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ORIGINAL RESEARCH PAPER

Freeze drying formulation using microscale and design of experiment approaches: a case study using granulocyte colony-stimulating factor

Yitzchak Grant · Paul Matejtschuk · Christopher Bird · Meenu Wadhwa · Paul A. Dalby

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Abstract The lyophilization of proteins in microplates, to assess and optimise formulations rapidly, has been applied for the first time to a therapeutic protein and, in particular, one that requires a cell-based biological assay, in order to demonstrate the broader usefulness of the approach. Factorial design of experiment methods were combined with lyophilization in microplates to identify optimum formulations that stabilised granulocyte colony-stimulating factor during freeze drying. An initial screen rapidly identified key excipients and potential interactions, which was then followed by a central composite face designed optimisation experiment. Human serum albumin and Tween 20 had significant effects on maintaining protein stability. As previously, the optimum formulation was then freeze-dried in stoppered vials to verify that the microscale data is relevant to pilot scales. However, to validate the approach further, the selected formulation was also assessed for solid-state shelf-life through the use of accelerated stability studies. This approach allows for a high-throughput assessment of excipient options early on in product development, while also reducing costs in terms of time and quantity of materials required.

Keywords Freeze-drying · Granulocyte colony-stimulating factor · Lyophilization · Microplates · Ultra scale-down

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Y. Grant · P. A. Dalby (✉)
Department of Biochemical Engineering, The Advanced Centre for Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK
e-mail: p.dalby@ucl.ac.uk

Y. Grant
e-mail: yitzchak.grant@kraftfoods.com

P. Matejtschuk
Standardisation Science Section, National Institute for Biological Standards & Control, Health Protection Agency, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK
e-mail: paul.matejtschuk@nibsc.hpa.org.uk

C. Bird · M. Wadhwa
Biotherapeutics Group, National Institute for Biological Standards & Control, Health Protection Agency, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK

C. Bird
e-mail: chris.bird@nibsc.hpa.org.uk

M. Wadhwa
e-mail: meenu.wadhwa@nibsc.hpa.org.uk

Introduction

Therapeutic proteins are available for the treatment of a wide range of diseases from cancers to rheumatoid

arthritis. These proteins are either available in a liquid formulation or as a lyophilised powder. The formulation of proteins is highly challenging as these are complex molecules, susceptible to chemical and physical degradation, which can result in diminished bioactivity and can also elicit serious immunological reactions. Therefore, stable lyophilised formulations that could preserve proteins at room temperature and avoid the need for cold storage are advantageous.

There is a need for a systematic approach to formulation development involving a deeper scientific understanding of the interaction between the protein and the excipients during lyophilization (Carpenter et al. 1997; Chang et al. 2005). Microscale approaches are powerful tools for assessment of protein properties such as stability (Aucamp et al. 2005), and rapid process optimisation, such as formulation for freeze-drying (Grant et al. 2009). The small volumes allow large numbers of excipients and their combinations to be screened while minimising the quantity of biological materials required. An alternative to microplates in freeze-drying is the use of V-well plates (Von Graberg and Gieseler 2006), or the commercially available VirTis microtube format (Genevac Ltd, Ipswich, UK) which use a customised metal plate that improves the thermal contact between the sample and the freeze-drier shelf. However, unlike the V-well plates, the microplate format provides wells with a cylindrical shape and flat base which closely mimics the geometry of glass vials.

While microplates can minimise the sample requirements during freeze-drying optimisation, to ensure that the protein retains its biological activity, monitoring of biotherapeutic proteins using biological functional assays is required. Here, we demonstrate that the microscale approach can be used for the rapid design of freeze-drying formulations of therapeutic proteins, in this case human granulocyte colony-stimulating factor (G-CSF), the non-glycosylated form of which has poor stability on lyophilization and at physiological pH (Carter et al. 2004).

