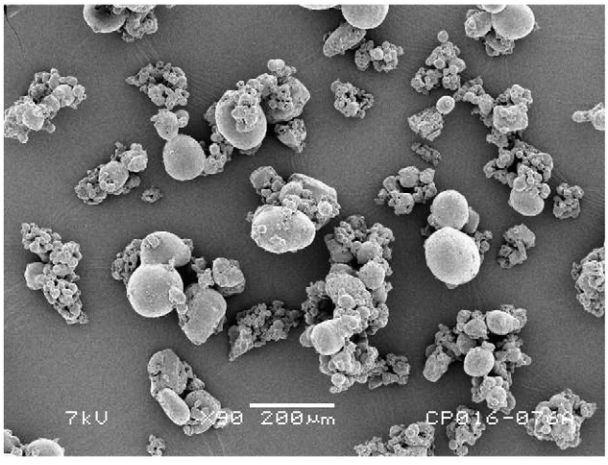

Fig. 1. SEM image of hGH microparticle formulation A.

The full particle size distribution and the d(10), d(50), d(90) and Vmd for the particle size distributions for all formulations are given in Table 2. For Formulation A, 90% of the particles were below about 100 µm and the Vmd was 61 µm indicating that these particles would be easily injectable after dispersion in a suitable injection vehicle. The remaining formulations all had higher d(90) and Vmd. The particle size of the PLGA microparticles produced here are similar to the PLGA microspheres produced by Costantino et al. [14] using a cryogenic method, and the microspheres produced by Cleland et al. [2] and Kim and Park [15] by an emulsion processes.

The encapsulation efficiencies for the formulations were found to be between 97.1% and 100% and the loading of the hGH between 5.5 and 6.4 w/w of the formulation (corresponding to about 10% hGH spray dried micronized bulk formulation). In comparison, the hGH encapsulation efficiency measured following encapsulation by an emulsion process was found to be 50% with a resultant loading of 2.0% hGH [2] and about 5% and representing considerable loss of recombinant protein [15]. In contrast, microspheres produced by a cryogenic method [14] showed encapsulation efficiencies between 70 and 100%. The encapsulation efficiency for the microparticles produced here using the supercritical fluid process was in all cases near 100%. In the current studies, the loading of hGH in the formulations were restricted to 10%w/w spray dried material in the interests of cost, but we have since achieved protein and peptide loadings of 20%w/w with other drugs. It is evident from the burst release seen *in vitro* and *in vivo* that some of the hGH is positioned on or close to the surface and readily diffuse out of the microparticles. It is also evident that a large amount of the hGH is encapsulated in the matrix of the microparticles which is shown by the sustained release obtained both *in vitro* and *in vivo* and the amount of drug measured in the microparticles after release.

3.2. In vitro release of hGH from the microparticles

In vitro release studies revealed an initial burst release from each of the formulations (Fig. 2). Formulation A showed the lowest 1 h burst and Formulation B produced the highest 1 h burst of 33.9% and 49.1%, respectively. Initial bursts of drug from Formulations C, D, and E were 36.1, 42.3 and 39.5% respectively. The hGH present on or near the surface of the microparticles, the large surface area to volume ratio and the presence of pores in the microparticles account for the initial release observed in all formulations. Following the burst release, hGH was released slowly from each of the formulations at different rates over the 14 day study period. Modifications to the polymer ratios and excipients

Table 2
Particle size distribution as measured by laser diffraction.

Formulation	d10	d50	d90	Vmd
A	25.9	56.1	104.5	61.2
B	50.5	100.0	167.7	105.0
C	48.0	96.1	149.6	98.1
D	51.9	104.5	165.5	106.6
E	44.7	90.6	153.6	96.0

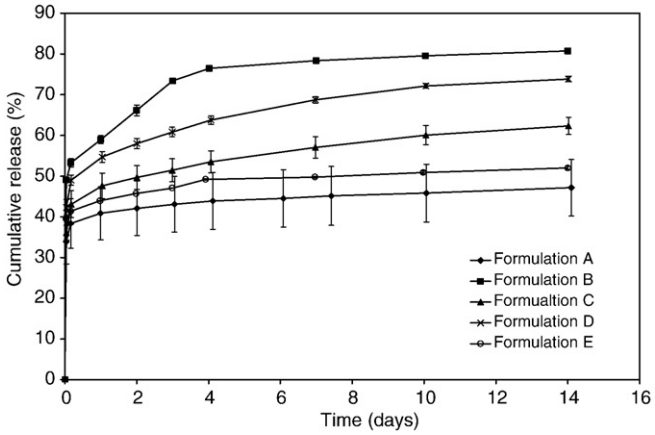

Fig. 2. Cumulative *in vitro* release of hGH from the microparticle formulations. The data represent mean values of triplicate experiments.

