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The effects of polyvinyl alcohol on the in vitro stability and delivery of spray-dried protein particles from surfactant-free HFA 134a-based pressurised metered dose inhalers

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Abstract

The objective of the present study was to investigate the physical stability of spray-dried proteins within surfactant-free hydrofluoroalkane (HFA) pressurised metered dose inhalers (pMDIs) during prolonged storage. Two model proteins (lysozyme and catalase) were spray-dried and stabilised in the presence of excipients, and subsequently suspended within HFA 134a. The pMDIs were stored valve-up for 6 months at room temperature (ca. 25 °C). Activities of the proteins were determined using biological assays and the fine particle fraction of the pMDIs was measured using a twin-stage impinger. The biological activities of catalase and lysozyme were found to be preserved in the presence of sugars and/or 80% hydrolysed polyvinyl alcohol (PVA) during spray drying. In addition, suspending the stabilised proteins within HFA for up to 6 months had little effect on their activity. The aerosolisation performance of lysozyme or catalase formulations containing either sucrose or trehalose as stabilisers appeared to deteriorate as a function of storage time. However, those formulations containing PVA were found to generate the greatest fine particle fraction, which in some cases was up to 50%, and to possess excellent physical stability during storage. The results indicated that the presence of PVA in the spray-dried stabilised protein particles could enhance the physical stability of particles, when suspended in the surfactant-free HFA MDI formulations, without affecting the protein stability upon prolonged storage.

Keywords: Spray-drying; Protein stability; Metered dose inhaler; Pulmonary delivery; Catalase; Polyvinyl alcohol; Lysozyme; Hydrofluoroalkane

1. Introduction

Proteins provide several unique treatments for chronic diseases including, diabetes, cancer, and cystic fibrosis (Chi et al., 2003). However, the clinical use

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of these therapeutic agents usually requires repetitive dosing, which makes parenteral administration undesirable. As a result, drug delivery technologies that allow the administration of proteins and peptides by non-parenteral routes, including via oral, nasal, transdermal, buccal, rectal, vaginal and pulmonary delivery, have drawn increasing interest (Wearley, 1991). Of these routes, pulmonary delivery seems to be most promising due to the large surface area and relatively high absorption efficiency of macromolecules at this site. Numerous studies have demonstrated the feasibility of using nebulisers and dry powder inhalers (DPIs) for pulmonary protein delivery (Prime et al., 1997; Yamashita et al., 1998). For example, both human and experimental animal studies have shown the success in delivering insulin, calcitonin, somatostatin and growth hormones systemically using DPIs whilst insulin has also been successfully delivered to the lung using a nebuliser (Agu et al., 2001; Patton, 1997).

Due to numerous technical challenges, there have been few studies that document the successful delivery of proteins using pressurised metered dose inhalers (pMDIs) (Zhu et al., 2002). Unlike small molecular weight compounds that have been successfully delivered using pMDIs since the 1950s, proteins when stored within hydrophobic vehicles, are thought to be physically unstable. Thus, formulating such molecules within hydrophobic pMDI propellants (e.g. hydrofluoroalkane (HFA) 134a) is perceived to be problematic. However, it has been reported that proteins such as calcitonin and DNase I, can be formulated so as to retain their biological activity within pMDI formulations, although the full details of the strategies employed have not been declared (Fulton et al., 2002; Oliver et al., 2000). In addition, preliminary studies by Quinn et al. (1999) have found that lysozyme undergoes no immediate physicochemical change in the presence of propellant HFA 134a (using Fourier transform Raman spectroscopy).

Apart from protein stability, excellent delivery efficiency is also required for successful pulmonary protein delivery, in particular for those formulations intended to elicit systemic effects. Proteins are typically hydrophilic and therefore, poorly soluble within non-polar HFA propellants. To enable the delivery of these agents via pMDIs, the therapeutic protein must be incorporated within a particulate carrier that can be suspended within the propellant. In order to overcome the problems of drug particle stability in the HFA propellants, Williams and Liu (1999) utilised ethanol as a co-solvent to increase the solubility of commonly used surfactants to effect the physical stabilisation of protein particles. However, the presence of alcohol might affect the stability of proteins or the solubility of stabilisers present in the protein particles. In addition, the presence of surfactants does not guarantee optimal aerosolisation properties because drug particles may become aggregated as a function of surfactant concentration (Hickey et al., 1988). The production of self-dispersible particles in HFA propellants in the absence of soluble surfactants is therefore desirable (Smyth, 2003). The aim of this study was to investigate the feasibility of developing spray-dried protein particles, which were physically stable in a surfactant-free HFA-based pMDI without loss of biological activity. Lysozyme and catalase were selected as model proteins for assessing both the suspension stability and concomitant biological activity during storage.

