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Insulin nanoparticles: Stability and aerosolization from pressurized metered dose inhalers

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ABSTRACT

Nanoparticles containing insulin have been produced by emulsification processes followed by freeze-drying. Purified nanoparticles were suspended in hydrofluoroalkane (HFA) 134a, using essential oils (cineole and citral) as suspension stabilizers to form pressurized metered dose inhaler (pMDI) formulations. The retention of insulin integrity after formulation processing was determined using high performance liquid chromatography (HPLC), size exclusion chromatography (SEC), circular dichroism (CD) and fluorescence spectroscopy. The results indicated that the native structure of insulin was retained after formulation processing. Aerosolization properties of the manufactured pMDI formulations were determined using a multi-stage liquid impinger. The results showed that the nanoparticles were suitable for peripheral lung deposition, with a fine particle fraction ($FPF_{<1.7\,\mu m}$) of approximately 45% (w/w). In conclusion, the pMDI formulations with nanoparticles containing insulin developed in this study have the potential to deliver protein therapeutics via inhalation for systemic action.

1. Introduction

The first marketed inhalable insulin preparation was Exubera® (Aventis and Pfizer), a dry powdered inhaler indicated for the treatment of type 1 and type 2 diabetes. Although a technically innovative solution for achieving needleless insulin delivery, it was withdrawn from the market in 2007 as the product had failed to gain acceptance from both clinicians and patients resulting in disappointing sales (Mack, 2007), and was not considered cost-effective (Black et al., 2007).

Other inhalable insulin products in various stages of development include Aerodose (Aerogen), AIR (Alkermes and Lilly) and Technosphere Insulin (Mannkind) and AERX Insulin Diabetes Management System (Aradigm) (Mastrandrea and Quattrin, 2006). These systems are dry powder or aqueous solution inhaler formulations. To date, there have been no reported investigations into insulin nanoparticles suspended in hydrofluoroalkane (HFA) propellants for delivery from pressurized metered dose inhalers (pMDIs).

pMDIs are the most widely prescribed aerosol devices for pulmonary drug delivery and as such may prove acceptable for protein administration. In these devices, propellant such as HFAs and chlorofluorocarbons (CFCs) provide the energy for aerosolization of the drug formulation into respirable particles. The ability of the particles to disperse and form a stable suspension in HFA is one of the major properties that controls dose reproducibility in a suspension type pMDI (Byron, 1992). Unstable suspensions can result in uncontrolled emitted dose and size characteristics, which lead to poor inhalation therapy.

Non-ozone depleting HFA propellants, i.e. HFA 134a and HFA 227ea, now replace ozone depleting chlorofluorocarbon propellants. Due to different physical properties between CFCs and HFAs, e.g. higher polarity of the HFA propellants, a direct substitution into existing pMDIs has proved to be difficult leading to less favourable formulations involving co-solvent systems. Previously, alcohols such as ethanol have been used as co-solvents to increase the solubility of surfactants commonly used to improve the physical stability of pMDI, utilizing HFA as propellants.

Application of ethanol as a co-solvent in pMDI formulations containing protein has previously been reported (Williams et al., 1998). However, the presence of ethanol may affect the stability of protein or the solubility of stabilizers present in protein pMDI formulation during storage.

Food grade natural flavours have been extracted using HFA 134a as the extraction solvent (FactFile, INEOS Flour, www.ineosflour.com, USA). This suggests that the active ingredients of these flavours that are mainly aldehydes and/or ketones are soluble in HFA 134a. However, the application of these molecules

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in pMDI formulation and particularly their influence on particle suspension characteristics has not previously been investigated (Nyambura et al., 2008). In this work, cinnamaldehyde, cineole and citral were investigated. The group of cinnamyl derivatives and citral are regarded as safe based on their self-limiting properties as flavouring substances in food. They are rapidly absorbed, detoxified, and excreted in man. No adverse effects have been reported and they lack a significant carcinogenicity, genotoxic and mutagenic potential (Adams et al., 2004).

The manufacturing of nanoparticles containing a model protein (lysozyme) and subsequent dispersion in HFA 134a has previously been demonstrated (Nyambura et al., 2009). However, it is necessary to determine the application of these procedures with a therapeutic protein, which could be delivered via the pulmonary route. In this study, insulin was chosen because the molecule's physicochemical characteristics are well studied and they can be used in a general manner to exemplify complex structural aspects of interest while assessing changes to manufacturing and/or formulation parameters for proteins and their corresponding products (Defelippis and Larimore, 2005).

This paper investigates procedures for manufacturing a pMDI formulation comprising insulin-containing nanoparticles suspended in HFA 134a; assesses insulin stability in HFA 134a and the aerosolization properties of the pMDI formulation produced. In addition, novel materials (such as essential oils) are investigated for their ability to aid pMDI suspension stability as well as improving the aerosolization properties of the formulation.

2. Materials and methods

2.1. Materials

Dichloromethane and absolute ethanol were obtained from VWR International Ltd. (Poole, Dorset, UK) while HFA 134a was purchased from Solvay Flour and Derivate GmbH (Hannover, Germany). HPLC grade acetonitrile was purchased from Fisher Scientific Ltd. (Loughborough, UK). Water was distilled (Option 4 water purification system, Elga Ltd., UK). Lactose, citral, cinnamaldehyde, cineole and bovine insulin (≥27 USP units per mg) were obtained from Sigma–Aldrich Company Ltd. (Gillingham, UK). Lecithin (egg) and glycerol monooleate (GMO) were kindly donated by Lipoid GmbH (Ludwigshafen, Germany) and Danisco A/S, DK-7200 (Grindsted, Denmark) respectively.

