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

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A B S T R A C T

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.

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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 quantify 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 to give a clear solution. 600 ml of each aliquot was transferred into labelled Wilmad 5 mm NMR tubes and 10 ml of 10% acetone-d6 was added.

2.1.2. Freeze drying of influenza antigen preparations

Influenza antigen ampoules were filled 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 lyophilisation.

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 (D2O) (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 D2O 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 ampoules were backfilled with nitrogen gas, stoppered in situ and sealed manually using an Adelphi flame sealer.

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 integrated areas of a specifically chosen peak in the sugar spectrum and acetone-d6 signal (δH 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 of each 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 DSC 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.
Fig. 1. $^1$H nuclear magnetic resonance spectra of 1% sugar in PBS: Lactose (A), Mannitol (B), Trehalose (C) and Sucrose (D). All spectra are referenced to the internal reference standard 10% acetone-d$_6$ signal at 2.2 ppm. The deuterated water signal is observed at 4.70 ppm.
2.4. Residual moisture analysis (Karl Fischer method)

Residual moisture analysis was performed using coulometric Karl Fischer methodology (Mitsubishi CA-100, A1-Envirotech GmbH, Cramlington, UK) operated within a dry box maintained at low relative humidity <300 ppm. Ampoules were opened inside the dry box and reconstituted with anolyte which was then returned to the coulometer. Three ampoules were tested per formulation option and the results expressed as mean moisture relative to the dry weight of the lyophilized product. The unit was calibrated daily before use with a check solution of known water content (Solution P, A1-Envirotech) [8].

2.5. Single radial immunodiffusion (SRD) assay

SRD assay was performed according to the European Pharmacopeial method [9]. In the SRD assay, specific antiserum raised against purified influenza haemagglutinin (HA) protein is included in an agar matrix; the vaccine to be tested is added into wells cut into the agar matrix [1]. Specific antibodies react with the haemagglutinin, leading to the formation of a precipitin ring that can be visualised by staining. The size of the ring depends on the amount of HA present in the vaccine.

3. Results

3.1. NMR

All four sugar structures (lactose, mannitol, sucrose and trehalose) were unambiguously identified (Fig. 1) using correlation spectroscopy (COSY), and total correlation spectroscopy (TOCSY) 2D NMR techniques. NMR spectra were compared with published data [10–13].

Each type of disaccharide (sucrose, trehalose and lactose) and the alditol mannitol have individual unique chemical shift patterns, displayed on a part per million (ppm) scale (Fig. 1). Acetone was chosen as the independent internal reference material as this peak was well separated from the other resonances present in the spectra. From each spectrum of the four sugars one signal was chosen and integrated for quantitation. This signal was chosen to be in a region which does not include other signals and has a flat baseline. In the case of lactose (Fig. 1A), trehalose (Fig. 1C) and sucrose (Fig. 1D) the integral of the signal from the internal anomeric proton, H1, was used and for D-Mannitol (Fig. 1B) the combined resonances arising from H3 and H4 were chosen. Fig. 1 provides the structure of the four sugars along with Table 1

| Ampoules | Acetone integral | H1 integral | H1/ Acetone | % Sucrose calculated using equation from graph |
|----------|------------------|-------------|-------------|-----------------------------------------------|
| 1        | 786.83           | 213.17      | 0.27        | 1.06                                          |
| 2        | 797.54           | 202.46      | 0.25        | 0.99                                          |
| 3        | 802.53           | 197.47      | 0.25        | 0.96                                          |
| 4        | 793.77           | 206.23      | 0.26        | 1.01                                          |
| 5        | 804.18           | 195.82      | 0.24        | 0.95                                          |
| 6        | 799.56           | 200.44      | 0.25        | 0.98                                          |
| 7        | 792.02           | 207.98      | 0.26        | 1.02                                          |
| 8        | 804.15           | 195.85      | 0.24        | 0.95                                          |
| Standard deviation | 6.28 | 6.28 | 0.01 | 0.04 |
| Mean value % | 797.57 | 202.43 | 0.25 | 0.99 |