2. Materials and methods

The buffer phosphate salts, sucrose, trehalose, polyvinyl alcohol 80% hydrolysed (PVA, M_w 9000–10,000), hydrogen peroxide, catalase (2× crystallised, aqueous suspension >46,000 units/mg), lysozyme (3× crystallised, dialysed and lyophilised, >48,000 units/mg), and *Micrococcus lysodeikticus* were purchased from Sigma–Aldrich Co., UK. A Micro BCA kit™ including reagents A–C and standard bovine serum albumin (BSA, 2 mg/ml) was obtained from Perbio Science UK Ltd., UK and HFA 134a, polyethyleneterephthalate (PET) pMDI canisters (∼20 ml) fitted with 25 μl metering valves actuators were kindly donated by AstraZeneca, UK.

2.1. Spray-drying samples

The enzymes (0.5 or 1.0 g) and excipients were dissolved in 100 or 200 ml of buffer and spray-dried using a Model 190 Büchi mini spray-dryer. The processing parameters comprised a feed rate of 3 ml/min, an atomising air-flow rate of 700 l/h and an inlet temperature of 95 °C. Outlet temperatures were found to range from 65 to 69 °C. The solutions employed to dissolve lysozyme and catalase were 5 mM potassium phos-

phate buffer (pH 6.2) and 5 mM potassium phosphate buffer (pH 7.0), respectively and all the enzyme concentrations were maintained at 5 mg/ml. Spray-dried particles were collected after the drying procedure had been effected and manufactured into MDI formulations immediately.

2.2. Particle size analysis by laser diffraction

Several milligrams of the spray-dried powders were dispersed in 1 ml of 0.1% (w/v) lecithin cyclohexane solution. The particle suspension was sonicated in a water bath (Model F5100b; Decon Laboratories, UK) for 30 s to disperse any possible agglomerates before being added to a stirred sample cell. The particle size of the sample was measured by a Malvern 2600 laser diffraction analyser (Malvern Instruments, UK) using a 63 mm focal length lens at an obscuration of 0.165–0.25. Particle size distributions were expressed in terms of volume median diameter (VMD) and span. The VMD was the diameter at the 50% point of the entire volume distribution whilst the span was defined as [D(v,90)-D(v,10)]/D(v,50), where D(v,90), D(v,50), and D(v,10) were the respective diameters at 90, 50 and 10% cumulative volumes.

2.3. Scanning electron microscopy (SEM)

Particle size and morphology were investigated by using SEM. Double-sized adhesive tape was place on an aluminium stub and after stripping off the upper side of the adhesive protection, a small amount of particles was scattered on the stub and dispersed by tapping lightly on the edge of the stub with a spatula to break agglomerates. The particles were then coated with approximately 15–20 nm gold using a sputter coater (Polaron E5100, Polaron Equipment Ltd., Watford, UK) operated at an electrical potential of 15.0 kV, 20 mA. Several photomicrographs were produced by scanning fields, selected randomly, at several magnifications with a Philips SEM501B scanning electron microscope (Einhoven, The Netherlands).

2.4. Quantification of proteins using the Pierce Micro BCA protein assay™

The protocol of the Pierce Micro BCA protein assay™ (supplied by Perbio Science UK Ltd., UK) as provided by the manufacturer was employed. Briefly, an aliquot of 150 μl of each standard or test sample was transferred into a 96-well microplate in duplicate. Subsequently, 150 μl of the working reagent, which was prepared by mixing 25 parts of Micro BCA reagent A and 24 parts of reagent B with 1 part of BCA reagent C, was added to each well and the solutions immediately mixed on a plate shaker for 30 s. The plate was covered and incubated at 50 °C for 90 min, after which it was cooled to room temperature and the absorbance in each well measured at 562 nm using a UV reader (SpectroMax, Molecular Devices Ltd., UK). BSA was used as the protein standard and a set of protein calibration solutions between 2 and 20 μg/ml was prepared by appropriately diluting a 2.0 mg/ml (BSA) stock solution using water. The response of each enzyme was determined by comparing the nominal concentration with the measured amount using the BCA assay. The Micro BCA assay was validated as 'fit for purpose' prior to use, with the relative standard deviation (R.S.D.) of both intra- and inter-run samples found to be less than 5%.

2.5. Determination of enzyme biological activity

Lysozyme was assayed as previously reported (Liao et al., 2001). The activity of catalase was measured by a published method (Aebi, 1974), in which the first-order degradation of hydrogen peroxide was monitored by measuring a decrease in absorbance at 240 nm as a function of time. Briefly, 100 μl of catalase solution, the concentration of which was between 0.4 and 1.2 μg/ml, was transferred to 2.9 ml of substrate (420 mcg/ml, H_2O_2 solution) contained in a silica cuvette, which was incubated in a thermostatic cell (25 °C) for 3–4 min to reach temperature equilibration. The A_{240} of the substrate was always initially between 0.520 and 0.550 and the time required for A_{240} to decrease from 0.450 to 0.400 was recorded. A calibration curve was constructed using catalase standards of known activity and the unknown samples were compared to this. The biological assays for catalase and lysozyme were validated as 'fit for purpose', with the inter-run and intra-run being 2–5% in terms of R.S.D.