2.2. Methods

2.2.1. Production of insulin-containing nanoparticles by a nanoprecipitation method

Insulin nanoparticles were produced by a nanoprecipitation process followed by freeze-drying (Nyambura et al., 2009). Insulin (80 mg) and lactose (20 mg) were weighed into a glass vial to form a composition of 80% (w/w) insulin and 20% (w/w) lactose. 1 mL of 0.1 M HCl was added to the vial and the powder dissolved to form the aqueous phase. Lecithin (200 mg) was weighed into a separate vial and dissolved into 7 mL of ethanol to form an oily phase. The aqueous phase was then added to the oil phase dropwise while homogenizing at low speed (10,000 rpm) and 5 min at high speed (24,000 rpm) using a Ultra Turrax T25 homogeniser (IKA-Werke GmbH, Staufen, Germany). The solution was snap-frozen using liquid nitrogen and freeze-dried (Drywinner 110, Heto-Holten A/S, Gydevang, Denmark) for a minimum of 12 h to remove water and ethanol. This resulted in dry matter containing nanoparticles covered with lecithin which was suspended in 0.5% (v/v) triethylamine (TEA) in dichloromethane, in which insulin and lactose were insoluble while lecithin was freely soluble, thus preserving the structure of the nanoparticles. The suspension was separated from free surfactant by centrifugation (3K30 Refrigerated centrifuge, Sigma Laborzentrifuges GmbH, Osterode am Harz, Germany). The sedimentation conditions were set as 17,000 rpm (equivalent to approximately 39,000 × *g*) at 25 °C in Oakridge Teflon® centrifuge tubes (50 mL, Nalge-Nunc Inc., Rochester, NY, USA). The solvent plus surfactant were decanted and the sediments comprising nanoparticles were collected. The process was repeated twice to ensure maximum purification of the nanoparticles (Cook et al., 2005).

2.2.2. Production of insulin-containing nanoparticles using an emulsification method

Insulin (80 mg) and lactose (20 mg) were dissolved in 1 mL of 0.1 M HCl. Lecithin was weighed in separate vials and 7 mL of chloroform was added to form the oily phase. The content of lecithin in the oily phase was varied from 0.5 to 2 g to investigate the optimal concentration for nanoparticle production. Both solutions (oily and aqueous phase) were homogenized for 5 min at 24,000 rpm to form a water in oil emulsion. The emulsion formed was snap-frozen to immobilize the emulsion into the solid state using liquid nitrogen and then freeze-dried overnight to remove water from frozen microscopic aqueous droplets and the nanoparticles purified as previously described.

Inclusion of an anti-foaming agent (glycerol monooleate) was investigated, with the objective of reducing the particle size of dispersed emulsion droplets. Briefly, in addition to the optimised lecithin concentration, 1 g of glycerol monooleate was added to form the oily phase of the emulsion with other processing parameters as described.

2.2.3. Particle size analysis of nanoparticles

Particle size distribution was determined using photon correlation spectroscopy (Malvern Zetasizer 3000, Malvern Instruments, UK). The instrument measures hydrodynamic diameter, expressed as *Z*-average diameter and a polydispersity index, determined by cumulant analysis as described in the International Standard on dynamic light scattering. The polydispersity index gives information regarding the width of the size distribution and the values range between 0 and 1. The nanoparticles were suspended in chloroform that was filtered through a 0.1 μm nylon membrane filter (Whatman, UK) and sonicated for 5 min. The sample concentration was maintained at 5 mg of nanoparticles/mL of chloroform, which was sufficient to provide the required analytical count rate in the spectroscopy (>50 kilocounts per second). The suspension was transferred into a non-frosted quartz cuvette and placed in the sample holder of the instrument. Three particle size determinations were performed for each sample.

2.2.4. Scanning electron microscopy of nanoparticles

Insulin-containing nanoparticles suspended in dichloromethane were placed on the sample holder and dichloromethane was allowed to evaporate. The samples were splutter coated with gold using an Emitech K550 (Ashford, UK) and then visualized with a Philips XL20 (Eindhoven, Holland) scanning electron microscope.

2.2.5. Dispersion of nanoparticles in HFA 134a

Dispersions of 1, 2, 3, 4, 5, and 10% (w/w) cinnamaldehyde, cineole and citral in HFA 134a were made by the cold filling process (Brown and George, 1997). The required amount of essential oil sample was placed in a chilled plastic coated glass vial (Wheaton, USA). The propellant (HFA 134a) was passed through a condenser chilled with dry ice before adding the required weight into the chilled vials containing the essential oil sample. A 63 μL/actuation metering valve (Valois DF60 MK42; Valois, France) was immediately crimped onto the vial (using manual bottle crimper 3000,

Aero-Tech Laboratory Equipment Company, USA) in order to avoid evaporation of propellant. The mixtures were shaken well and left to stand at ambient temperature. The solutions were visually inspected for the solubility of the essential oils.

Batches of nanoparticles (100 mg) were prepared, and after washing off excess lecithin as previously described, the nanoparticles were suspended in 5 mL of dichloromethane. 0.5 mL of cineole or citral was added and the suspension was vortexed to ensure homogeneity. Dichloromethane was removed by evaporation under vacuum using a Rotavapor® (Büchi, Switzerland) set at 35 °C for 5 min. A paste of nanoparticles moistened by cineole or citral was obtained which was subsequently suspended in HFA 134a by the cold filling process previously described. The pMDIs were then sonicated (XB6 Grant Instruments Ltd., UK) for approximately 1 min and investigations were performed on each aerosol unit within 2 days of manufacture. The solid concentration of the filled vials was maintained at 1% (w/w) and the suspension stability was visually inspected with time.

2.2.6. Analysis of insulin structure integrity

In this work, biophysical methods of structure determination have been employed to determine the integrity of insulin at three levels (primary, secondary and tertiary structures) after the production process.

