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Physicochemical characterization of commercial freeze-dried snake antivenoms

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ABSTRACT

Freeze-drying is a process used to improve the stability of pharmaceutical proteins, including snake antivenoms. This additional step confers these with a higher stability in comparison to liquid formulations, especially in tropical regions where high temperatures could affect the activity of immunoglobulins. Currently, the knowledge about freeze-drying process conditions for snake antivenoms is very limited. Some of the scarce scientific works on this subject reported reconstitution times up to 90 min for these preparations, which could imply a delay in the beginning of the antivenom therapy at the clinical setting. Therefore there is a reasonable concern about whether freeze-dried antivenoms exhibit the desired attributes for solid pharmaceutical proteins. In this work, a physicochemical characterization of seven commercial freeze-dried snake antivenoms was performed based on tests recommended by the World Health Organization (WHO). No significant differences were observed between the products regarding macroscopic appearance of the solid cakes, reconstitution times, residual humidity and monomers content. On the other hand, total protein concentration, turbidity and electrophoretic profile were different among samples. Microscopic analysis by scanning electron microscopy showed no collapsed structure and, instead, most of the samples showed a characteristic protein morphology composed of smooth plates and channels. All the parameters tested in this study were according to literature recommendations and evidenced that, in spite of slight variations found for some products, formulation and freeze-drying conditions chosen by manufacturers are adequate to prevent aggregation and generate, in physicochemical terms, freeze-dried antivenoms of acceptable quality.

1. Introduction

Snakebite envenomation is an important neglected tropical disease in many regions of the world, particularly in sub-Saharan Africa, Asia, Latin America and Papua-New Guinea (Gutiérrez et al., 2011). Snake antivenoms are considered the only scientifically proven therapy against snakebite envenomation (WHO, 2010a). Currently, around 45 public and private laboratories manufacture snake antivenoms in the world (WHO, 2010b, http://apps.who.int/bloodproducts/snakeantivenoms/database/). The majority of these antivenoms are commercialized as liquid formulations, which represents a drawback in remote areas of developing countries, where high temperatures are common and the cold chain is poor.

Freeze-drying is a process that confers stability to proteins, particularly at high temperatures. However, during this procedure antibodies can experience physical and chemical modifications, which may cause irreversible changes and have a negative impact on protein activity and aggregation, as well as on rehydration of the product (Sarciaux et al., 1999; Maury et al., 2005). Aggregation is the main evidence of antibody degradation (Mahler et al., 2009), which could affect the efficacy and safety of these proteins. Nonetheless, it is known that this effect could be prevented during freeze-drying by the addition of excipients like sugars and polyols (Chang et al., 2005; Herrera et al., 2014).

The stability and efficacy of freeze-dried antivenoms depend, among other things, on the lyophilization process and the selection of an adequate formulation. Quality control of freeze-dried

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antivenoms must include the entire set of tests for liquid antivenoms, in addition to specific assays for solid formulations, such as residual humidity and reconstitution time (WHO, 2010a). Despite the relevance of this issue, there is a limited published literature about physical and chemical characterization of freeze-dried antivenoms.

Concerning lyophilized monoclonal antibodies, studies have evaluated the effect of stabilizers, residual moisture and glass transition temperature on the freeze-drying cycle development and on the stability of these pharmaceutical proteins (Andya et al., 2003; Breen et al., 2001; Cleland et al., 2001; Colandene et al., 2007; Duddu and Dal Monte, 1997). In the case of snake antivenoms, the thermal characterization and the stability of different freeze-dried antivenoms formulated with sorbitol, sucrose or mannitol have been reported (Herrera et al., 2014). It was found that antivenom antibodies conserved their neutralizing potency, but most of the formulations presented destabilization when they were incubated at 40 °C for six months, underscoring the need for more stable thermal formulations.

On the other hand, the reconstitution time of freeze-dried antivenoms has received attention due to the relevance of this parameter at the clinical setting, and the importance of providing a prompt and timely antivenom therapy on the evolution of the envenomation has been emphasized (Otero et al., 2002; Iliyasu et al., 2015). Prolonged reconstitution times is a commonly reported problem in some commercial freeze-dried antivenoms (Hill et al., 2001; Quan et al., 2010); this characteristic may reflect an inadequate freeze-drying process that could end up in denaturation of antibodies and loss of neutralizing activity (Theakston et al., 2003). However, other studies showed that is possible to produce freeze-dried antivenoms of rapid reconstitution (Herrera et al., 2014) or to shorten the reconstitution time, using a modified reconstitution method (Gerring et al., 2013).