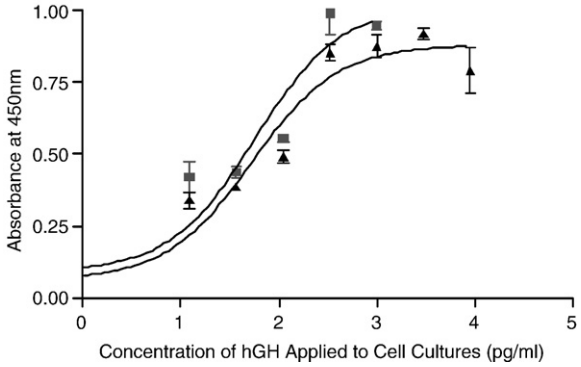

Fig. 4. Cell proliferation assay of a hGH standard and hGH after processing, encapsulation and release. ■ hGH standard; ▲ hGH formulated using CriticalMix and released *in vitro*.

made observable differences to the release of hGH. Release of hGH from the microparticle formulations A, E and C were significantly ($P<0.001$) slower compared to Formulation B which showed the most rapid rate of release between days 1 and 6.

Similar burst releases of hGH from PLGA microspheres were found in work by Cleland et al. [2,16] of more than 30% or 20%, respectively. Proteins encapsulated in PLGA formulations are often released in a multiphasic manner with an initial burst release of drug close to the surface of the microparticles followed by a much slower release period principally determined by the rate of degradation of the polymer.

The principle advantages of the $scCO_2$ process used in the current study are that polymers can be plasticised at a temperature just above 32 °C, no solvents are required and proteins have low inherent solubility in $scCO_2$. As a result, proteins are not exposed to the processing stresses known to degrade the drug and the structure and function of the hGH is preserved. In the current study, SEC-HPLC and RP-HPLC analyses found that no aggregation or degradation of hGH occurred following encapsulation or release, and the hGH comfortably passed the E.P limits (data not shown). Far UV CD analysis showed no significant change to the hGH secondary structure after processing with the α-helix remaining intact, further confirming the stability of hGH in $scCO_2$ (Fig. 3). Lastly, the *in vitro* bioassay showed that 100% of the biological activity was maintained upon encapsulation and release when compared to a standard (Fig. 4).

It has been shown by other authors that microsphere emulsification technology can cause proteins to aggregate [17]. For example, for α-chymotrypsin it was found that 34% of the protein had aggregated and that the specific activity of the enzyme was reduced to 50% within 24 h after encapsulation into PLGA microspheres using this technique [18]. This problem can be overcome by forming a stable freeze dried hGH-Zn complex to be encapsulated into the microspheres [19,20].

3.3. In vivo studies in rats

A single subcutaneous injection of each of the microparticle formulations (A–E) led to sustained elevated plasma levels of hGH in the rat model compared to a single subcutaneous administration of soluble hGH, where serum hGH levels dropped to baseline levels within 12 h (Fig. 5). All formulations showed a T_{max} of 1 h which corresponds to the initial burst release observed *in vitro* after 1 h. A summary of the rat pharmacokinetic data is shown in Table 3. Formulations A and B showed the highest levels of hGH in the serum after 24 h (1.98 ng/ml and 3.8 ng/ml, respectively) and 48 h (1.49 and 2.1 ng/ml respectively) with bioavailabilities of 85.7% and 47.9%, respectively, compared to intravenous administration of hGH. This indicates that protein still remains in the microparticles and may be released slowly from the microparticles. Formulations A and B showed significantly higher hGH serum levels ($p<0.05$) up to 48 h after administration compared to the other formulations tested with formulation B showing the highest hGH serum concentration after