The particles emitted from the pMDI formulation were dissolved directly into 25 ml aqueous buffer (using the method described below) prior to the determination of biological activity. Following this, the

concentration of enzyme in each sample was determined using the Micro BCA assay. The activity of each pMDI sample was determined as units per mg and expressed relative to the activity of a control sample. The control sample comprised of a spray-dried enzyme stored in a freezer, which had not been in contact with HFA.

2.6. DSC analysis

The melting enthalpies (ΔH_m) of spray-dried powders were determined using a model 2920 modulated DSC (TA Instruments, UK), which was calibrated with indium prior to analysis. Approximately 5 mg of dry sample was placed in an aluminium pan, which was hermetically sealed, and then equilibrated at −25 °C in the sample compartment. Data were collected whilst the sample was heated at a rate of 10 °C/min between −25 and 200 °C. For liquid samples, approximately 30 μl of protein solution was hermetically sealed, and data were collected between 5 and 95 °C at a heating rate of 10 °C/min.

2.7. Preparation of protein pMDI formulation

Samples (approximately 50 mg) of each spray-dried formulation were accurately weighed into individual PET canisters. The canisters were stored open in a desiccator containing phosphorus pentoxide, thereby exposing the formulation to a relative humidity of 0% for 24 h, so as to reduce the moisture content to approximately 1.5% (w/w) (thermogravimetric analysis (TGA) results showed that the moisture content of either catalase or lysozyme particles was at approximately 1.5% (w/w) upon exposure in a desiccator containing phosphorus pentoxide for a period of 24 h or longer). Subsequently, the canisters were capped by crimping metering valves into place and filling through the valves with ca. 10 g of HFA 134a. The integrity of the crimping of the canisters was tested by monitoring the weight change, when a weight change less than 10 mg was regarded as meeting the integrity requirement. Both integrity and other tests were carried out on formulations which were stored for ∼2 days, 6 weeks, 12 weeks and 26 weeks at room temperature and the results obtained after these time intervals were designated as week-1, week-6, week-12 and week-26 respectively. Due to the limited availability of the spray dried proteins several of the samples could not be tested at 26 weeks.

2.8. Deposition test

An in vitro determination of the fine particle fraction (FPF) of enzyme from pMDI formulations was carried out using a twin stage impinger (TSI). Purified water was introduced into the two stages as the entrapment solvent. Each canister was mounted onto the end of the glass throat via a rubber mouthpiece adapter and an actuator. The vacuum pump was adjusted so that an air flow of 60 l/min was established through the TSI set-up and the pMDI was discharged three times to waste prior to each test. The vacuum pump was switched on and the pMDI actuated 20 times for each test. After the final actuation, the pump was left on for a further 5 s, air flow was then stopped and the set-up dismantled.

After aerosolisation of the formulation using this procedure, the inhaler device and the mouthpiece adapter (device) were washed with deionised water. The amount of enzyme remaining in the device, stage 1 or stage 2 was determined using the Micro BCA assay as described above. The fraction from each stage was defined as the percentage of the total recovered amount of enzyme, with the FPF defined as the percentage depositing on stage 2 of the device.

2.9. Determination of dose delivered through the valve and the recovery of deposition

The dose delivered through the valve of a canister was determined using the following protocol. A holder with three legs and central indentation with a hole tapered in a downward direction was placed into 50 ml glass beaker with 25 ml purified water. A canister was shaken, discharged three times to waste with >5 s between each activation and the device washed using purified water. After the canister and the valve assembly were completely dried, the canister was shaken for ∼5 s and then placed inverted in the beaker vertically downwards and immediately discharged once. Subsequently, 19 more puffs were actuated into the beaker, after shaking for 5 s between each actuation. The resultant protein solution was diluted to 200 ml with purified water for subsequent quantification. The percentage deposition at various points in the impinger set up was expressed as the total amount of enzyme recovered from the three stages relative to the dose delivered through the valve.

Table 1
The recovered biological activity of spray-dried lysozyme and catalase microparticles prior to suspension in hydrofluroralkane propellant (mean ± S.D., $n = 3$)

Formulation	Relative activity (%)
Lysozyme alone	87.2 ± 2.1
Lysozyme:sucrose 1:1	97.2 ± 3.0
Lysozyme:trehalose 1:1	96.9 ± 3.2
Lysozyme:PVA:trehalose 5:0.5:5.5	100.4 ± 2.2
Lysozyme:PVA:trehalose 5:1:6	97.1 ± 3.7
Lysozyme:PVA:trehalose 5:2:7	97.3 ± 4.8
Lysozyme:PVA:trehalose 1:1:2	95.9 ± 3.0
Catalase alone	54.4 ± 4.1
Catalase:sucrose 1:1	92.3 ± 2.8
Catalase:trehalose 1:1	93.38 ± 2.1
Catalase:PVA:trehalose 5:1:6	99.8 ± 2.8