2.2.6.1. Reverse phase chromatography (primary structure). The HPLC method for insulin analysis was modified from Kunkel et al. (1997) by lowering the mobile phase pH to 1.9 using TFA so that a peak due to bovine insulin could be eluted with a retention time of approximately 9 min. The HPLC settings were: mobile phase—50 mM sodium sulphate buffer and acetonitrile (ratio 70:30), pH 1.90 adjusted using trifluoroacetic acid; oven temperature—30 °C; flow rate—1.0 mL/min; column—ODS 5 μm, 4.6 mm × 150 mm (Hichrom, UK); injection volume—100 μl; detector—UV at 214 nm. Selectivity was determined by allowing the solution containing insulin (standard) to degrade for 7 days while stored at room temperature. The solution was then analysed, with extra peaks observed due to insulin degradation. The sensitivity of the method was determined and the limit of quantification was found to be 2.0 μg insulin/mL. A calibration curve was constructed by plotting peak area versus the concentration of insulin. Accuracy was determined by calculating the relative standard deviation (RSD) of peak areas of three consecutive standard injections of each data point used to construct the calibration curve. The RSD values were in the range of 1.0–1.6 and were within the range recommended by International Committee on Harmonisation guidelines for content assay by HPLC. All sample and standard solutions were freshly prepared. The chromatograms of unprocessed insulin (standard material) and that of processed insulin (samples from pMDI formulations) were evaluated for differences, such as the presence of extra peaks and the retention time of the main peak due to insulin.

2.2.6.2. Size exclusion chromatography (SEC) (primary structure). Nanoparticles were collected from the pMDI canisters as follows: three pMDI vials containing nanoparticle suspensions in HFA 134a were placed in a beaker with dry ice. Once cold, the vials were removed from the beaker and their valves removed to allow HFA 134a to escape slowly, leaving the nanoparticles at the base of the vials. The nanoparticles were suspended in dichloromethane, centrifuged and freeze-dried for 30 min to remove traces of cineole and dichloromethane. Approximately 4 mg/L sample and standard solutions were prepared by dissolving nanoparticles and insulin standard in 0.1 M HCl. The samples were analysed by SEC with the following conditions: injection volume—100 μL; flow rate—0.25 mL/min; run time—100 min; temperature—ambient; detector—UV at 214 nm; column—SuperoseTM12 10/300GL (TricornTM high performance columns, Amersham Biosciences, UK); mobile phase: 50 mM NaCl, 0.15 M phosphate buffer (pH 7.0).

2.2.6.3. Circular dichroism (CD) (secondary and tertiary structures). Nanoparticles were collected from three pMDI vials and rinsed as described previously.

Insulin (control and nanoparticles) was weighed in triplicate on a Metler-Toledo microgram balance (Sartorius Laboratory Balances, UK) and made up to a concentration of 0.2 mg/mL using phosphate buffer (50 mM, pH 2). The CD spectra were then acquired using a Jasco J720 spectropolarimeter (Jasco Corporation Milestones, UK) using the following settings: cell pathlength—0.5 mm far UV, 10 mm near UV; wavelength range—260–190 nm far UV, 400–230 nm near UV; bandwidth—2 nm; speed—10 nm/min; time constant—4 s; step size—0.2 nm. All spectra were corrected for solvent baseline and far-UV CD spectra were normalized for concentration and pathlength using a mean peptide molecular weight of 113. Where appropriate, secondary structure estimation was calculated using the Principal Component Regression method (Malik, 1997) and the results tabulated.

2.2.6.4. Fluorescence spectroscopy (tertiary structure). Nanoparticles were collected from three pMDI vials and rinsed as described previously.

10 mL of 0.2 mg/L sample and control insulin solutions were prepared in triplicate by dissolving in acidified (using HCl) 0.9% (w/v) NaCl (pH 2). Insulin in both sample and control solutions was unfolded by pipetting 5 mL of each sample and control solutions into separate vials each containing 5 mL of 6 M guanidine hydrochloride. Subsequently, 5 mL of acidified 0.9% NaCl solution was added to the remainder of each sample and control solutions containing the unfolded protein to maintain uniform concentration.

Fluorescence emission intensities of the sample and control (containing folded and unfolded insulin) solutions were measured using a PerkinElmer LS50-B fluorescence spectrophotometer (PerkinElmer, UK) and fluorescence cell with a 1 cm pathlength. The excitation wavelength was set at 275 nm while emission intensities were scanned from 200 nm to 450 nm. Excitation and emission slit widths were set at 10 nm and 2.5 nm respectively, while scan accumulation was set at 5 to maximize signal to noise level ratio.

2.2.7. Aerosolization properties of insulin-containing pMDI formulations

The aerosolization characteristics of pMDI formulations were determined using a multi-stage liquid impinger (MSLI; Copley Scientific Instruments, UK) operated at 60 L/min to determine their deposition profile (European Pharmacopoeia). The effective cut-off diameters were: Stage 1 = 13.0 μm, Stage 2 = 6.8 μm, Stage 3 = 3.1 μm, and Stage 4 = 1.7 μm. Stages 1–4 of the impinger were filled with 20 mL of acidified 0.9% (w/v) NaCl (pH 2) while a total collection filter was fitted in Stage 5. A USP throat fitted with a device-specific rubber mouthpiece was used. The rubber mouthpiece was designed and moulded by Copley Scientific (UK).

The pMDI was thoroughly shaken and 5 shots were actuated to waste (using an actuator with an orifice diameter of 0.5 mm, GlaxoSmithKline, UK) and subsequently a dose was fired into the MSLI via the mouthpiece. The inhaler was removed from the mouthpiece and thoroughly shaken for 5 s prior to actuating the second dose into the MSLI. The procedure was repeated until 10 doses were fired into the MSLI with an interval of 10 s between doses. After actuating the tenth dose, the airflow was continued for 10 s. The throat plus mouthpiece were rinsed with 20 mL of dissolving solvent. The filter was carefully retrieved and rinsed with 20 mL of dissolving

solvent. The other part of the MSLI was swirled to ensure thorough mixing of the contents in each stage and carefully avoiding inter-stage mixing of the sample. The content of insulin in each stage was assayed using reverse phase high-pressure liquid chromatography as described previously.