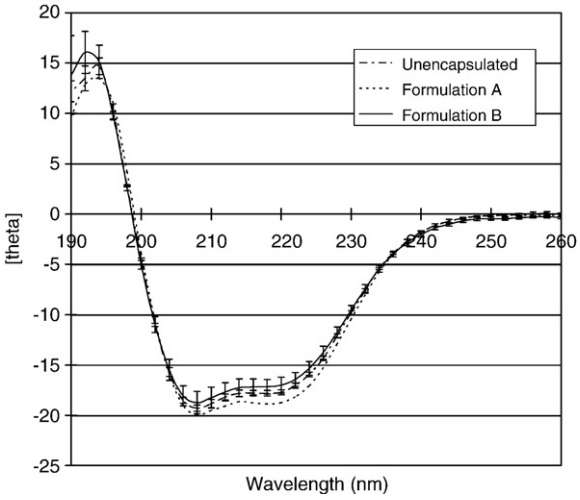

Fig. 3. Circular dichroism analysis of hGH extracted from formulations A and B.

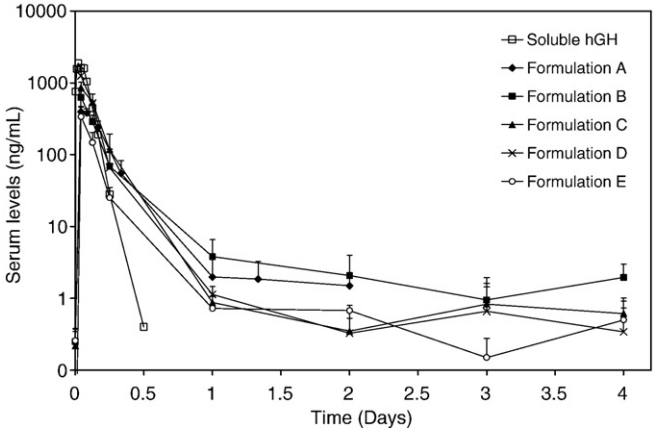

Fig. 5. Serum hGH levels in Sprague Dawley rats after administration of microparticle formulations and a soluble hGH formulations.

Table 3
Pharmacokinetic evaluation of the rat serum data.

Test item	Dose mg/kg	C_{max}* ng/ml (SD)	T_{max} h	$AUC_{0-\infty}$* ng h/ml (SD)	F** % (SD)	Fvs sc** % (SD)
Formulation A	5	395.0 (±60.6)	1	2255 (±490.0)	85.7 (±18.3)	107.5 (±22.9)
Formulation B	5	313.8 (±71.2)	1	1211 (±350.3)	47.8 (±13.1)	60.0 (±16.4)
Formulation C	5	852.7 (±207.0)	1	1934 (±814.1)	73.0 (±30.3)	92.0 (±38.1)
Formulation D	5	625.8 (±144.2)	1	1951 (±646.3)	73.9 (±24.1)	92.8 (±30.3)
Formulation E	5	338.9 (±115.7)	1	1148 (±396.1)	43.9 (±14.7)	55.1 (±18.5)

* Significant difference between formulations $P = 0.0005$.
** No significant differences between formulations $P > 0.05$.

96 h. hGH concentrations in the rat serum for formulations C and D fell below 1 ng/ml after 24 h and the high C_{max} for both formulations and the similar bioavailabilities of around 92% at the 168 h time point, indicated that a large percentage of the encapsulated hGH was released in the initial burst.

After an initial burst release, similar to that of Formulation A and B, hGH remained unreleased in Formulation E with F% ~ 43.9% compared to subcutaneous injection. A similar hGH-zinc complex was investigated by Cleland et al. [20] and Costantino et al. [14] who found increased stability of the hGH during the microsphere production process, decreased initial burst and subsequent decreased rate of drug release. It was found here that the use of a zinc complex did not improve the performance of Formulation E over Formulations A and B. It was demonstrated earlier by Takada et al. [21] in a solid-in-oil-in-water emulsion process that as the particle size of the encapsulated hGH decreased, the burst effect in rats decreased and the subsequent serum levels in the sustained release phase were higher. The hGH-zinc complex used in Formulation E was less than 650 nm in diameter [14] whereas the micronized hGH used in Formulations A–D were between 2 and 5 μm, thus surprisingly a similar effect was not seen in the present studies.

It has been reported by Mordenti et al. [22] that the total body clearance of hGH in the rat model (0.959 l/h) is faster than for humans (0.124 l/h) and dogs (0.194 l/h) due to the more extensive metabolism in the rat model and hence a sustained release of the drug over longer periods would not be expected in the rat. Takada et al. [21] obtained sustained release over 14 days in the rat model with their PLGA microsphere system. However, the dose given to the rats was about 24 mg/kg as compared to the present dose of 6 mg/kg.