Human G-CSF, manufactured recombinantly as a non-glycosylated form in *Escherichia coli* (international non-proprietary name, Filgrastim) or as a glycosylated form using CHO cells (Lenograstim), stimulates the proliferation of neutrophils and is licensed for several indications relating to neutropenia (Wadhwa and Thorpe 2008). G-CSF is a monomeric cytokine consisting of a four α-helical bundle (Brems 2002) and whose conformational stability in solution is greatest at pH 4.0, but which can aggregate within days at pH 7.0 (Raso et al. 2005). The current formulation for Filgrastim is in 10 mM sodium acetate pH 4.0 buffer with 5% (w/v) mannitol and 0.004% Tween 80 in which it is stable for over two years at 2–8°C (Herman et al. 1996).

Materials and methods

Materials

Trehalose, phenylalanine, RPMI-1640 media, sodium phosphate (mono- and di-basic) and L-histidine were from Sigma-Aldrich. Mannitol and arginine were purchased from VWR, sucrose from FSA (Loughborough, UK), Tween 20 from Bio-Rad Laboratories and human serum albumin (20% w/w) (HSA) was from Grifols (Barcelona, Spain). Tritiated thymidine (thymidine [methyl-^{3}H] 1 mCi (37 MBq)/ml) was purchased from Perkin Elmer (Boston, MA, USA). Bacterially expressed recombinant G-CSF was generously donated to NIBSC as a solution at 1 mg/ml (pH 4.0) for research/evaluation purposes by the Serum Institute of India Ltd (Pune, India).

G-CSF freeze-drying in microplates

A fractional factorial design (Design Expert 7 Software, Stat Ease, Minneapolis, MN, USA) was used to screen the impact of seven excipients and two buffer types on the preservation of G-CSF during freeze drying. Each combination of excipients and buffers, was prepared at 200 µl in different wells of a 96-well microplate (Greiner, Stonehouse, UK), with 100 ng G-CSF/ml. Due to the edge effects observed previously for sublimation (Grant et al. 2009), all outside edge wells were filled only with buffer. Microplates had the bottom lips trimmed off to place the well bottoms in direct contact with the freeze dryer shelves (Virtis Genesis 25EL, Biopharma Process Systems, Winchester, UK), and the samples freeze-dried with primary drying at −40°C for 10 h (75 mTorr) and secondary drying at 20°C (5 h at the same vacuum). Samples were then resuspended in PBS and assayed for biological activity. Subsequent optimisation experiments were developed in Design Expert using random surface modelling to produce a central composite

faced design with each factor investigated at three levels using the microplate method described above.

GNFS-60 cell-based bioassay for G-CSF activity

The biological activity of G-CSF was determined using a bioassay based on the G-NFS-60 cell line which proliferates in response to G-CSF (Wadhwa et al. 2000). Briefly, a doubling dilution series (ranging from 1,000 pg/ml to 7.8 pg/ml) of the various G-CSF samples (lyophilized samples and unlyophilized G-CSF control stored in the supplied formulation) and the WHO International Standard for G-CSF (88/502) were prepared in 100 µl volumes in 96-well microtitre plates. Exponentially-growing G-NFS-60 cells were washed three times, resuspended to a concentration of 10^5/ml in RPMI 1640 containing 5% (v/v) FCS and 100 µl was added to each well. The plates were incubated for 48 h, pulsed for 4 h with ^{3}H-thymidine (0.5 µCi/well), harvested and the radioactivity incorporated into DNA estimated by scintillation counting using Microbeta Trilux (Perkin Elmer). Data was plotted and parallel line analysis conducted to calculate the relative potency of the tested samples relative to the untreated G-CSF control samples.

Freeze-drying in vials

Granulocyte colony-stimulating factor samples were formulated to a 1 ml fill-volume in 22 mm diam. 10 ml brim-fill volume stoppered Type I glass vials (Adelphi Tubes, Haywards Heath, UK) part-stoppered with 20 mm diam. cruciform halobutyl closures and lyophilized using −35°C as the primary drying (15 h) and +20°C as the secondary drying conditions (9 h), the slightly extended times ensured each step reached completion on scale up.