2.10. Statistical analysis

All data are expressed as means ± standard deviations (S.D.). Statistical analysis was performed using a Kruskal–Wallis test, Mann–Whitney test or Student's *t*-test (Minitab, Minitab Inc., CA, USA). In all cases, *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. Relative biological activity preservation after spray-drying

The biological activity of the spray-dried enzymes (measured immediately after spray-drying) prior to suspension in HFA propellant is shown in Table 1. Lysozyme, spray-dried alone lost just over 10% of its original biological activity. However, the incorporation of either sucrose, trehalose or a combination of trehalose and polyvinyl alcohol with lysozyme preserved approximately 100% of the enzyme's biological activity. Catalase lost 45% of its biological activity upon spray-drying, but this reduction in activity was reduced to approximately 7% when either sucrose or trehalose was used to stabilise the enzyme during the manufacture process. Catalase retained almost full activity when it was stabilised using a mixture of PVA-trehalose. Spray-dried catalase with or without trehalose (at a sugar to enzyme mass ratio 1:1) was found to have ΔH_m values of 26.8 and 9.2 J/g, which was approximately 95.0 and 32.6%, respectively of the ΔH_m of the native structure (28.2 J/g). Such results suggested that the native structure of the enzymes was preserved by the presence of stabiliser during spray-drying. The stabilisation of lysozyme and catalase conferred by excipients during spray-drying and the use of ΔH_m to determine protein denaturation has been reported previously (Liao et al., 2002; Quader et al., 2002).

3.2. The effect of HFA on the biological activity of spray-dried enzymes during storage

Spray-dried lysozyme, either alone or stabilised using a number of different excipients including sucrose, trehalose, PVA and mixtures of trehalose and PVA displayed no detectable reduction in biological activity after being stored within a HFA based-pMDI canister for up to 12 weeks (Table 2). Due to a lack

Table 2
The retained biological activity of HFA based pMDI-formulated lysozyme and catalase particles relative to the corresponding control powders after storage for 12 weeks at room temperature ($n = 1$ or 3)

Formulation	Activity of pMDI-formulated enzyme at week-1 (%)	Activity of pMDI-formulated enzyme at week-12 (%)
Lysozyme alone	99.0	98.1
Lysozyme: trehalose 1:1	96.2 ± 4.8	98.1 ± 10.0
Lysozyme:PVA:trehalose 5:0.5:5.5	96.8	100.0
Lysozyme:PVA:trehalose 5:1:6	102.9	95.3
Lysozyme:PVA:trehalose 5:2:7	98.9	99.0
Lysozyme:PVA:trehalose 1:1:2	97.6	93.0
Catalase alone	98.9 ± 2.7	20.8 ± 6.8
Catalase: trehalose 1:1	100.5 ± 0.8	101.3 ± 12.3
Catalase:PVA:trehalose 5:1:6	101.1 ± 4.7	98.2 ± 1.8

of available spray-dried protein, only a single biological activity measurement was performed for several of the MDI formulations and therefore it is difficult to make statistical comparisons across this data set. However, it was apparent even from this limited data set that lysozyme was a very robust protein that did not loose its biological activity either during spray drying or suspension within HFA propellants. Only the lysozyme and sugar combinations were tested beyond 13 weeks i.e. up to 26 weeks and in a similar manner there was no change in the biological activity of the protein over this extended time period, which again confirms our previous findings (Fig. 1).

The biological activity of catalase when spray-dried alone was reduced from ∼100 to ∼20% during 12 weeks of storage in HFA (Table 2). However, a similar reduction in biological activity was not observed when catalase was stabilised with sucrose, trehalose or the combination of trehalose and PVA. Overall, no significant change in the biological activity of lysozyme or catalase occurred over 26 weeks in the presence of either sucrose or trehalose as a function of storage (Fig. 1, $p > 0.05$, Kruskal–Wallis test).

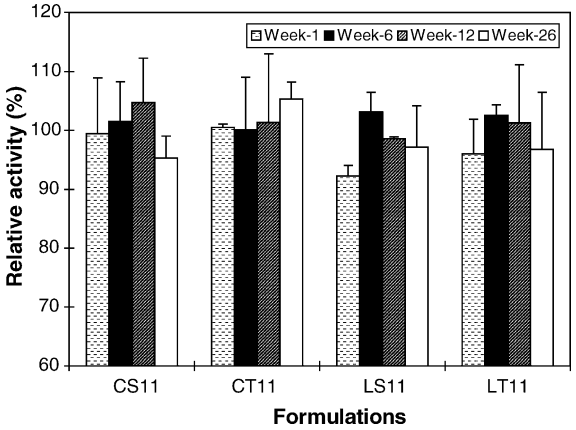

Fig. 1. The retained biological activity of HFA-based pMDI formulations of lysozyme and catalase particles relative to the corresponding control powders (which were the corresponding spray-dried enzyme powder as stored in the freezer without being dispersed in HFA), after storage up to 26 weeks at room temperature (mean ± S.D., $n = 3$). CS11, CT11, LS11 and LT11 represent spray-dried catalase:sucrose 1:1, catalase:trehalose 1:1, lysozyme:sucrose 1:1 and lysozyme:trehalose 1:1 particles, respectively.