| Ampoules | Acetone integral | H1 integral | H1/ Acetone | % Sucrose calculated using equation from graph |
|----------|------------------|-------------|-------------|-----------------------------------------------|
| 1        | 624.23           | 375.77      | 0.6         | 2.4                                           |
| 2        | 648.36           | 351.64      | 0.54        | 2.16                                          |
| 3        | 605.15           | 394.85      | 0.65        | 2.61                                          |
| 4        | 662.35           | 337.65      | 0.51        | 2.03                                          |
| 5        | 664.55           | 335.45      | 0.5         | 2.01                                          |
| 6        | 670.24           | 329.76      | 0.49        | 1.96                                          |
| 7        | 633.56           | 366.44      | 0.58        | 2.31                                          |
| 8        | 644.43           | 355.57      | 0.55        | 2.2                                           |
| Standard deviation | 22.28 | 22.28 | 0.05 | 0.22 |
| Mean value % | 644.11 | 355.89 | 0.55 | 2.21 |

patterns, displayed on a part per million (ppm) scale (Fig. 1). Acetone was chosen as the independent internal reference material as this peak was well separated from the other resonances present in the spectra. From each spectrum of the four sugars one signal was chosen and integrated for quantitation. This signal was chosen to be in a region which does not include other signals and has a flat baseline. In the case of lactose (Fig. 1A), trehalose (Fig. 1C) and sucrose (Fig. 1D) the integral of the signal from the internal anomeric proton, H1, was used and for D-Mannitol (Fig. 1B) the combined resonances arising from H3 and H4 were chosen. Fig. 1 provides the structure of the four sugars along with Table 1

Table 2

| Ampoules | Acetone integral | H1 integral | H1/ Acetone | % Sucrose calculated using equation from graph |
|----------|------------------|-------------|-------------|-----------------------------------------------|
| Flu Antigen 1 | 668.55 | 331.5 | 0.27 | 1.42 |
| Flu Antigen 2 | 684.2 | 315.8 | 0.46 | 1.3 |
| Flu Antigen 3 | 685.7 | 314.3 | 0.46 | 1.3 |
| Flu Antigen 4 | 703.35 | 296.7 | 0.42 | 1.19 |
| Flu Antigen 5 | 679.35 | 320.7 | 0.47 | 1.33 |
| Flu Antigen 6 | 659.94 | 304.1 | 0.46 | 1.3 |
| Standard deviation | 15.03 | 12.26 | 0.02 | 0.07 |
| Mean value % | 680.18 | 313.82 | 0.46 | 1.31 |

Patterns, displayed on a part per million (ppm) scale (Fig. 1). Acetone was chosen as the independent internal reference material as this peak was well separated from the other resonances present in the spectra. From each spectrum of the four sugars one signal was chosen and integrated for quantitation. This signal was chosen to be in a region which does not include other signals and has a flat baseline. In the case of lactose (Fig. 1A), trehalose (Fig. 1C) and sucrose (Fig. 1D) the integral of the signal from the internal anomeric proton, H1, was used and for D-Mannitol (Fig. 1B) the combined resonances arising from H3 and H4 were chosen. Fig. 1 provides the structure of the four sugars along with Table 1

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| 4        | 793.77           | 206.23      | 0.26        | 1.01                                          |
| 5        | 804.18           | 195.82      | 0.24        | 0.95                                          |
| 6        | 799.56           | 200.44      | 0.25        | 0.98                                          |
| 7        | 792.02           | 207.98      | 0.26        | 1.02                                          |
| 8        | 804.15           | 195.85      | 0.24        | 0.95                                          |
| Standard deviation | 6.28 | 6.28 | 0.01 | 0.04 |
| Mean value % | 797.57 | 202.43 | 0.25 | 0.99 |