Stability of liquid formulated G-CSF

Three formulations of G-CSF were prepared as a 1 ml fill in standard stoppered vials and incubated at 22°C for the period of the experiment. For set 1 ("freeze-dried"), 100 ng G-CSF/ml was prepared in 0.28% (v/v) Tween 20, 3.5% (w/v) HSA and PBS, lyophilized and then duplicate sets each resuspended in water on days 0, 2, 4 and 7 (to give 0, 3, 5, 7 days in resuspension). For set 2 ("liquid formulated"), the formulation was prepared as set 1 but without the G-CSF which was then spiked to 100 ng G-CSF/ml into duplicate samples on days 0, 2, 4 and 7. Set 3 ("unformulated liquid") was prepared as set 2, except the G-CSF was spiked into PBS only. On day 7, all samples were assayed as above for activity.

Accelerated degradation study of lyophilised G-CSF formulations

Formulated G-CSF [100 ng/ml in 0.28% (v/v) Tween 20 and 3.5% (w/v) HSA in PBS] was lyophilized as above and stored in controlled temperature cabinets at −20, 4, 20, 37, 45 or 56°C. Vials were assayed after 4 and 6 months alongside a lyophilized control stored at −70°C. The fraction of activity lost was used to estimate the rate of product loss (v), using the Arrhenius equation and assuming first order decay (Kirkwood 1977; Kirkwood and Tydeman 1984).

Results

G-CSF freeze drying and activity measurement in microplates

Seven different excipient and two buffers, selected based upon the reviews of Wang (2000); and Constantino (2004), were screened in a fractional factorial design with each constituent varied at two levels, to rapidly identify those which had a positive effect in maintaining the biological activity of G-CSF during lyophilization. These included trehalose (0–2% w/v), human serum albumin, HSA (0–2% w/v), mannitol (0–0.5% w/v), sucrose (0–2% w/v), Tween 20 (0–0.1% v/v), arginine (0–0.5% w/v) and phenylalanine (0–0.5% w/v) (Supplementary Table S1), selected for their common use as cryoprotectants or lyoprotectants. As a buffer choice, 30 mM histidine, pH 7.0 or 50 mM sodium phosphate, pH 7.0 were selected since they are commonly used in liquid and freeze-dried protein formulations (Constantino 2004). Twenty runs were required for the initial fractional factorial design which included four centre-point repeats (Supplementary Table 1). A half-normal plot of actual data versus predicted data identified the most significant factors and interactions (Fig. 1). The prediction assumes that all factors are not significant

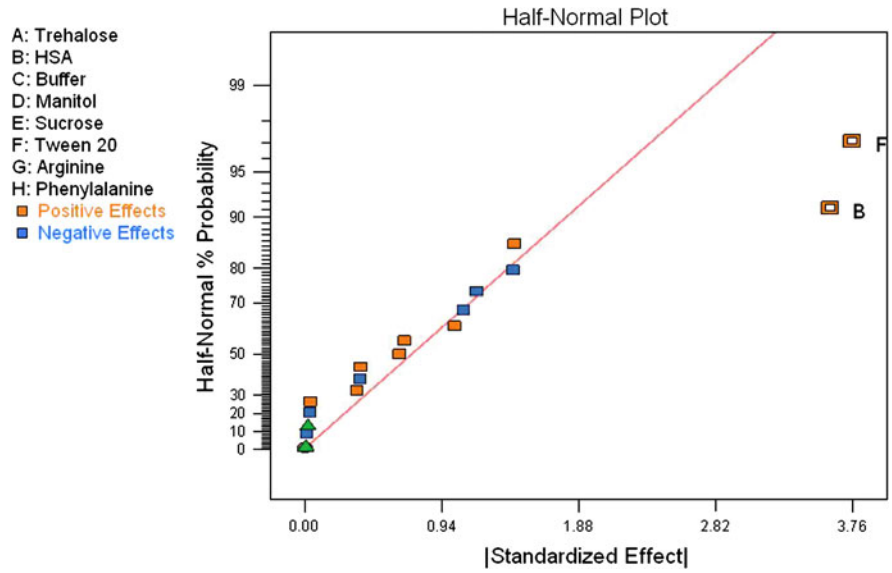

Fig. 1 Half-normal plot of the initial fractional factorial screen data. Each point represents a factor under investigation and any point not on the *straight line* signifies a significant factor

where the outcomes are expected to give a normal distribution around zero. Deviations from this normal distribution (the straight line) therefore represent significant effects.