2.2.8. Effect of cineole concentration on deposition of insulin nanoparticles

Batches (100 mg) of insulin-containing nanoparticles were prepared and after washing off excess lecithin (and where appropriate glycerol monooleate) the nanoparticles were suspended into 5 mL of dichloromethane. Cineole (0.1, 0.25 and 0.5 mL) was added to one of each batch and the suspensions were vortexed to ensure homogeneity. Dichloromethane was removed by evaporation under vacuum using a Rotavapor® (Büchi, Switzerland) set at 35 °C for 5 min. A paste of nanoparticles moistened by cineole was obtained which was consequently suspended in 10 g of HFA 134a by the cold filling process to form pMDI formulations with different amounts of cineole. The nanoparticle concentration in the filled vials was in the range of 1–5% (w/w). Aerosolization properties were determined as previously described.

3. Results

3.1. Production of insulin nanoparticles

It was not possible to produce insulin nanoparticles using a nanoprecipitation method. This is because a homogeneous solution with no defined nanoprecipitates was formed when the aqueous phase was added to the oily phase. Efforts to freeze-dry the solution followed by attempts to purify insulin proved impossible as the dry matter dissolved in the washing solvent. This made it difficult to produce insulin-containing nanoparticles using this procedure and therefore, no further work was undertaken using this method of production.

Using an emulsification method, initially large particles were produced. However, increasing the lecithin concentration in the oily phase resulted in reduced mean particle size (Fig. 1) and polydispersity index (Fig. 2) of nanoparticles produced. Although increasing the lecithin concentration from 0.2 to 0.4 g/mL had minimal effect on nanoparticle size, there was however a substantial decrease in the polydispersity index.

Typical size distribution curves indicated a narrow distribution range for the nanoparticles (Fig. 3) and SEM revealed the nanoparticles to be approximately spherical and 500 nm in diameter (Fig. 4).

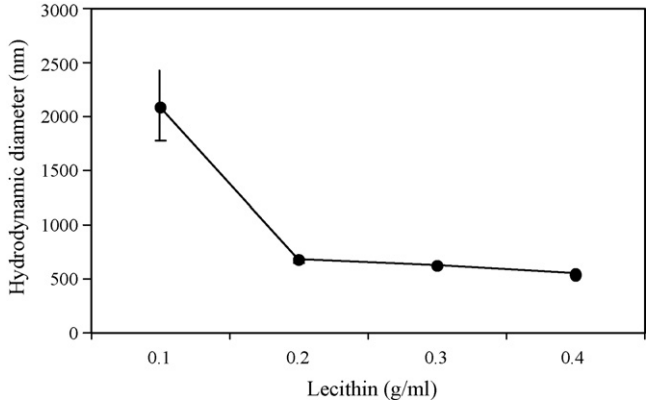

Fig. 1. Effect of lecithin concentration on insulin-containing nanoparticle size. Each point is the mean (±s.d.) of 5 preparations.

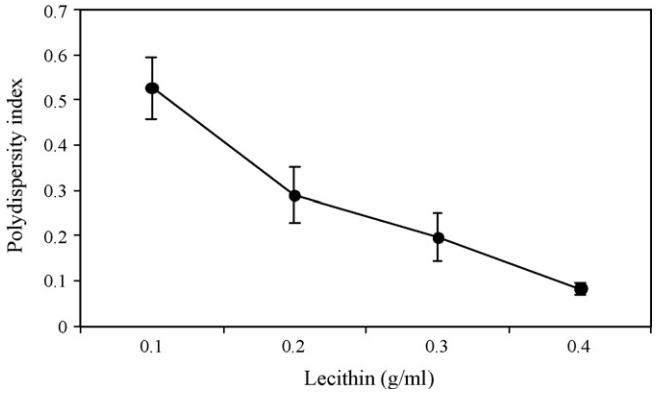

Fig. 2. Effect of lecithin concentration on the polydispersity index (PI) of insulin-containing nanoparticles. Each point is the mean (±s.d.) of 5 preparations.

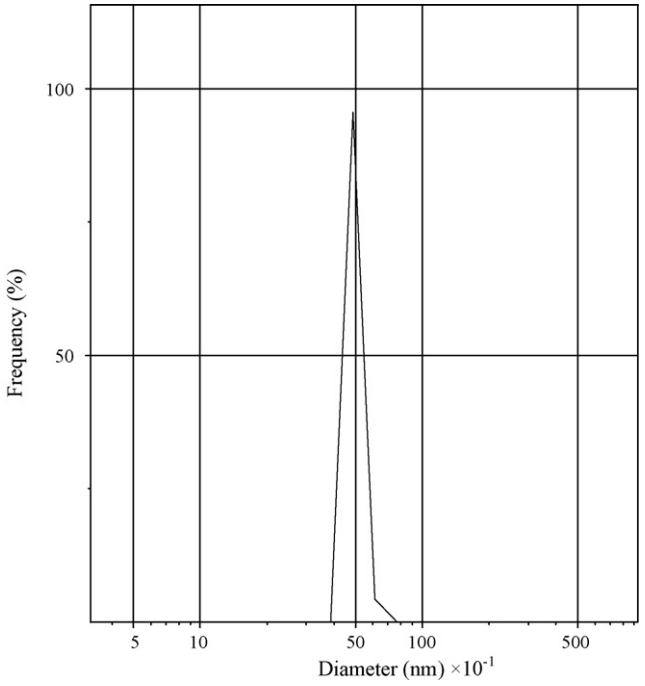

Fig. 3. Typical particle size distribution curve of insulin-containing nanoparticles (lecithin concentration = 0.4 g/mL, Z_{ave} = 545.9 nm, PI = 0.084).

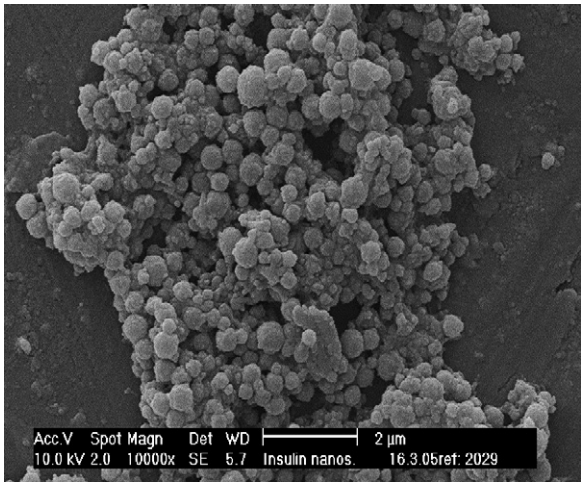

Fig. 4. SEM of insulin-containing nanoparticles.