3.3. Particle size and morphology of spray-dried particles

The scanning electron microscopy (SEM) results demonstrated that the morphology of spray-dried particles appeared to be similar regardless of the type and quantities of excipents used to stabilise the protein. Due to the lack of discrimination between the microparticles although all the spray dried lysosyme and catalase particles were analysed using SEM only two representative images are shown in Fig. 2. Spray-dried lysozyme (with or without excipient) presented smooth surfaces containing characteristic dimples, typical of a spray-dried material.

The particle size and size distribution of the spray-dried protein particles are shown in Table 3. The volume median diameters (VMD) of all spray-dried batches were found to be between 2.48 and 3.43 μm.

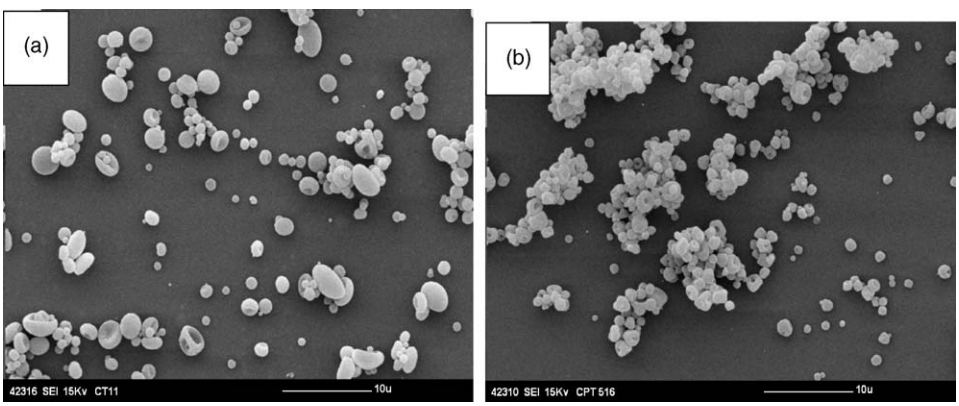

Fig. 2. Scanning electron micrographs of spray-dried (a) catalase in the presence of trehalose at a mass ratio 1:1 and (b) catalase in the presence of PVA-trehalose at a mass ratio 5:1:6.

Table 3
Particle size and distribution of spray-dried lysozyme and catalase formulations

Formulation	Volume median diameter (μm)	Span
Lysozyme alone	3.09	0.77
Lysozyme:sucrose 1:1	3.43	0.92
Lysozyme:trehalose 1:1	3.31	0.91
Lysozyme:PVA:trehalose 5:0.5:5.5	2.48	1.15
Lysozyme:PVA:trehalose 5:1:6	2.67	1.03
Lysozyme:PVA:trehalose 5:2:7	2.78	1.18
Lysozyme:PVA:trehalose 1:1:2	2.89	1.13
Catalase alone	3.12	1.18
Catalase:sucrose 1:1	2.95	1.04
Catalase:trehalose 1:1	2.96	1.15
Catalase:PVA:trehalose 5:1:6	2.77	1.13

In addition, the particle size span for these batches lay between 0.77 and 1.18, which indicates that the manufacturing method generated particles within a narrow size distribution.

3.4. Deposition of pMDI-formulated enzyme particles in the absence of PVA

The mean dose of protein delivered through the valve from four formulations, lysozyme:sucrose, 1:1 (LS 1:1), lysosyme: trehalose 1:1 (LT 1:1), catalase:sucrose (CS 1:1), catalase:trehalose (CT 1:1) each of which comprised three independent samples, was found to be between 1395 and 1524 μg per 20 actuations. The coefficients of variation between samples of each formulation and between formulations were less than 10% (data not shown). Such results indicated that the uniformity of the pMDI formulations was satisfactory and was independent of formulation. The recovery (relative to the theoretical dose) of protein from the four formulations was found to range from 94.8 to 101.6% with a mean of $97.4 \pm 6.8\%$ ($n = 12$). As a result, the emitted dose appeared to represent the theoretical dose.