Figure 1 shows that only two factors, HSA and Tween 20, significantly influenced the preservation of biological activity of G-CSF during the freeze drying. A model was built with the DoE software from the fractional factorial results and then assessed for its statistical significance using ANOVA. The model gave a *P*-value less than 0.0001 and an *F*-value of almost 24 indicating a highly significant fit. This initial model predicted the greatest preservation of activity at 2% (w/v) HSA and 0.1% (v/v) Tween 20, but the resultant plot (not shown) suggested that an optimum had not yet been found and so the ranges were increased up to 5% w/v HSA and 0.5% v/v Tween 20, for an experimental optimisation.

Optimisation of G-CSF formulation from excipient screen

An optimisation experiment was developed in Design Expert using random surface modelling to produce a central composite faced design using Tween 20 and HSA, each investigated at three levels (Supplementary Table 2), and also two different buffer types. Current commercial liquid formulations of G-CSF are typically buffered at pH 4 so 10 mM acetate, pH 4 was studied for comparison as the second buffer category.

Due to the categorical nature of pH as a factor, the optimisation results were plotted separately for demineralised water, pH 7 (Fig. 2a) and acetate, pH 4 (Fig. 2b). The response curve modelled data in Fig. 2 confirmed the results of the initial screening investigation and also identified that the optimum region existed outside the initially screened range of concentrations for HSA and Tween 20. Excess reagents did not continue to increase the retained activity and so their optimal concentrations were limited to within the region delineated by the 100% contour shown. An optimum formulation of 0.1 µg G-CSF/ml in water (pH 7) was at 0.3% (v/v) Tween 20 and 3.5% (w/w) HSA. Interestingly, given that G-CSF is most stable in liquid forms at pH 4, the optimum formulation of 0.1 µg G-CSF/ml in acetate, pH 4, was 0.17% (v/v) Tween 20 and 3.5% (w/v) HSA, but the maximum recovered activity was only 70% that observed for the optimum at pH 7.

Verification of optimal freeze-drying formulation in vials

Following identification of the optimal region of operation for the formulation in microscale trials, it was necessary to confirm that the predictions made by the model remained true during scale up to the types of vials typically used in manufacturing. Three formulations [(0.22% (v/v) Tween 20, 2.6% (w/v) HSA in water, pH 7; 0.28% (v/v) Tween 20, 3.5% (w/v) HSA