3.2. Dispersibility of the nanoparticles in HFA 134a

Clear yellow solutions were formed when 1–10% (w/w) cinnamaldehyde was dissolved in HFA 134a, with the intensity of colour increasing with cinnamaldehyde concentration. After the solutions were left to stand overnight, milky suspensions were observed which suggested that a separation between cinnamaldehyde and HFA 134a occurred. Consequently, no further investigations were carried out using cinnamaldehyde.

Homogeneous solutions were formed when citral and cineole were dissolved in HFA 134a at all concentrations examined. No changes were noted after leaving the solutions to stand overnight at room temperature. It was concluded that citral and cineole remained soluble in HFA 134a and therefore further investigations were carried out using cineole and citral as model essential oils.

Both cineole and citral were found to stabilize the dispersion of insulin-containing nanoparticles in HFA 134a. Homogeneous dispersions were formed immediately after shaking and remained visibly stable for at least 1 min. The rate of particle separation depended on whether citral or cineole was used in the formulation. The time to visually detect separation was shorter when citral (approximately 2 min) was used compared to cineole (approximately 3 min). However, the formulations readily re-dispersed upon shaking which is important for dose reproducibility.

3.3. Insulin stability as determined by the primary structure of insulin

Insulin, like other proteins, is an unstable entity and is vulnerable to modification by chemical reactions with molecules in its vicinity. Thus, during the production of the nanoparticles containing insulin and subsequent dispersion of nanoparticles in HFA 134a, insulin may be degraded by hydrolytic reactions (deamidated products) or transformed by formation of intermolecular covalent bonds with other insulin molecules leading to higher molecular weight products (Oliva et al., 2000). Reverse phase HPLC and SEC techniques were therefore used to assess the retention of primary structures of insulin after processing.

Chemical degradation of insulin occurs mainly by hydrolysis and intermolecular transformation reactions. The degradation products have different molecular weights as well as chemical characteristics. Previously, monodesamido-A21-insulin and monodesamido-B3-insulin have been reported as the major chemical degradation products of insulin (Oliva et al., 2000). Insulin degrades rapidly in an acid medium at residue Asn A21, while in slightly acid or alkaline medium deamidation takes place at residue Asn B3 (Brange et al., 1992). Reverse phase chromatography helps to separate, detect and quantify these degradants, where they are observed as extra peaks resolved from the main peak representing the main insulin compound. The chromatograms from both unprocessed insulin (authentic standard material) solutions and processed insulin (samples from pMDI batches collected from MSLI stages) showed similar profiles characterized by one peak with a retention time around 9 min (Fig. 5). This was an indication that insulin was not aggregated or chemically degraded by hydrolysis and intermolecular reactions during the manufacture of pMDI formulations.

The main aggregation products formed by insulin are covalent insulin dimers, where the two insulin molecules in the dimer are held together by non-polar forces and four hydrogen bonds arranged as a β-sheet structure between the two anti-parallel COOH terminal strands of the B chain (Brange et al., 1992).

In this study, SEC was not calibrated since it was designed for qualitative work to separate and detect the different aggregation products of insulin only and not for quantification. However, insulin standard material was analysed on every occasion to monitor system performance by determining the retention time of the main peak due to the insulin monomer.

Table 1
Composition of secondary structures of control insulin and extracts from three pMDI batches. Each result is the mean (±s.d.) of 3 preparations.

Solution	α-Helix (%)	β-Sheet (%)	Other (%)
Insulin (standard)	33.8 ± 0.3	20.3 ± 0.3	45.9 ± 0.1
pMDI sample	32.3 ± 1.0	20.7 ± 0.4	47.0 ± 0.6

The chromatograms from both unprocessed insulin (standard material) and those of processed insulin (samples from pMDI batches) showed similar profiles characterized by one peak at retention time around 66 min (Fig. 6). No extra peaks due to insulin aggregation products were observed in the sample chromatograms. Therefore, it was concluded that the manufacturing process for the pMDI formulations containing insulin nanoparticles did not cause insulin aggregation.

3.4. Insulin stability as determined by secondary structures of insulin

The CD spectrum of bovine insulin has been studied previously and is characterized by three far-UV CD signals that are dependent on molecular conformation and not side-chain groups (Mercola et al., 1967). The three major far-UV CD signals are noted as a large positive peak at 194 nm, and two well-defined negative peaks at 209 nm and 222 nm. However, when insulin is degraded using trypsin, the far-UV CD signal associated with the 222 nm region is lost and the absolute residual dichroism decreases in a monotonic fashion from 210 nm to 230 nm (Mercola et al., 1967). The far-UV CD results (Table 1) indicated that the secondary structures of insulin were retained after processing. There was no significant difference ($p < 0.05$) between the secondary structural composition of unprocessed insulin (standard material) and processed insulin (from the pMDI batches).

3.5. Stability of insulin determined from tertiary structures of insulin

Insulin molecules are devoid of tryptophan and therefore, their near-UV CD signal originates from the tyrosyl side chains. These tyrosyl side chains on the surface of the insulin monomers are important in self-association of insulin as they are involved in the formation of dimers, tetramers and hexamers. The near-UV CD signal of insulin is sensitive to the degree of self-association, with the tyrosyl CD signal at 275 nm being enhanced as monomers interact to form dimers and as dimers interact to form hexamers (Strickland and Mercola, 1976). Thus, perturbation of the tyrosyl side chains can lead to loss of tertiary structure (removal of tyrosyl side chains from the surface of the molecule) that is observed as loss or reduced near-UV CD signal at 275 nm.

The unprocessed insulin and processed insulin from the pMDI formulation both had a large negative CD signal at around 275 nm. This indicated that the overall tertiary structure of insulin was not affected by manufacturing conditions, as well as the harsh environment when nanoparticles were suspended in HFA 134a.