Fig. 2. a. Freeze dried products showing (1%, 1.2%, 1.4%, 1.6%, 1.8% and 2% sucrose in PBS respectively) robust (far left) and collapsed cakes (right). b. Lyophilized influenza antigen preparations showing 1% sucrose in PBS robust (left) and 2% sucrose in PBS collapsed cake (right).
the labelled integration regions. The ratios of the integral values of the four sugars to the integral of the acetone-d₆ signal from each dilution were used to create a calibration graph and from this plot a linear equation was calculated (see Supplementary Data S1). Two batches of excipient in ampoules containing either 1% or 2% sucrose were submitted for NMR analysis. ¹H spectra were acquired for each set of duplicate samples and the results listed in Table 1. The percentage of sucrose was calculated from the equation in plot D shown in Supplementary Data S1. The data in Table 2 shows that concentrations of different sugar excipients can successfully be estimated in lyophilized influenza antigen formulations also.

3.2. Freeze drying

The mean fill weights and CV measured for the excipients were 1.0168 g, CV = 0.31% and 1.0186 g, CV = 0.26%, (n = 3 for each) for the 1% and 2% sucrose in PBS respectively.

Table 3
The table below shows the potency results by SRD for freeze dried flu antigen samples.

| Batch   | Sugar contents | Potency (µg HA/mL) | Precision (%) |
|---------|----------------|-------------------|---------------|
| SS-266  | (2% sucrose in PBS) | 50 | 87 |
| SS-266  | (1% sucrose in PBS)  | 44 | 87 |

Fig. 3. a. mDSC profile of 1% and 2% sucrose in PBS (n = 3 for range of three repeat runs. Mean of events = −24.58 °C). b. mDSC profiles of influenza excipients with varying sucrose content.
The freeze dried excipients gave rise to robust loose cakes but the appearance of the freeze dried cakes got progressively worse with increasing amounts of sucrose (Fig. 2a). 1.4% w/v sucrose preparations gave rise to robust cakes but the appearance of the cakes began to deteriorate from 1.6% w/v. At 2% w/v sucrose in PBS, the products had completely collapsed. The mean residual moisture for the 1% w/v sucrose in PBS was 0.25% w/w, CV = 7.9%, n = 3 and for the 2% w/v sucrose in PBS 1.08% w/w, n = 2.

Visual monitoring of the freeze drying process clearly showed that the collapse of 2% sucrose in PBS occurred during primary drying (see supplementary data S2).

The freeze dried influenza antigen with either 1% or 2% sucrose in PBS gave rise to robust loose cakes and product of collapsed appearance respectively (see Fig. 2b).

### 3.3. mDSC

MDSC analysis was performed to determine eutectic event temperatures for the excipients. These events were in the range of −24 to −27 °C. The event in 1% sucrose/PBS formulation was compared to 2% sucrose/PBS, the former had a strong eutectic event while the latter gave a much weaker event (Fig. 3a). In addition, the size of these thermal events varied with the different amounts of excipient (Fig. 3b).

MDSC analysis performed on production scale influenza antigen batches also showed eutectic events ranging from −24 °C to −26 °C (Table 4).

### 3.4. Standard DSC

Dry state DSC analysis was performed on freeze dried influenza excipients to determine the glass transition (Tg) temperatures. Tg events were observed at about 54–55 °C for the 2% sucrose in PBS and 87–90 °C for the 1% sucrose in PBSA. There were also endotherms observed for each excipient at 121–122 °C and 130–132 °C respectively (Fig. 4). Both preparations showed a strong exothermic event at about 200 °C.

### 3.5. SRD potency results for influenza antigen

SRD assay showed 50 μg HA/ml for the influenza antigen with 2% sucrose in PBS and 44 μg HA/ml for the 1% sucrose in PBS formulation with a precision of 87% for both formulations (Table 3). These results are not significantly different given the fiducial limits of the assay and so no difference in the potency of the reagent was detected.

### 4. Discussion

The content of sucrose in the sucrose/PBS formulation is critically important for the appearance and residual moisture content of the freeze dried material.

