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Optimization of the formulation for a candidate lyophilised tetanus toxoid reference preparation

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Abstract

Tetanus toxoid is a vital primary reference material used for standardization of assays required to establish the antigenic purity of tetanus toxoid for vaccine production. Several formulations were assessed and ampouled fills of each formulation lyophilised. The relative Lf content determined by Ramon flocculation, SRD, and ELISA assays was measured. The stability of the tetanus toxoid activity in each formulation was assessed by accelerated degradation studies. Formulations containing glycine were not suitable in flocculation tests but both sorbitol and trehalose formulations were. The trehalose/sodium chloride formulation had a good appearance, showed good activity in all assays and maintained its activity best under stress conditions. This formulation has been applied to a large scale batch of ampoules prepared as a WHO candidate replacement standard, evaluated in a collaborative study and accepted as a replacement WHO IS for use in flocculation test (WHO ECBS, October 2007, ref no BS/07.2061). The stability of this formulation was also excellent for the large scale batch. The benefits of using thermal analysis and freeze drying microscopy coupled with small scale lyophilisation trials in order to screen formulations for the preparation of batches of biological reference materials are demonstrated.

Keywords: Tetanus toxoid; Lyophilisation; Formulation; Lf assay; Reference materials

1. Introduction

Tetanus, caused by the action of the neurotoxin from *Clostridium tetani*, is effectively controlled by mass immunization in the western world but remains a problem in the Third World, due to poor compliance with vaccination schedules to allow full immunization [1]. Antigenic strength and the purity of tetanus toxoid, prior to its use in the production of vaccine must be determined by a flocculation test (to express Lf unit in toxoids). WHO provides calibrated tetanus toxoid as a primary international standard to help in standardization of assays used to determine the Lf unit of toxoids. It is also used as an antigen for independent *in vitro* quality control assessments of vaccines and anti-tetanus preparations. The National Institute for Biological Standards and Control (NIBSC) develops, produces and distributes international reference materials on behalf of the World Health Organisation (WHO) for use as primary standards in the quantitation of Lf units (from the Latin Limes flocculationis) of tetanus toxoid preparations [2].

The first WHO international standard for tetanus toxoid (derived from material kindly donated by the Statens Serum Institut (SSI), Copenhagen, Denmark) for use in tetanus flocculation test (coded TEFT) [3,4] is soon to be exhausted and a candidate replacement reference material was prepared, derived from Sanofi (formerly Aventis) Pasteur MSD, France, formulated with 5% glycine (coded 02/232). The material had

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good activity in MAb capture ELISA and SRD assays, but was found to work sub-optimally in the Ramon flocculation test, the primary method used by most laboratories and all vaccine manufacturers [4–6].

The aim of this study was to reformulate the purified tetanus toxoid antigen kindly donated by SSI, Denmark so as to provide a reference material which was compatible with the Ramon flocculation test as well as in all other immunoassays that could be used to quantify the Lf activity of toxoids.

Trehalose is widely used as a stabiliser in the lyophilisation of proteins [7], and sorbitol was reported to counteract aggregation of lyophilised tetanus toxoid by Schwendeman et al [8]. Sodium chloride was a required excipient for the assay (0.1 M concentration) and glycine was initially evaluated as a stabiliser and performed well in both the single radial diffusion (SRD) and ELISA assays, but was found to give poorly interpretable responses in the Ramon flocculation assay. This study was carried out with alternative formulants to select a formulation which gave acceptable results with the Ramon flocculation test and a range of other assays.

2. Methods

2.1. Tetanus toxoid formulations

Tetanus toxoid (batch 95, with 1970 Lf/mg protein nitrogen) was supplied by Statens Serum Institut (Denmark) at an initial concentration of 750 Lf/ml. To avoid large dilution of the tetanus activity solid excipients were added to the toxoid solution (50 ml aliquots) where possible, and fully dissolved before filling. Each preparation was adjusted to a final volume of 52 ml (721 Lf/ml) prior to filling. All chemicals were of analytical or equivalent grade. Six formulations were prepared (Table 1).