There were no unscheduled deaths during the study and no rats showed any ill effects after administration of the sustained release hGH. Histology of the area of the injection site showed some needle trauma but negligible inflammatory responses in the subcutaneous tissue, which shows that the formulations were well tolerated. This lack of local toxicity is at least partly due to the complete lack of residual solvents due to the manufacturing process used.

Wagner–Nelson modelling was performed on the rat serum data in order to establish an *in vitro*/*in vivo* correlation. The data showed that the *in vitro* release of formulations A and E correlated well with the rat serum levels where both formulations have a low burst and C_{max} whereas there was a less clear correlation between the other three formulations. This may indicate that the current *in vitro* model may need adapting with changes to the formulation to accurately predict the *in vivo* performance.

3.4. In vivo studies in primates

As discussed above, the total body clearance of hGH in rats is higher than in humans and hence it is difficult to test the full potential of a sustained release formulation in this animal model [23]. The two selected formulations (Formulations A and B) were therefore further tested in a non-human primate model (Cynomolgus monkeys), with a daily injection of soluble hGH as a control. Blood samples were collected after administration of the formulations to determine the pharmacokinetics of and the resultant pharmacodynamics after hGH release from the microparticle formulations (Fig. 6, Table 4).

Soluble hGH with a C_{max} of 252.7 ng/ml at 120 min after s.c. injection, was rapidly cleared from the circulation and below detection limits at 24 h. An initial burst of drug was seen in the plasma of the primates after injection of Formulation A and B. The AUC_{0-24} of this burst was similar to the AUC_{0-24} obtained from the soluble hGH formulation given as daily injections. The C_{max} for the formulations were 338 ng/ml and 245 ng/ml, and T_{max} were 3.5 h and 1.5 h, respectively. There was no significant difference in the initial burst and subsequent release from formulations A and B ($P = 0.784$), and both were significantly higher compared to background levels ($P < 0.001$). Plasma levels remained elevated between 1 and 5 ng/ml for the 7 days samples were collected. The rate of release was increasing in both formulations after 5 days, possibly due to hydrolysis of the polymer matrix releasing more of the encapsulated hGH. The bioavailability of the formulations after 7 days release was 30% and 18%, respectively for the sustained release formulations A and B compared to the subcutaneous administration of soluble hGH indicating that a substantial amount of hGh was still left in the microparticles. The low bioavailability found for the formulations was most likely due to the termination of the study after 7 days. hGH was still being released after 7 days and would potentially have carried on until day 14, and possibly longer.

The pharmacodynamic effect of hGH was measured by serum IGF-1 concentration. IGF-1 is an endocrine hormone mainly produced by the liver. The production is stimulated by the presence of growth hormone in the blood and hence IGF-1 can be used as a biological marker of hGH activity. IGF-I levels were elevated for up to 7 days after both the daily and the single sustained release injections of hGH. Increases in IGF-1 were determined as the AUC0-168 h and were

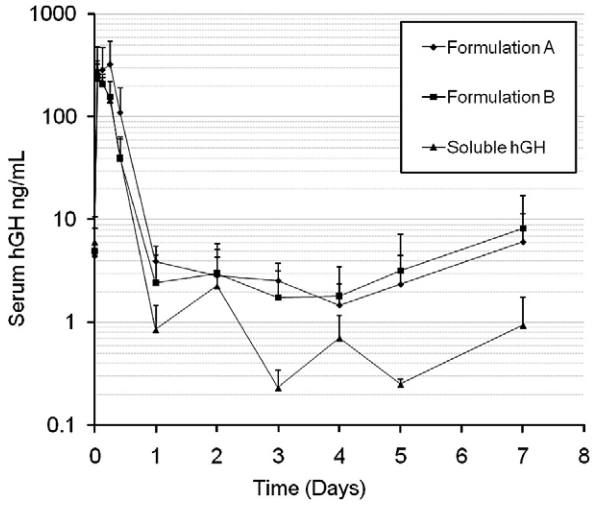

Fig. 6. Serum hGH levels in primates after daily subcutaneous injections of soluble hGH and a single subcutaneous injection of microparticle formulations A and B.

Table 4
Pharmacokinetic evaluation of the primate serum data.