The physical stability of pMDI formulations was primarily evaluated in terms of changes in fine particle fraction as a function of storage time. Only the protein formulations containing stabilisers were assessed using the twin-stage impinger as the biological activity measurements for all these systems, irrespective of the protein or stabiliser employed, was shown to be ca. 100%. For formulations prepared using spray-dried lysozyme in the presence of either sucrose or trehalose, the aerodynamic properties of the resultant pMDI formulations were significantly affected by storage ($p < 0.05$, Kruskal–Wallis test, Fig. 3a and b). Analysis at week 1 determined the stage 2 fraction of the lysozyme:sucrose 1:1 pMDI as 27.2% (recovered dose) whilst the fractions recovered from the device and stage 1 were 21.7 and 51.3%, respectively. After 6 weeks storage at room temperature, the fraction depositing in stage 2 decreased significantly to approximately 8%, however with further storage for up to 26 weeks, there appeared to be no further reduction in the stage 2 fraction. The pMDI formulated lysozyme:trehalose 1:1 particles displayed a similar aerodynamic performance to those emitted from the lysozyme:sucrose 1:1 formulation at the first week after preparation. However, the storage suspension stability of the former proved to be significantly better than the latter ($p < 0.05$, paired Student *t*-test). Nonetheless, the fine particle fraction (stage 2 fraction) of trehalose-based pMDI formulation was susceptible to decrease as a function of storage time and at 26 weeks, the fine particle fraction decreased to 12.7% whilst the stage 1 fraction increased to 61.4% (Fig. 3b).

The fine particle fractions generated by the spray dried catalase particles in the presence of either sucrose or trehalose appeared to significantly decrease as a function of storage time ($p < 0.05$, Kruskal–Wallis test, Fig. 3c and d). The formulation, which incorporated trehalose, was found to deposit a higher fine particle fraction in stage 2 after 6–26 weeks of storage than the similar formulation containing sucrose. For example, after 26 weeks storage at room temperature, the stage 2 fraction of the pMDI formulation containing sucrose was 6.0%, relative to the 18.7% recovered from the pMDI formulated using trehalose. The reductions in the fine particle fractions were compensated by increases in the stage 1 fractions, whilst the device fractions were consistently found to be approximately 20% of the recovered dose and independent of formulation and storage time.

3.5. Deposition of pMDI-formulated enzyme particles in the presence of PVA

Combining lysozyme with both PVA and trehalose during the spray-drying process produced microparticles that when suspended in HFA to produce pMDI

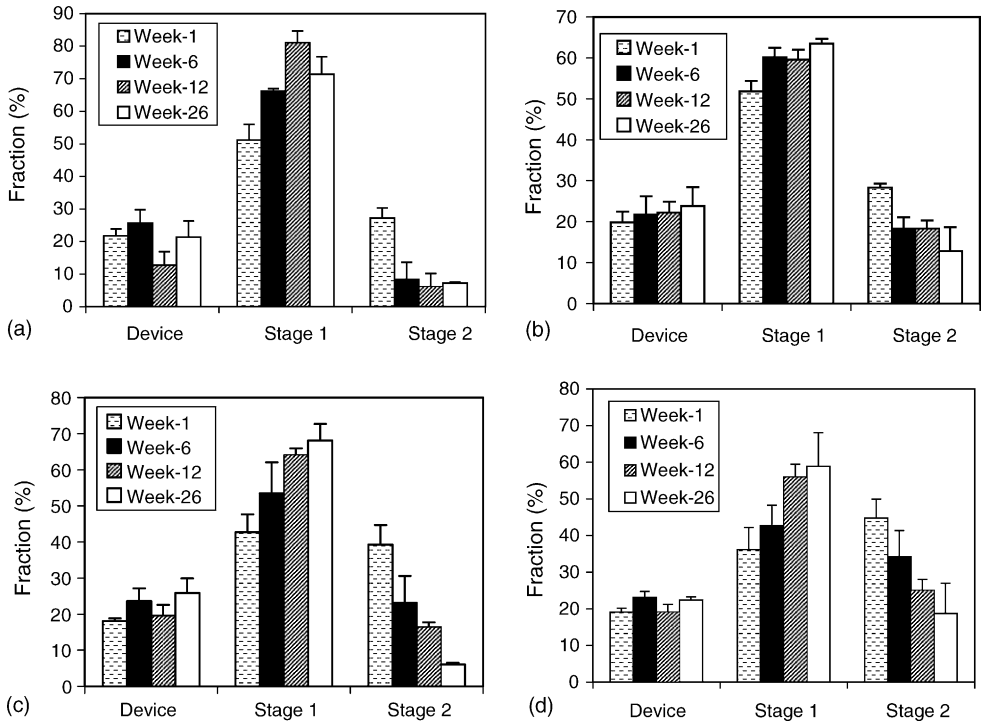

Fig. 3. The aerosol performance of HFA based pMDI-formulated spray-dried (a) lysozyme:sucrose 1:1, (b) lysozyme:trehalose 1:1, (c) catalase:sucrose 1:1 and (d) catalase:trehalose 1:1 particles as a function of storage time, evaluated by a twin stage impinger (mean ± S.D., $n = 3$).