To verify the near-UV CD results, the tertiary structure of insulin was further studied by fluorescence spectroscopy. The environment of the tyrosine amino acids influences the intensity of fluorescence signal. The samples (pMDI formulations) and control insulin spectra (not shown) indicated that the tyrosine amino acids were distributed over the surfaces of the insulin monomers, which was in agreement with the near-UV CD results. The emission intensity was highest before the insulin was denatured using 6 M guanidine hydrochloride, as the fluorescence signal was not quenched. After

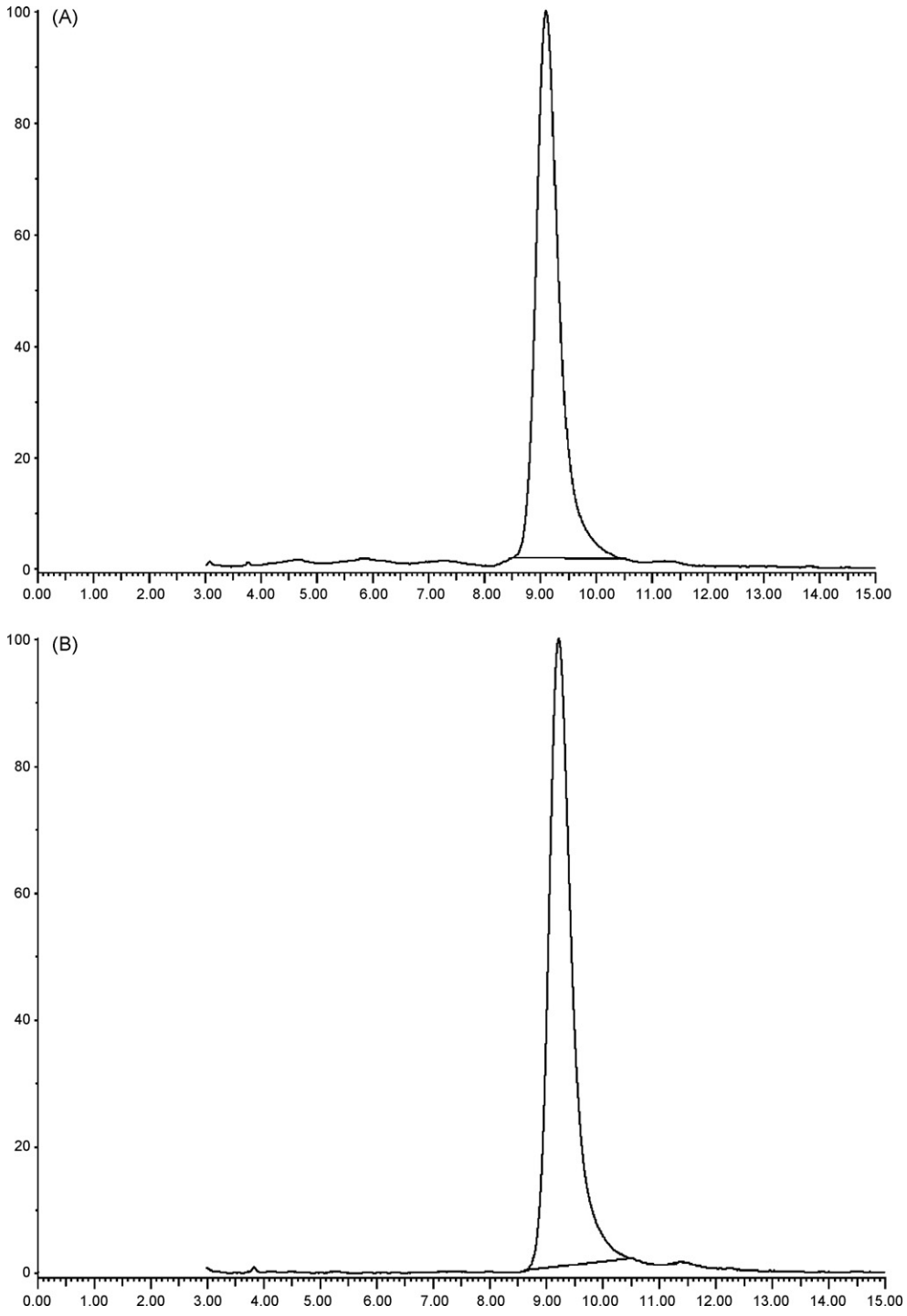

Fig. 5. Typical HPLC chromatograms for (A) standard and (B) typical sample insulin solutions.

insulin monomers were denatured, the tyrosine amino acids that were located on the surface were buried (internalized) within the molecule and their fluorescence signal was quenched.

The quenching of the fluorescence signal from the buried tyrosine residues originates from other parts of the molecule surrounding it. Neutral aspartic and glutamic acid residues, charged lysine and arginine residues, amide and peptide groups are effective dynamic quenchers. However, disulphide is one of the strongest quenchers, although a single cysteine can also be an effective quencher (Ladokhin et al., 2000).

The fluorescence spectroscopy results indicated that there was no significant perturbation of the tyrosyl side chains on the surface of insulin molecule. This provides further evidence that the 3D structure of insulin was retained after manufacturing, suggesting that insulin present in the pMDI nanoparticle formulation was likely to be biologically active.