Test item	Dose	C_{max}* ng/ml (SD)	T_{max}* H (SD)	$AUC_{0-24\ h}$* ng h/ml (SD)	$AUC_{0-\infty}$* ng h/ml (SD)	Fvs sc* %
Soluble hGH	0.21 mg/kg b.w./day	258.7 (±72.6)	2.00 (±1.2)	1774 (±219.8)	1777 (±220.1)	–
Formulation A	1.5 mg/kg b.w.	343.3 (±225.1)	3.50 (±2.9)	3246 (±2132)	3265 (±2137)	30
Formulation B	1.5 mg/kg b.w.	250.2 (±95.2)	1.50 (±1.0)	1797 (±519.8)	1810 (±511.9)	18

* No significant difference between groups.

477.8, 453.9 and 442.8 μg/h/ml for the soluble hGH, formulation A and formulation B, respectively. Based on the primate hGH serum data, levels greater than 1 ng/ml are required to induce an IGF-I response in these animals. Studies in the literature have suggested this level in Rhesus monkeys (2–3 kg) to be nearer 5 ng/ml [2]. This group injected the monkeys subcutaneously with 24 mg hGH (~10 mg/kg hGH) encapsulated in 200 mg PLGA microspheres produced by a double emulsion technique. The formulation provided a large initial burst release of protein (in line with the *in vitro* burst release of more than 30%) followed by a lag phase of 10 days and then a continuous release phase of 30 days [23]. In the present study, a much lower dose of 1.5 mg/kg hGH was given and hence it is likely that a higher dose would have produced a more significant IGF-1 response.

A similar study was reported by Kim et al. [24] where 1 mg/kg hGH encapsulated into hyaluronate microparticles and injected subcutaneously into cynomolgus monkeys (~2 kg) was found to give a T_{max} of 2 h and a C_{max} of 188 ng/ml and a release that continued for 30 h. Surprisingly, the IGF-1 levels stayed elevated for 6 days at a level similar to that after daily injections of 0.15 mg/kg soluble hGH, however no control group was reported in the study.

In the present study, the primates received a single dose of hGH microparticles at 1.5 mg/kg hGH which was higher than the daily approved dose of hGH for growth hormone deficient children who currently receive between 0.026 and 0.043 mg/kg daily equivalent to a monthly dose of between 0.8 and 1.3 mg/kg [1]. As the clearance of hGH in children is similar to that in monkeys, a similar dose of microencapsulated hGH should produce similar serum levels of hGH and IGF-I in children. For adults the clearance will be slower and hence a lower dose per kg would be needed [23].

4. Conclusion

A hGH sustained release PLGA microparticle formulation was successfully manufacture by a supercritical fluid process (CriticalMix™) and achieved an encapsulation efficiency of 100%. No degradation or aggregation of the hGH was found following encapsulation and release, and the protein remained as active in an *in vitro* bioassay as an hGH standard. The process is based on the ability of $scCO_2$ to liquefy polymers at near ambient temperatures, significantly lower than the polymer glass transition temperature. The liquefied polymer can then be mixed efficiently with the protein in the dry state. The mixture is depressurised through a nozzle whereby the CO_2 returns to its gaseous state and diffuses out of the droplets resulting in the production of microparticles containing the hGH. The preparation procedure is a one-step process. The sustained release formulations were tested *in vivo* in rats and in cynomolgus monkeys. Due to a much faster clearance rate of hGH from the blood in rats than in humans, the rat is not a good model for evaluation of a sustained release hGH formulations. However, the rat studies did identify two formulations superior to the others consisting of blends of PLGA and PLA with poloxamer 407 or 188 as processing aids and 6% hGH encapsulated as a spray dried micronized powder. The formulations maintained the hGH in the rat plasma in a sustained manner for 3 days. When administered to cynomolgus monkeys the formulations were found to give low initial burst of hGH followed by at least 7 days sustained release at a level between 1 and 5 ng/ml hGH. Due to financial constraints the primates were only bled up to 7 days. However, the microparticles had only released about 18% or 30% of their hGH at 7 days and at this point the plasma curves suggested that the release would continue for at least another week. The microparticles were well tolerated both by the rats and the monkeys with no evidence of subcutaneous inflammation or a chronic inflammatory response and fibrosis. The results show that the CriticalMix™ technology has provided two promising hGH sustained release formulations that warrant further development.

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