formulations appeared to have significantly better aerosol performance, indicated by a higher fine particle fraction than those formulated using either trehalose or sucrose alone ($p < 0.05$, Mann–Whitney test, Fig. 3a and b). The fine particle fraction of pMDI formulations of lysozyme containing PVA were found to range from 47.1 to 52.7% during the first week of preparation (Fig. 4a). However, the stability of the aerodynamic properties was found to independent of the PVA content in the spray-dried particles (Fig. 5). For example, although after storage for 12 weeks at room temperature, the formulation (lysozyme:PVA:trehalose 5:0.5:5.5) containing the lowest PVA content was found to produce a slightly

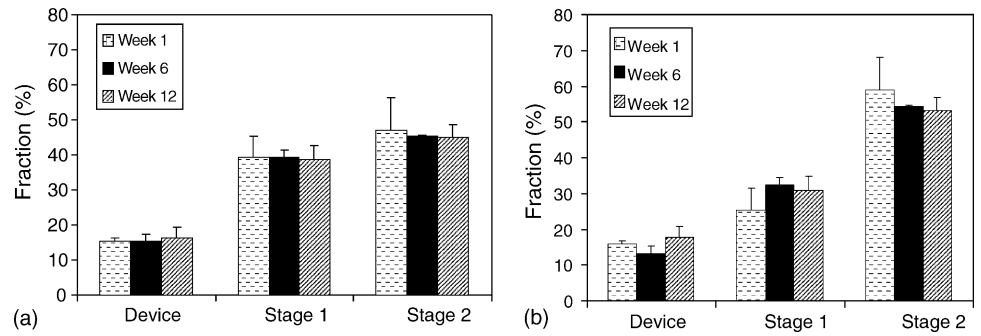

Fig. 4. The aerosol performance of HFA based pMDI-formulated spray-dried (a) lysozyme:trehalose:PVA 5:1:6 and (b) catalase:trehalose:PVA 5:1:6 particles as a function of storage time, evaluated by a twin stage impinger (mean ± S.D., $n = 3$).

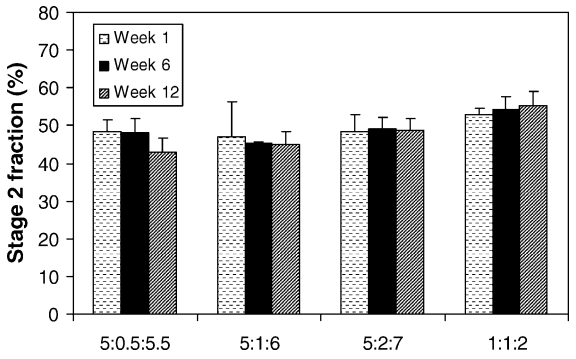

Fig. 5. The aerosol performance of HFA based pMDI-formulated spray-dried lysozyme:trehalose:PVA formulated at various relative weight:weight ratios.

decreased fine particle fraction, 42.8%, in comparison to the 48.3%, obtained during week-1 this apparent reduction was not statistically significant ($p > 0.05$, ANOVA). Furthermore, all the PVA containing pMDI formulations displayed a significantly better storage suspension stability in terms of fine particle fraction than either of the pMDI formulations containing lysozyme:sucrose 1:1 or lysozyme:trehalose 1:1 alone ($p < 0.05$ Mann–Whitney test).

The fine particle fraction of the PVA-containing catalase pMDI formulation was found to be 58.9% (Fig. 4b), which was significantly higher than that of the pMDI formulated catalase:sucrose 1:1 or catalase:trehalose 1:1 particles (Fig. 3c and d, $p < 0.05$, Mann–Whitney test). The device and stage 1 fractions from the PVA-containing catalase formulations accounted for only 15.9 and 25.2% of the recovered does respectively, as evaluated during the first week after preparation (Fig. 4b). After storage for 6 weeks at room temperature, a slight decrease in fine particle fraction was found. However, after storage for a further 6 weeks, the recovered fine particle fraction appeared to be the same and the effect of storage on the fine particle fraction appeared to be insignificant ($p > 0.05$, Kruskal–Wallis test). When catalase was spray-dried in the presence of a PVA-trehalose mixture, the resultant pMDI formulations appeared to always display a markedly better aerosol performance in comparison to the pMDI formulated catalase:sucrose 1:1 or catalase:trehalose 1:1 particles during storage ($p < 0.05$, Mann–Whitney test) as shown in Figs. 3c and d and 4b.

4. Discussion

In this study, spray-dried lysozyme with or without additional excipients was found to be biologically stable in HFA 134a contained within a pMDI for up to 26 weeks. Such results were broadly in agreement with a previous study, which reported that the secondary structure of lysozyme underwent no change after dispersal in HFA propellants, although the effects of storage time on structure conformation were not investigated in the earlier investigation (Quinn et al., 1999). In addition, the results of the current study showed that spray-dried catalase, in the presence of stabiliser(s), was stable in the propellant upon storage. Therefore, these results indicate that HFA propellants are compatible with these proteins when the latter are present in a particulate form. However, spray-dried catalase without stabiliser was found to undergo rapid inactivation after dispersal of the protein in HFA 134a. The inactivating effect might be attributable to the unfolding of the protein. Indeed, the spray-dried catalase alone appeared to be considerably denatured, as indicated by the change in melting enthalpy (ΔH_m), obtained by DSC. The denaturing of proteins leads to the exposure of the reactive side chains such as hydrophobic groups, which might be expected to increase the rate of aggregation, degradation and inactivation (Manning et al., 1989). The present results support the hypothesis reported previously (Carpenter et al., 1999), which states that to maintain the protein stability in formulations (in the present study, the biological activity of pMDI-formulated proteins), the native structure is required to be intact.