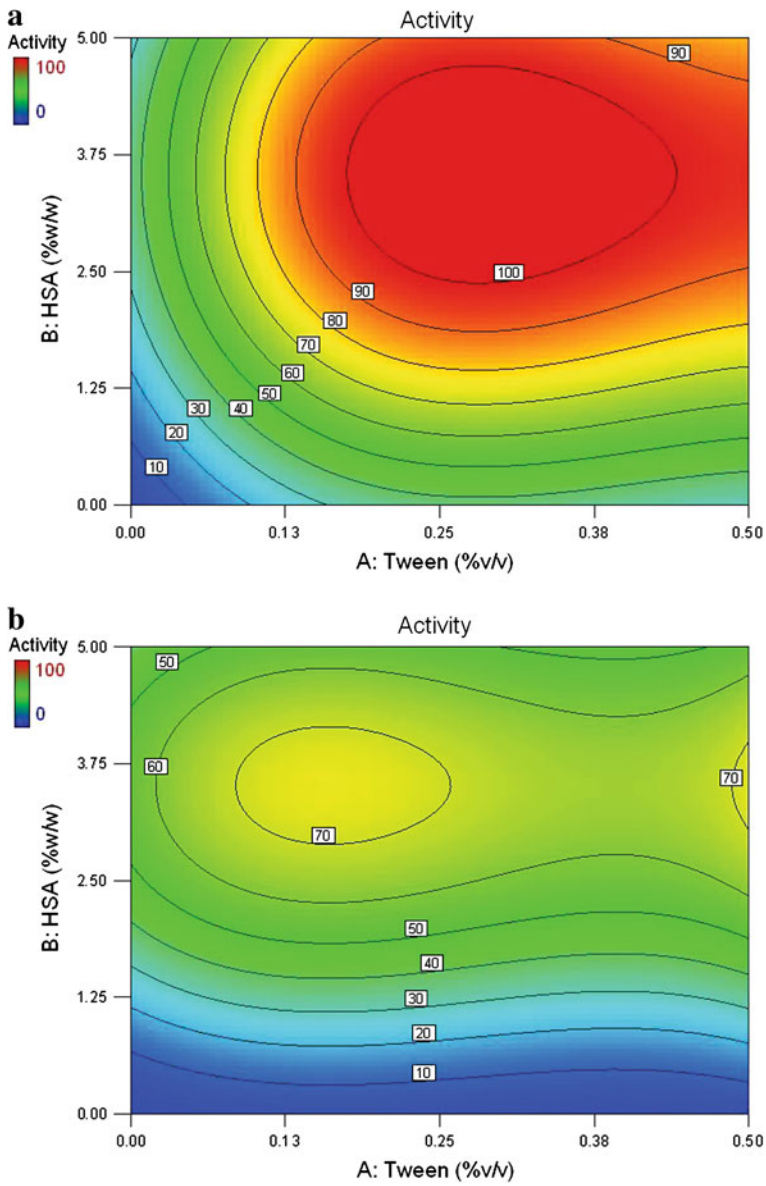

Fig. 2 Contour graph showing the effect of varying Tween 20 and HSA on the retention of G-CSF biological activity in water at **a** pH 7 and **b** in acetate buffer at pH 4

in water, pH 7; and 0.17% (v/v) Tween 20, 3.5% (w/v) HSA in 10 mM acetate, pH 4)] at 1 ml fill-volume in 10 ml stoppered vials were lyophilized using the cycle described previously (Supplementary Table 1). As shown in Table 1, the predicted values closely matched those of the experimental values obtained in stoppered-vials, thus demonstrating the high level of accuracy for the model. The experimental results were all well within the confidence interval ranges, and given that the variability between different runs of the bioassay was often up to 20%, were reasonably accurate. To further investigate that no additional buffer was necessary in this system, a comparison was made between two identical formulations: 0.33% (v/v) Tween 20, 2% (w/v) HSA in 30 mM histidine, pH 7; and 0.33% (v/v) Tween 20, 2% (w/v) HSA in water, pH 7. The relative activities retained for samples resuspended after lyophilization, obtained from five repeat vials of each formulation, were 110 ± 8% and 108 ± 7% for water and histidine buffer, respectively. This confirmed that there was no requirement for a buffer in addition to the excipients present, and that the samples, which are at pH 7, must be sufficiently self-buffering at this concentration of G-CSF.

Table 1 Comparison of activities of G-CSF freeze-dried in 10 ml vials to those predicted from DoE at microscale

Run	Tween	HSA	pH	Activity (%)	
				10 ml vial scale	Microscale DoE predicted
1	0.22	2.64	7	99.9	100 (81–121)[a]
2	0.28	3.54	7	97.3	109 (84–137)
3	0.17	3.51	4	75.6	73.5 (65–74)

[a] ±95% confidence intervals are given in parentheses

Efficacy of formulation at higher G-CSF concentrations

Since all studies had been performed at low (0.1 μg/ml) G-CSF concentration (as this was a concentration more typical for the development of a biological reference material), we investigated the effect of higher G-CSF concentrations which are more representative of therapeutic formulations. G-CSF was prepared in the unbuffered 0.33% (v/v) Tween 20, 2% (w/v) HSA formulation at concentrations of 1, 10, 100, 250 and 500 μg/ml with a 1 ml fill-volume dispensed into 10 ml stoppered vials. The drying cycle was applied, then the samples resuspended and assayed as above.