2.2. Thermal analysis

The freezing properties of the formulations were assessed by thermal analysis and freeze drying microscopy so as to identify critical glass transition (Tg′)/collapse temperatures (Tc) for the formulations. These temperatures must not be exceeded in the product during early primary drying if collapse of the lyophilised cake is to be avoided. Thermal analysis was performed by modulated temperature differential scanning calorimetry (mDSC), electrical resistivity and differential thermal analysis (DTA).

Table 1
Formulation variants selected for the study of tetanus toxoid reference standard

Code	Formulation	Composition
A	0.1 M NaCl 50 mg/ml glycine	Formulation as for candidate 02/232
B	0.1 M NaCl 13.5 mg/ml glycine	A reduced glycine content
C	0.1 M NaCl 10 mg/ml trehalose	Alternative formulation
D	0.05 M NaCl 10 mg/ml trehalose	Lower salt alternative formulation
E	0.1 M NaCl 10 mg/ml lysine	Alternative amino acid stabiliser
F	0.1 M NaCl 10 mg/ml sorbitol	Alternative sugar alcohol stabiliser

2.2.1. Modulated temperature differential scanning calorimetry (mDSC)

This was performed on a TA2920 calorimeter (TA Instruments, Crawley, West Sussex, UK). Samples were prepared by dispensing 80 µl of the sample into a stainless steel pan (part 900825.902, TA Instruments) which was then hermetically sealed by crimping on a lid containing an O-ring. Samples were cooled by a liquid nitrogen cooling accessory (LNCA) and analysis was carried out by heating at 1.5 °C/min with heat only modulation (0.23 °C/min) from −70 °C to 20 °C. Profiles were analysed using Universal Analyst software (TA Instruments) and eutectics and glass transitions (Tg′) identified where present. The instrument was calibrated regularly against indium (melting point 156 ± 1 °C).

2.2.2. Differential thermal analysis (DTA)

DTA was performed using the Lyotherm analyser (Biopharma Technology Ltd, Winchester, UK) on duplicate 1 ml samples of formulation in plastic tubes against 1 ml aliquots of distilled water as a reference. Temperature probes were inserted in each sample and reference aliquots which were then frozen in a sample holder by immersion of the holder in liquid nitrogen, and then warmed through to ambient temperature in a heating block. The product temperatures were recorded and analysed using proprietary software (Biopharma Technology Ltd). A 5% w/v solution of sodium chloride was run as a check calibrant (eutectic temperature = −21 ± 2 °C) for both DTA and resistivity assays prior to the analysis of samples.

2.2.3. Electrical resistivity

This was also measured using the Lyotherm on 5 ml aliquots of the sample dispensed into a glass cell into which a temperature probe and electrode assembly was then inserted. The cell was frozen in liquid nitrogen and warmed in a heating block as for the DTA and data generated were analysed by the same software.

2.2.4. Freeze drying microscopy (FDM)

FDM was performed on 5 µl aliquots of the sample using a Linkam FDCS 196 cryostage (Linkam, Epsom, Surrey, UK) fitted on a BX51 microscope (Olympus) as described in Matejtschuk et al [9]. Freeze drying conditions were selected based upon the thermal analysis and trial lyophilisation.

2.3. Filling and lyophilisation

Initially, 1 ml aliquots of the different excipient formulations (without tetanus toxoid, code 04-027-PM) were filled into 5 ml DIN glass ampoules (Adelphi Tubes, Haywards Heath, Sussex, UK) fitted with purpose-designed adaptors [10] and 13 mm diameter closures, and lyophilised in a laboratory scale freeze dryer (Virtis Advantage, Biopharma Process Systems, Winchester, UK). Once lyophilisation was completed the samples were assessed based on physical appearance. Subsequently, 50 ampoules of each formulation including tetanus toxoid (04-028-PM) were prepared to tight coefficient