NMR was used to firstly provide structural fingerprints for four different sugars commonly used as excipients in freeze drying (sucrose, lactose, trehalose and mannitol). Once sucrose had been

| Batches  | 12/106 | 12/168 | 13/112 | 13/124 | 13/234 | 14/112 |
|----------|--------|--------|--------|--------|--------|--------|
| Eutectic °C | 25.3   | 25.6   | 25.6   | 24.3   | 25.2   | 25.1   |
| Freeze drying outcome | Successful | Successful | Successful | Successful | Successful | Successful |

Table 4

| Batches  | Eutectic °C | Freeze drying outcome |
|----------|-------------|-----------------------|
| 12/106   | −25.3       | Successful            |
| 12/168   | −25.6       | Successful            |
| 13/112   | −25.6       | Successful            |
| 13/124   | −24.3       | Successful            |
| 13/234   | −25.2       | Successful            |
| 14/112   | −25.1       | Successful            |

Fig. 4. Dry state DSC profiles of freeze dried influenza excipients.
unambiguously identified, quantitation of the amount of sugar in the formulation was calculated using an internal reference standard.

NMR spectroscopy is a powerful analytical technique that is non-destructive to samples, enabling the entire sample to be used for other purposes after quantification is complete. Proton NMR experiments are relatively quick and high resolution spectra are achievable. Proton NMR spectroscopy is not the simplest method for the quantification of sucrose, but offers the advantage of unambiguous identification of the sugar present in the formulation without the need for prior sample processing; and the quantification of the sugars without purification from the formulation.

MDSC was used to measure the glass transition ($T_g$) of amorphous materials and eutectic ($T_{eu}$) event temperatures (for crystalline materials) for each of the formulations. Using mDSC, samples can be analysed rapidly. MDSC is especially useful for the measurement of small $T_g$ and $T_{eu}$ because of the deconvolution procedure which allows separation of the relaxation endotherm from the glass transition compared to conventional DSC [14,15]. There is a relationship between the size of the mDSC eutectic temperature event and the freeze dried appearance of the products. Influenza standards made up in formulations containing 1–1.4% sucrose in PBS are more likely to give rise to products with a good appearance whereas formulations containing 1.6–2% sucrose in PBS give rise to product with unacceptable cakes. This is thought to be due to the suppression of the crystallisation of the sodium chloride in this formulation by the increasing concentration of sucrose, which is amorphous. This can be seen in Fig. 3a where there is no obvious sodium chloride eutectic event for the 2% sucrose in PBS. Products with collapsed appearance as with 2% sucrose in PBS (see Fig. 2b, right) are generally unacceptable, although this does not necessarily correlate to loss of activity of the material. The residual moisture for the 2% sucrose in PBS (1.08% w/w) was considerably higher than for the 1% sucrose in PBS (0.25% w/w). This is to be expected for products with collapsed appearance as sublimation of water would have been hindered by the loss of structure. This also has implications for long term storage/stability as water lowers the $T_g$ as seen in Fig. 4 [14,16]. The $T_g$ for the 2% sucrose in PBS was 54–55 °C while that of 1% sucrose in PBS was 87–90 °C. Other techniques could be employed in the quantification of sugars, but mDSC and NMR spectroscopy have been used in this study as these techniques are readily available at NIBSC. NMR spectroscopy is a more expensive technique than mDSC, but the two techniques provide complementary results, and NMR spectroscopy is particularly useful where the identity of the excipient is uncertain. Using the mDSC, samples can be analysed rapidly with results available within a short period (about 90 minutes) and so can predict fill acceptability before proceeding. Influenza batches tested in this way prior to scale up have shown eutectic events ranging from −24 to −26 °C and all resulted in successful freeze dried reference materials. Screening of influenza samples by DSC is now being routinely done at NIBSC prior to scale up to avoid valuable product loss.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biologicals.2014.12.001.

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