It is thought that sugars stabilise proteins during rapid dehydration through either vitrification or water substitution mechanisms. Water substitution involves the formation of hydrogen bonds between the sugar and protein, which is believed to be responsible for the inhibition of the unfolding of the proteins, whereas vitrification depends upon the immobilisation of protein molecules that accompanies glass formation (Arakawa et al., 1993; Carpenter et al., 1999). The vitrification mechanism is kinetic in origin but, the water substitution hypothesis describes a thermodynamic mechanism, dependent upon the free energy of unfolding. Although the exact mechanism of trehalose stabilisation was not identified in this work it was shown that the sugar was able to conserve the biological activ-

ity of the proteins both during spray-drying and suspension in HFA propellants. However, in addition to heat-induced dehydration, the process of spray-drying exposes macromolecules to high shear and rapidly forming air-water interfaces. Whilst trehalose is known to protect DNase I against the removal of water, there is little evidence that it is effective in protecting therapeutic agents against the surface effects caused by atomization. Simply measuring the biological activity of proteins may not determine such effects and further work should go onto investigate these effects in more detail.

Without the inclusion of PVA in the spray-dried enzyme particles, the resultant pMDI suspensions delivered a low fine particle fraction. This poor aerosol performance is likely to be attributable to differences in the physicochemical properties of the suspended enzyme particles within the HFA propellant. The protein/sugar microparticles probably formed aggregates within the pMDI which were not re-dispersed upon dose actuation. Thus, whilst the combination of the proteins with a sugar may have conferred significant advantages in terms retaining functional integrity during spray-drying they did not facilitate the production of a physically stable suspension of the enzyme in HFA propellant.

The enhanced suspension stability conferred by the inclusion of PVA presumably involves the modification of the surface composition of spray-dried particles such that the interparticle interactions favouring particle agglomeration were reduced. The effects of excipients on the surface composition of spray-dried protein particles have been reported to depend upon the differences between the adsorptivity of proteins and excipients at the air-water interfaces of the droplets (Landstrom et al., 2000; Millqvist-Fureby et al., 1999). It is possible that the PVA, a polymeric surfactant, preferentially adsorbed at the surface of the droplets during the spray-drying procedure, displacing protein and/or sugars from the surface of the resultant particles. Previously, Tarara et al. (2004) reported that coating budesonide microcrystals with surfactants (lipid) resulted in the enhanced suspension stability. Therefore, when the formulated protein particles are dispersed in HFA, the PVA might by steric stabilisation, reduce interparticle attractions, which might otherwise be expected to decrease the physical stability of pMDI suspensions. However, adsorption at the air-liquid interface within a droplet is dependent upon both diffusion and convection. Whilst the PVA used in this work is significantly smaller than either catalase or lysosyme and should therefore diffuse to the droplet surface more rapidly, this is based on the assumption that the two molecules do not interact. In addition, both compounds are macromolecules containing a number of discrete confirmations and it is difficult to predict if certain functionalities within the molecules will adsorb to the interface preferentially. Although using current analytical technology it is difficult to determine the interactions within a dynamic environment such as a spray-drier's spray-plume, further work, perhaps using a model system would be beneficial to truly understand the effects of such a process on complex macromolecules such as proteins.

5. Conclusion

During microparticulate fabrication methods such as spray-drying, peptides and proteins can be susceptible to inactivation. However, the different susceptibility of the two model proteins in this study indicated that the extent of inactivation is molecule specific. In the present study, the spray-dried protein particles were found to display different physical characteristics when suspended within HFA pMDIs. The aerosolisation performance and thus, the suspension stability was shown to be dependent upon the composition of the protein microparticles. Those formulations containing PVA were found to physically stabilise suspensions of the test proteins and limit the potential for irreversible aggregation more effectively than those that did not contain the polymer. In addition the biological activity of the protein was preserved for a minimum of 12 weeks when PVA and a sugar was used in combination regardless of the type of protein or relative quantities of excipients. Importantly, PVA conferred excellent suspension stability and aerosolisation performance without the requirement for the inclusion of surfactants. This may be an additional advantage for pMDI formulations since surfactants, which are usually presented as dissolved excipients can lead to the production of aerosolised aggregates during the evaporation of sprayed droplets. In particular, such detrimental effects can increase with increasing suspension concentration.

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