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The importance of formulation in the successful lyophilization of influenza reference materials

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ABSTRACT

Lyophilized Influenza antigen reference reagents are a critical resource in the quality control of influenza vaccines. A standard formulation has been used successfully at NIBSC for many years however, following the unexpected occurrence of a collapsed appearance in a particular batch a study was carried out to establish the impact of the sugar concentration in the formulation using modulated differential scanning calorimetry (mDSC) and nuclear magnetic resonance spectroscopy (NMR).

There was a correlation between the presence and size of the mDSC eutectic temperature events and the freeze dried appearance of the cakes, which became progressively worse with increasing amounts of sugar. NMR spectroscopy could be used to positively identify and quantify the sugars in the formulations. MDSC can rapidly predict if the freeze dried appearance will be acceptable so as to assure the successful lyophilization of influenza reference preparations.

1. Introduction

Inactivated influenza vaccines have been tested for potency by the single radial immunodiffusion (SRD) assay, following a WHO recommendation, since 1979. The assay requires standardised reagents: an antigen and antiserum reagent for each vaccine component. The haemagglutinin (HA) content of an influenza vaccine is calculated by comparison of the precipitin ring formed by the vaccine with that formed by the antigen standard [1]. Therefore, calibrated standard antigen reagents are needed to quantitate the amount of HA present in a vaccine. The antigen standard is a freeze-dried preparation of partially purified, inactivated, whole influenza virus that is calibrated for its HA content (μg/ml) in a collaborative study between the Essential Regulatory Laboratories (ERLs).

NIBSC, in its role as an ERL within the WHO Global Influenza Programme, produces these reagents whenever vaccine strains are changed and supplies them to vaccine manufacturers and National Control Laboratories worldwide.

The method of extraction of the influenza virus involves centrifugation through a 20–60% w/v sucrose gradient [2] resulting in residual sucrose contents left in the preparation and so the effect of varying the sucrose content on the freeze drying was assessed.

Influenza antigens are usually freeze dried while the antibody preparation is in liquid form. The standard formulation for influenza antigen reference materials supplied by NIBSC has been 1% sucrose in PBS [3]. Phosphate buffers are known to show marked pH shifts on freezing [4] and studies have shown that the secondary and tertiary structures of HA are affected on freezing in PBS alone [5] compared to when HBS (HEPES buffered saline) was used. Changes in structure however are not seen when freeze-drying with PBS in the presence of a cryoprotectant e.g. sucrose [6]. The presence of sucrose in the NIBSC formulation presumably helps to stabilize the HA structure on freeze drying. Freeze dried preparations usually give rise to robust loose cakes in trial lyophilization studies and at definitive fills. However on scale up to production, there had been a case of a batch with collapsed appearance. Products with collapsed appearance are generally unacceptable due to issues with high residual moistures and poor stability on storage.

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Modulated Differential Scanning Calorimetry (mDSC) is a valuable tool for comparing the thermal properties of formulations for the optimisation of lyophilisation conditions. In this study, in order to ascertain the cause of the poor appearance, the thermal properties of the formulations were studied using mDSC [7] while the sucrose/sugar identity and concentration were determined by nuclear magnetic resonance spectroscopy (NMR).

2. Materials and methods

2.1. Freeze drying

2.1.1. Freeze drying of excipients

Influenza excipients were made up with 1% or 2% (w/v) of sucrose, lactose, trehalose dihydrate or mannitol (obtained as analytical grade reagents from VWR or Fisons) in PBS. Ten ampoules of each material (1 ml per ampoule) were filled in 5 ml DIN glass ampoules using a Gilson pipette P1000 at ambient temperature, 13 mm diameter igloo halobutyl stoppers were used (Adelphi Group, Haywards Heath, UK). The tray containing the filled ampoules was loaded onto the top shelf of the Virtis Genesis 25 EL (Biopharma Process Systems, Winchester, UK) freeze dryer which had been precooled to +4 °C. Following a ramped freeze from 4 °C to −50 °C, the products were held at −50 °C for a further 2 h. Primary drying was for 20 h at −35 °C with a vacuum of 100 μbar and secondary drying was for 10 h at 25 °C. The products were then held at these conditions until the run was stopped. The ampoules were backfilled with nitrogen gas, stoppered *in situ* and sealed manually using an Adelphi flame sealer.

2.1.2. Freeze drying of influenza antigen preparations

5 mL of antigen concentrate (from a bulk of A: /Wisconsin/15/2009 x-183 FA376743VED, 190 μg HA/ml containing ~1.5% w/v of sucrose) was added aseptically in a laminar flow hood to 14 ml of PBS (to give a final concentration of 50 μg HA/ml of antigen), more sucrose was added to correct for the dilution for each of the fill options (1% and 2% sucrose in PBS). The fill volumes and freeze drying procedure for the antigen containing preparations were the same as for the excipient only. Samples were stored at −20 °C after lyophilization.