The retained activities remained constant at approx. 80% within limit of error, as G-CSF was increased to 100 μg/ml. However, as G-CSF was increased further to 500 μg/ml, the retained biological activity decreased slightly to just below 70% (data not shown).

Effect of optimum formulation conditions on activity assays

It was important to verify that the formulation was not having a spurious stabilising effect due to altering the response to the assay itself. It is unlikely that at the low concentrations present Tween 20 [present at 0.003% (v/v) in the first and 0.0004% (v/v) in the final well] or HSA [present at 0.035% (w/v) in the first and 0.0044% (w/v) in the final well] could have an effect on cell growth. However, although present in low concentrations in the assay media, the ratio of HSA and Tween 20 to hG-CSF remains constant throughout the dilutions. Therefore, any protection interaction mechanism that exists may continue to act throughout the assay. Furthermore, the presence of excipients may have protected the G-CSF from rapid non-specific binding to the walls of the initially prepared sample vials.

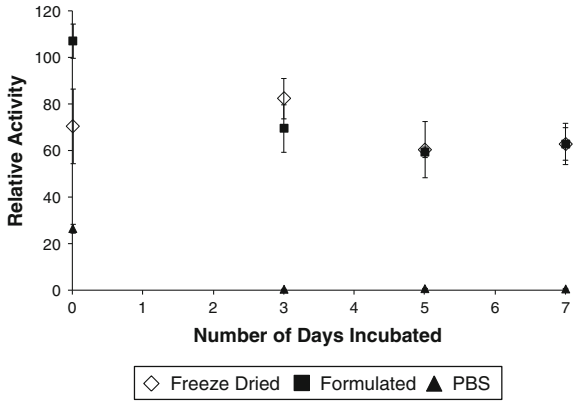

Fig. 3 Data showing biological activity in samples prepared at set intervals (Days 0, 2, 4 and 7). One set (*open diamonds*) were vials containing lyophilized G-CSF in a formulation containing 0.28% Tween 20 and 3.5% HSA in water which were resuspended in PBS (on days 0, 2, 4 and 7). One set (*filled squares*) were vials containing G-CSF at 100 ng/ml in a formulation containing 0.28% (v/v) Tween 20 and 3.5% (w/v) HSA in PBS. These vials were formulated as liquids on days 0, 2, 4 and 7. The final set (*filled upward triangles*) contained G-CSF at 100 ng/ml in PBS and were formulated on days 0, 2, 4 and 7. All vials were incubated at 22°C and were assayed on day 7 relative to G-CSF formulated directly into assay media

Stability of liquid formulated G-CSF

The stability of the formulation in the liquid state was investigated over a period of 7 days stored at ambient temperature. The stability in terms of the relative reactivity in the bioassay was assessed for the formulated material, the freeze dried and reconstituted formulated material and for G-CSF diluted in PBS alone (Fig. 3). G-CSF stored in PBS alone lost activity rapidly whereas the formulated samples maintained a similar level of activity from 3 to 7 days.

Accelerated degradation study of lyophilised G-CSF formulations

Accelerated degradation studies were performed on lyophilized material at a range of temperatures in order to assess their stability. Such studies are widely used to rapidly assess the likely stability of biopharmaceuticals (Kirkwood and Tydeman 1984) when stored over long periods at indicated storage temperatures. Two vials were assayed immediately after

Table 2 Estimated shelf life of 100 ng/ml G-CSF lyophilized in Tween 20 and HSA at pH 7, and stored at various temperatures

Temperature (°C)	Loss per month (%)	Loss per year (%)
−20	0.046	0.547
4	0.322	3.778
20	0.984	11.157
37	2.821	29.06

lyophilization as a baseline for comparison. By converting the data to give an Eyring plot and fitting a linear regression line (not shown), it was possible to estimate the product shelf life at various temperatures. The predicted stability derived from this data (Table 2) shows that at 20°C storage, losses of just over 11% per annum were expected, and at 4°C losses of 3.7% per annum were expected.