3.6. Effect of cineole and suspension concentration on nanoparticle deposition in the MSLI

Cineole concentration did not affect throat deposition and $FPF_{<1.7\,\mu m}$ for the range investigated (Fig. 7). A ratio of 0.25 mL cineole to 100 mg of insulin-containing nanoparticles was selected for further investigation and was applied in the

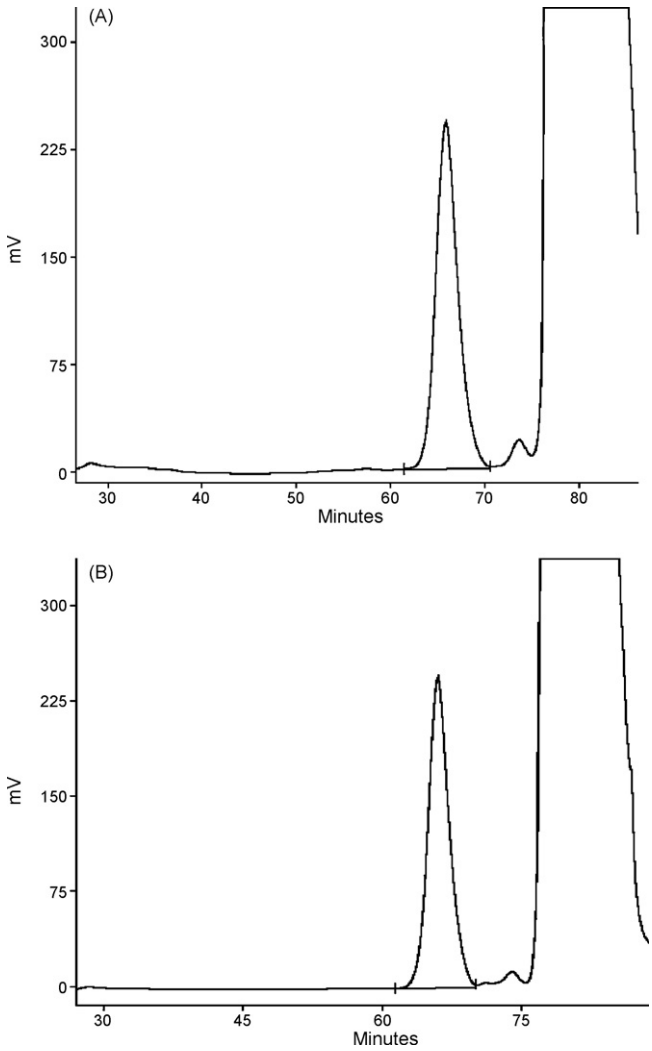

Fig. 6. Typical SEC chromatogram for (A) standard and (B) typical sample insulin solutions.

further studies of aerosolization characteristics of pMDI formulations.

The performance of the pMDI formulations was found to be dependent on the suspension concentration. The results (Fig. 8) indicated that the pMDI formulation containing the lowest concentration (1%, w/w) insulin nanoparticles had the lowest throat deposition and the best aerosol performance characterized by a $FPF_{<1.7\,\mu m}$ of around 20% (w/w) emitted dose. Therefore, further studies were undertaken to further optimise the formulation parameters in order to improve the $FPF_{<1.7\,\mu m}$.

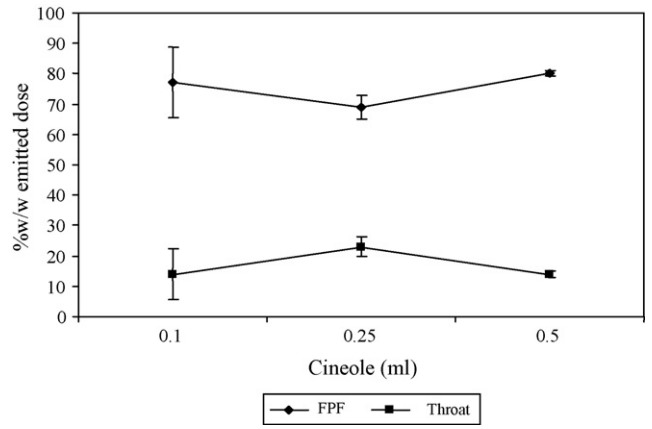

Fig. 7. Effect of cineole concentration (mL per 100 mg insulin-containing nanoparticles) on $FPF_{<1.7\,\mu m}$ and throat deposition of nanoparticles. Each point is the mean (±s.d.) of 3 preparations.

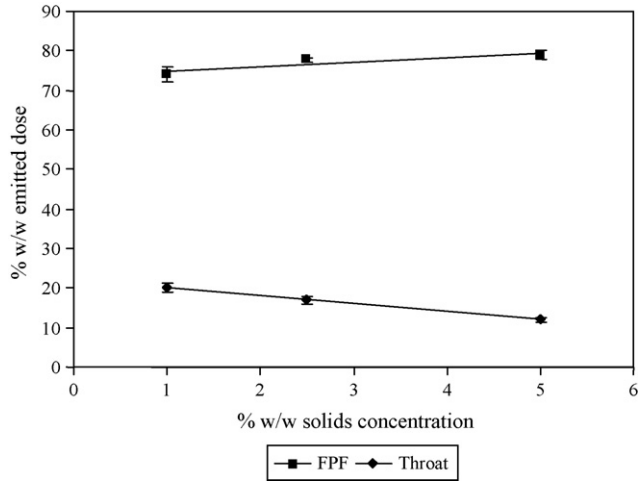

Fig. 8. FPF and throat deposition of pMDI formulations containing 1, 2.5 and 5% concentration of insulin nanoparticles in HFA 134a. Each point is the mean (±s.d.) of 3 preparations.

3.7. *Effect of glycerol monooleate on aerosolization properties*

Glycerol monooleate, generally regarded as a safe excipient, was employed in this study as an anti-foaming agent as well as a co-surfactant. Co-surfactants act in conjunction with a surfactant to further lower the interfacial energy of the emulsion (Attwood and Florence, 1983). GMO has previously been reported to reduce the hydrophilicity of an emulsifier system by encouraging the formation of a water in oil emulsion (Rybinski and Hill, 1998). These properties led to the reduction of the droplet size of the emulsion and consequently ensure a reduction in nanoparticle size.

The inclusion of glycerol monooleate resulted in a significant reduction ($p < 0.001$) in nanoparticles size from 545.9 ± 12.7 nm to 346.1 ± 9.0 nm. However, the polydispersity index increased from 0.084 ± 0.013 to 0.21 ± 0.1 when glycerol monooleate was used in the formation of the emulsion.