In the present study, a physicochemical characterization of several commercial freeze-dried antivenoms from different countries was performed to determine whether the different formulations and process conditions in the manufacturing of snake antivenoms could have an impact on the pharmaceutical quality of these freeze-dried products.

2. Materials and methods

2.1. Antivenoms

The antivenoms used in this study were: 1) Snake venom antiserum I.P, Bharat Serums and Vaccines Ltd. (India), Batch Number A5309094; 2) Antivipmyn® Fabotherapic Polyvalent Antivenom, Instituto Bioclon (Mexico), Batch number B-2H-18; 3) Neuro Polyvalent Snake Antivenin, Queen Saovabha Memorial Institute (Thailand), Batch number NP00109; 4) Snake Venom Antiserum I.P, VINS Bioproducts Ltd. (India), Batch number 1081; 5) Fabotherapic Polyvalent Antivenom, Birmex (Mexico), Batch number SV162; 6) Polyspecific Antivenom, Instituto Clodomiro Picado (Costa Rica), Batch number 4770411POLF; 7) Sii Polyvalent Anti-snake Venom Serum, Serum Institute of India Ltd. (India), Batch number 045D0004. The antivenoms were codified with the letters A to G in no particular order.

2.2. Appearance of the freeze-dried antivenoms

Freeze-dried antivenoms were examined visually for macroscopic collapse, cake shrinkage, color homogeneity and adhesions to the vials.

2.3. Electrophoretic analysis

Electrophoretic analysis was performed by SDS-PAGE under non-reducing conditions (Laemmli, 1970). Twenty micrograms of total protein of each sample were separated using an acrylamide concentration of 7.5%, and the gel was stained with Coomassie Brilliant Blue R- 250.

2.4. Total protein concentration

Total protein concentration was determined by a modification of the Biuret method (Gornall et al., 1949). Briefly, 50 μL of antivenoms or protein standards were mixed with 2.5 mL of the Biuret reagent and incubated at room temperature during 30 min. Absorbances at 540 nm were recorded and protein concentration was calculated in a calibration curve obtained by plotting the absorbance of the standards as a function of their protein concentration.

2.5. Monomers concentration

The content of antibody monomers was assessed by FPLC gel filtration in a Superdex 200 10/300 GL column (GE, UK), using 0.15 M NaCl, 20 mM Tris, pH 7.5 as eluent, at a flow rate of 0.5 mL/min (Segura et al., 2013).

2.6. Reconstitution time

Reconstitution time was assessed by dissolving commercial freeze-dried antivenoms with the solvent supplied by the manufacturer. Dissolution was observed visually, as the vials were gently agitated by hand, and the time required to achieve complete dissolution was recorded.

2.7. Residual humidity

Residual humidity of freeze-dried antivenoms was assessed by the Karl Fischer titration method, using a TitroLine KF apparatus (Schott, Germany). At least 50 mg of pulverized antivenom cake were dispersed in a methanol bath (Merck, LiChrosolv® 106018) and titrated with Karl Fischer reagent (Fisher Scientific AL2000-1) until the end point was reached, as determined by the KF processor.

2.8. Turbidity

Turbidity was measured using a turbidimeter model 2020 (La Motte, USA) that was calibrated with standards (HACH, USA) prior to analysis. Turbidity was expressed as nephelometric turbidity units (NTU).

2.9. Scanning electron microscopy (SEM)

Scanning Electron Microscopy analysis was performed in a Hitachi S2360N microscope (Tokyo, Japan) at 15 kV. Freeze-dried antivenoms were removed intact from vials, sectioned vertically using a razor blade, mounted in a sample stub, and coated under vacuum with a platinum-palladium layer during 3 min at 10 mA. Images were obtained using a digital camera Pentax K100 (Tokyo, Japan).

2.10. Statistical analysis

The statistical analysis was performed using the software IBM®SPSS® 19.0 (New York, USA) and SigmaPlot® (San Jose, CA, USA). The results were expressed as the mean ± SD. Non-parametric Kruskal-Wallis test was used for data analysis.