Discussion

The current study aimed to show the value of ultra scale-down methods and the use of Design of Experiment (DoE) approaches to rapidly screen and optimise the formulation of a relevant therapeutic protein, G-CSF.

Using a rapid DoE screen, a number of potential excipients were assessed and only HSA and Tween 20 found to have a major impact in this system. A comparison of the freeze dried formulation at pH 4.0 and pH 7.0 showed that contrary to reports demonstrating that G-CSF is more stable in liquid formulation at pH 4 than pH 7 (Narhi et al. 1991) this was not the case during freeze drying of G-CSF at the lower concentrations used in this study. At pH 4, an optimum of approx. 70% of biological activity was obtained with 0.15% (v/v) Tween 20 and 3.5% (w/w) HSA, although >0.5% v/v Tween 20 could also potentially have achieved greater than 70% retention of activity.

Surprisingly, the addition of a buffer did not seem to provide any benefit. It was possible that the HSA provided sufficient buffering to obviate the need for an additional buffer. Clinical grade HSA also contains trace levels of buffer from its preparation (Peters 1996). Its amphiphilic properties also make it suitable as an additive to inhibit adsorption of the protein onto plastic and glass surfaces. This property is important because of the low concentration of G-CSF present in the formulations used here. HSA is also a known lyoprotectant (Hawe and Friess 2006). Tween 20 is commonly added in pharmaceutical formulations as it suppresses the denaturation of proteins at air/liquid interfaces which can occur during fill/finish procedures or through shaking in transit or on resuspension (in the case of lyophilized products). The presence of peroxides in Tween 20 can damage the protein through oxidization of methionine residues, as shown with G-CSF (Herman et al. 1996) although Tween 20 is less prone to this oxidation than Tween 80.

The optimal combination of Tween 20 with HSA, selected by DoE in the microscale experiments at pH 7, was further evaluated at the larger 10 ml vial scale and shown to give both a good recovery of G-CSF activity, and improved stability over several days storage at ambient conditions, both in the liquid state and post-reconstitution of the lyophilized protein. However a decline in biological activity was seen at higher concentrations of G-CSF (above 0.5 mg/ml) indicating that the formulation may not be optimal for these ranges (data not shown).

The impact of the formulants on the recovery of biological activity was also assessed. Loss of potency in the sample formulated in PBS alone may have been due to irreversible binding of the G-CSF at very low protein concentration to the walls of the vessel in which it was formulated. This problem was apparently overcome by the addition of HSA and Tween 20.

Finally, the long term stability of G-CSF freeze dried in the optimal formulation was assessed by accelerated degradation studies over six months. Loss of activity at 20°C storage was predicted to be 11% p.a. which is not ideal for reference materials (Wadhwa et al. 2011). However, it is important to note that the formulation was not optimised directly for long term storage. Inclusion of an additional test parameter of an accelerated storage period during the microscale DoE studies could help to address this attribute.

Conclusions

Microscale-down techniques and DoE experimental design have been successfully combined recently to accelerate the formulation design process for the freeze-drying of a non-therapeutic model protein

lactate dehydrogenase, for which a convenient colorimetric enzymatic assay was available in microplates to assess native function (Grant et al. 2009). We have shown here that these techniques can rapidly provide useful information on the suitability of potential excipients for biotherapeutic proteins also. Due to the greater number of excipient combinations which can be easily accommodated in a single microplate run it can significantly accelerate the development of freeze-drying formulations of an industrially manufactured biopharmaceutical, even where the assay for retention of the native protein is a biological assay, and not for example, a simple colorimetric enzyme assay. The results were also transferable to manufacturing scale vials, and provided a formulation for G-CSF at physiological pH, that also showed promising accelerated degradation characteristics for solid-state storage. The use of robotics to simply mix and blend the formulations created via the factorial design software would further increase the efficiency and accuracy, and the development of a tailor-made microscale freeze drier might also increase the viability of this method even further (Luthra et al. 2007).

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Conflicts of interest None.

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