Aerosolization results showed that an $FPF_{<1.7\,\mu m}$ of 45% (w/w) of emitted dose was achieved when nanoparticles with Z_{ave} diameter of 346.7 ± 9.0 nm were used in the formulation (Table 2). The emitted dose (ex-actuator) determined by assay of insulin content was 0.44 ± 0.04 mg/actuation. This indicated that approximately 0.55 mg of insulin-containing nanoparticle formulation was aerosolized from the pMDI device and the $FPD_{<1.7\,\mu m}$ was approximately 0.20 mg of insulin per actuation. The results showed that there was no difference between $FPF_{<1.7\,\mu m}$ (fraction predicted to reach the alveolar region) and $FPF_{<3.1\,\mu m}$ (fraction predicted to reach the peripheral airways of the lung) since there was no insulin detected in Stage 3 of the MSLI. The results also indicated

Table 2
Comparison of optimised pMDI formulations. Each result is the mean (±s.d.) of 3 preparations.

Property	GMO absent	GMO present
Nanoparticles hydrodynamic diameter (nm)	545.9 ± 12.7	346.7 ± 9.0
$FPF_{<1.7\,\mu m}$ (% (w/w) emitted dose)	20 ± 1.2	45 ± 3.5
Throat deposition (% (w/w) emitted dose)	74 ± 2.0	37.5 ± 4.5
Solid concentration (%, w/w)	1	1

that throat deposition was reduced to 37.5% (w/w) of the emitted dose.

4. Discussion

The emulsification stage in the manufacturing process for the insulin-containing water in oil emulsion required optimised parameters that would ensure reduction of particle size as well as narrowing of the particle size distribution. Effective droplet disruption was achieved by exposing the droplets to high shear stress using a homogenizer. This may be detrimental to insulin, as it could have led to both physical and chemical degradation. However, the results indicate that insulin was not degraded after processing. It can be deduced that excipients such as lactose (lyoprotectant) and lecithin (surfactant) protected insulin monomers during manufacturing.

An altered composition of secondary structures of insulin has previously been reported when a water in oil emulsion was formed by homogenization (Jørgensen et al., 2003). The authors suggested that exposure of insulin to the oil–water interface, followed by homogenization, could lead to altered distribution in the secondary elements, e.g. α-helix and β-sheet, measured by area overlap calculations of the infrared spectra. Non-polar side chains of proteins are more soluble in organic solvents than in water, while hydrophobic interactions are weakened by organic solvents. This causes the protein to unfold and therefore there is loss of native structures. Considering the manufacturing process used, there was a potential for insulin to be denatured while forming the water in oil emulsion. However, the results indicate that insulin was not denatured after processing. The possible explanation for this might be the protection of insulin from exposure to interfaces by lecithin (surfactant) in the emulsion. This is in agreement with Bam et al. (1998) who reported the protection, by surfactants, of protein from interface-induced denaturation.

Freezing was one of the critical manufacturing steps that could have caused insulin damage. The emulsion was frozen, subjecting the protein to increased concentrations of both protein and other additives from the formulation that could have led to aggregation of insulin (Eckhardt et al., 1991). Nucleation and crystallization of ice crystals formed during freezing can also disrupt the hydration shell of insulin molecules (Koseki et al., 1990). However, excipients such as lactose and lecithin that play the roles of cryoprotectant and lyoprotectant are concentrated but not crystallized, and therefore protected insulin from damage.

Removal of the functional water molecules during dehydration inactivates proteins, for example, lysozyme loses its activity upon removal of those water molecules located in the active sites (Nagendra et al., 1998). As no aggregation or degradation was demonstrated by the results obtained, it can be deduced that insulin was protected from degradation by the lyoprotectant (lactose) during drying.

The results have shown that a stable pMDI suspension was achieved with the aid of citral or cineole. The mechanism by which citral or cineole stabilized the pMDI suspension was not investigated. However, it is hypothesized that these molecules (citral or cineole) have surface-active properties that enable them to stabilize a colloidal suspension. It is suggested that the non-polar end of these molecules orientate towards HFA 134a while their polar end orientate towards the nanoparticle. This is thought to be possible because insulin-containing nanoparticles are made up of hydrophilic materials while HFA 134a is hydrophobic. This is in agreement with Meziani et al. (1997, 2000) who have reported the co-surfactant properties of aldehydes and ketones. Using ternary systems of water/AOT/aldehydes and water/AOT/ketones, these authors have shown the realms-of existence of stable monophasic, transparent and isotropic systems that are indicative of co-surfactant behaviour of both aldehydes and ketones.

The tests on the aerosolization characteristics of the compositions showed that stabilizer concentrations of 0.1–0.5 mL per 100 mg of insulin-containing nanoparticles were effective. It was also found that throat deposition decreased as the concentration of solid decreased, while $FPF_{<1.7\,\mu m}$ increased with decreasing solid concentration. However, after varying both stabilizer and solid concentrations in the formulation, $FPF_{<1.7\,\mu m}$ was still low (approximately 23% (w/w) of emitted dose).

The deposition profile of the nanoparticles in the MSLI was significantly improved when nanoparticles size was reduced from Z_{ave} diameter of 545.9 ± 12.7 nm to 346.7 ± 9.0 nm by inclusion of GMO. The amount of insulin deposited as $FPF_{<1.7\,\mu m}$ was increased from approximately 20 to 45% (w/w) of the emitted dose while throat deposition was reduced from approximately 74 to 37.5% (w/w) of the emitted dose. This was a successful formulation as only 10–20% peripheral lung deposition is achieved when a typical pMDI formulation is used (Anderson, 2001), whereas in the present pMDI formulation approximately 45% (w/w) would be predicted to deposit in the peripheral lung.

5. Conclusion

In conclusion, a freeze-drying process can produce bio-stable insulin-containing nanoparticles with the appropriate size for peripheral lung deposition. They are characterized by high retention of insulin native structural integrity and relatively narrow size distribution.

Essential oils (citral and cineole) can aid suspension stability of insulin-containing nanoparticles in the pMDI formulations using HFA134a as a propellant. The optimal pMDI formulation developed is characterized by high aerosol performance that may ensure an adequate insulin dose reaches the target sites of the lung for insulin absorption into the circulatory system.

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