2.2. NMR

2.2.1. NMR sample preparation

Four sugars were used for this study, D (−)-Mannitol (BDH, (VWR), Lutterworth, UK) 96.5% purity by HPLC), D (+) − Trehalose Dihydrate (Fluka-Garantie, Sigma Aldrich, Poole, UK) ⟩99.0% purity by HPLC, B-Lactose (Sigma Aldrich, Poole, UK) 99.0% purity by HPLC and Sucrose (Sigma Aldrich, Poole, UK) ⟩99.5% purity by GC. A 10% buffer stock solution of each of the four sugars in PBS was prepared in deuterium oxide (D_2O) (99.92% atom D, Apollo Scientific, Stockport, UK). For the internal reference, a 10% solution of acetone (Sigma Aldrich, Poole, UK) >99.9% HPLC grade in D_2O was used. Dilutions of varying concentrations (w/v), 0.25%, 0.5%, 1%, 2% and 5% of the four sugars were prepared in PBS buffer for each of the sugars from the 10% stock solution. 1 ml from each dilution was transferred to labelled vials and lyophilized. Post lyophilization the samples were reconstituted with 1 ml of D_2O and mixed to form clear solutions. 600 μl of each of the solutions were transferred into labelled Wilmad 5 mm NMR tubes. Before ^1H NMR acquisition, 10 μl of the 10% acetone-d_6 stock solution was added to the tubes.

A blind analysis was set up to show that NMR can discriminate between different simple sugar structures used in this investigation. Ampoules containing 1% and 2% of each of the four sugars alone in PBS buffer were submitted for NMR analysis. Each ampoule was reconstituted in 1 ml of D_2O and 600 μl of each of the solutions were transferred into labelled Wilmad 5 mm NMR tubes along with 10 μl of acetone-d6. Proton (^1H) spectra were acquired on the NMR spectrometer.

Eight freeze dried excipient-only ampoules (see 2.1.1) were allowed to equilibrate at room temperature before their contents were reconstituted in 1 ml of D_2O and spun for 10 min to separate the supernatant. 600 μl of each aliquot was transferred into labelled Wilmad 5 mm NMR tubes and 10 μl of the 10% acetone-d_6 was added.

Six freeze dried influenza antigen ampoules in 1% sucrose/PBS (see 2.1.2) were allowed to equilibrate at room temperature before their contents were reconstituted with 1 ml D_2O and spun for 10 min to separate the supernatant. 600 μl of each aliquot was transferred into labelled Wilmad 5 mm NMR tubes and 10 μl of 10% acetone-d_6 was added.

2.2.2. NMR ^1H parameters

All spectra were obtained using a 500 MHz Varian Inova spectrometer (Agilent Technologies, Oxford, UK) equipped with a 5 mm gradient triple resonance probe. Experiments were recorded at 303 K with a relaxation delay of 10s. Spectra were collected with 64 transients, a 90 °C pulse width of 5.0, receiver gain at 14 and acquisition time of 4 K. The NMR data were processed and integral areas of a specifically chosen peak in the sugar spectrum and acetone-d6 signal (δ_H 2.22 ppm) were normalised using MestReNova software (Mestrelab Research version 7.0, S.L Feliciano Barrera 9B − Bajo, 15706 Santiago de Compostela, Spain).

2.3. DSC

2.3.1. Modulated differential scanning calorimetry (mDSC)

One ampoule each of freeze dried influenza excipients, with different and varying amounts of sugars, was reconstituted in 1 ml of ultrapure water and 80 μL aliquots made into large volume hermetically sealed pans (part number 900825.902 TA Instruments, Elstree, UK) and analysed on TA Instruments Q2000 mDSC against an empty pan using a standard method, equipment had previously been calibrated against indium.

Samples were cooled to −90 °C at a ramp rate of 10 °C/min and then heated at a ramp rate of 1.50 °C/min with a modulation of 0.23 °C every 60 s back to room temperature. Heating and cooling rates were applied using a refrigerated cooling system (RCS 90).

Profiles were analysed with Universal Analysis software (TA Instruments) to determine the eutectic event temperatures of the formulations.

2.3.2. Standard DSC

One ampoule each of freeze dried influenza excipients (either 1% or 2% sucrose in PBS), were panned into large volume hermetically sealed pans (part number 900825.902 TA Instruments, Elstree, UK) in a dry bag (Captair pyramid, 2200A, #12847CN, Erlab, USA) purged with nitrogen gas and RH of less than 5% throughout panning. The samples were then analysed on TA Instruments Q2000 standard DSC against an empty pan using a standard method, equipment had previously been calibrated against indium.

Samples were equilibrated to 10 °C, kept isothermal for 5 min and then heated at a ramp rate of 5 °C/min to 250 °C. Heating and cooling rates were applied using a refrigerated cooling system (RCS 90). Profiles were analysed with Universal Analysis software (TA Instruments) to determine the eutectic event temperatures of the formulations.