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Freeze-dried snake antivenoms formulated with sorbitol, sucrose or mannitol: Comparison of their stability in an accelerated test

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ABSTRACT

Freeze-drying is used to improve the long term stability of pharmaceutical proteins. Sugars and polyols have been successfully used in the stabilization of proteins. However, their use in the development of freeze-dried antivenoms has not been documented. In this work, whole IgG snake antivenom, purified from equine plasma, was formulated with different concentrations of sorbitol, sucrose or mannitol. The glass transition temperatures of frozen formulations, determined by Differential Scanning Calorimetry (DSC), ranged between −13.5 °C and −41 °C. In order to evaluate the effectiveness of the different stabilizers, the freeze-dried samples were subjected to an accelerated stability test at 40 ± 2 °C and 75 ± 5% relative humidity. After six months of storage at 40 °C, all the formulations presented the same residual humidity, but significant differences were observed in turbidity, reconstitution time and electrophoretic pattern. Moreover, all formulations, except antivenoms freeze-dried with mannitol, exhibited the same potency for the neutralization of lethal effect of *Bothrops asper* venom. The 5% (w:v) sucrose formulation exhibited the best stability among the samples tested, while mannitol and sorbitol formulations turned brown. These results suggest that sucrose is a better stabilizer than mannitol and sorbitol in the formulation of freeze-dried antivenoms under the studied conditions.

1. Introduction

Freeze-drying or lyophilization is the most commonly used method for preparing solid proteins which are physically or chemically unstable in aqueous solution. However, most proteins are sensitive to lyophilization due to the stress of freezing and drying that can cause irreversible damage to the protein structure and biological activity (Heller et al., 1999; Sarciaux et al., 1999). The effectiveness of this technology in the stabilization of biopharmaceutical products is the result of the combination of optimizing the formulation and controlling the process. Formulation optimization is focused on the use of disaccharides as stabilizers, together with bulking agents, such as mannitol and glycine (Imamura et al., 2003; Sharma and Kalonia, 2004).

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On the other hand, the optimization of the process involves controlling the freezing and drying stages for each formulation developed.

Immunoglobulins are a group of proteins relevant as pharmaceuticals and as diagnostic agents. These proteins are prone to forming aggregates and undergo other physical and chemical modifications during manufacturing and long term storage, which may cause loss of activity (Maury et al., 2005). The immunoglobulins G (IgGs) constitute the active principle of antivenoms. Snake antivenoms are considered the only scientifically proven therapy against snakebite envenomation (Bon, 1996), and they are produced from the plasma of animals immunized with a venom or a mixture of venoms.

Snakebite envenomation is an important neglected tropical disease in many regions of the world, particularly sub-Saharan Africa, Asia, Latin America and Papua-New Guinea (Gutiérrez et al., 2011). The global crisis of antivenom supply and the need to distribute antivenoms in remote areas of developing countries, where an adequate cold chain cannot be guaranteed, underscore the importance of freeze-dried formulations for snake antivenoms, ensuring their stabilization during transport and storage.

Despite the need to produce more stable and easy to distribute antivenoms, this issue has received little attention by antivenoms manufacturers, and although many commercial formulations are freeze-dried, there is a very limited body of published literature on freeze-drying of snake antivenoms. To the best of our knowledge, the thermal properties of antivenoms and their stability after freeze-drying and during storage in the solid state have not been reported.

The thermal characterization of antivenom formulations, and particularly the determination of the glass transition temperature of the maximally freeze-concentrated solution (Tg'), are critical parameters in the development of the freeze-drying cycle (Wang, 2000). Tg' defines the maximally allowable temperature for the primary drying since, if the product temperature exceeds this critical temperature, amorphous collapse could occur (Kasper and Friess, 2011). The stability and activity of freeze-dried antibodies largely depend on the processing conditions and on the use of an adequate stabilizer at the optimum concentration (Sarciaux et al., 1999).

In this work, we performed a thermal characterization of snake antivenoms formulations, assessed the effect of freeze-drying on equine antibodies, and evaluated the effectiveness of sorbitol, sucrose and mannitol as stabilizers in antivenom samples subjected to an accelerated stability test during six months.

2. Materials and methods

2.1. Snake venom

Pools of venoms from adult specimens of the snakes *Bothrops asper, Crotalus simus* and *Lachesis stenophrys*, maintained in captivity at the Serpentarium of Instituto Clodomiro Picado, were obtained by mechanical stimulation of venom glands, stabilized by freeze-drying and kept at −20 °C until use. For neutralization studies, only the venom of *B. asper* was used, since this species is the most important venomous snake in Central America.

2.2. Antivenoms production and formulation with stabilizers

Plasma from horses immunized with a mixture of the venoms of *B. asper, C. simus* and *L. stenophrys* was used as a starting material. The antivenom immunoglobulins were purified by precipitation with 5% caprylic acid, followed by vigorous agitation for 1 h. Then the immunoglobulins were micro-filtered through an 8 μm retentive paper (Whatman N° 2, Kent, UK), dialyzed against distilled water, and formulated with deionized water at a total protein concentration of 8 g/dL and a pH of 7.0 (Rojas et al., 1994). Additionally, antivenoms were formulated with either 0.05 M, 0.5 M, 1 M or 2 M sorbitol (Sigma S-7547), 2% or 10% mannitol (Merck-5982), 2% or 5% sucrose (Sigma S-5016) or 0.9% NaCl (Sigma S-1679). Antivenom without excipient was used as a control.

2.3. Differential Scanning Calorimetry (DSC)

The glass transition temperature (Tg') was determined using a differential scanning calorimeter, model Q200 (TA Instruments, USA). Twenty mg of each antivenom formulation were placed in an aluminum pan that was hermetically sealed and frozen at −50 °C at scan rates of 5–10 °C/min, followed by an isotherm of 3 min, and heated at 25 °C at scan rates of 2.5–5.0 °C/min. All the Tg' were reported as the midpoint of the transition.

2.4. Freeze drying microscopy (FDM)

Collapse temperatures were measured using a freezing-drying cryo-stage FDCS 196 (Linkam Scientific Instruments, UK) equipped with a liquid nitrogen cooling system LNP94/2 (Linkam Scientific Instruments, UK), a programmable temperature controller, and a vacuum pump Edwards E2M1.5 (Linkam Scientific Instruments, UK). Samples were placed on a 16 mm quartz cover slip and were frozen to −50 °C at 10 °C/min. Each sample was heated under vacuum (about 1 Pa) at 3 °C/min up to 0 °C. Direct observation of microscopic collapse was done by using a Nikon Eclipse E600 (Nikon, Japan) polarized microscope with a condenser extension lens.

2.5. Freeze-drying of antivenoms

Ten milliliter vials were filled with 5 mL of each formulation and loaded on a freeze-dryer Benchmark 1100 (Virtis, USA). The samples were frozen at −40 °C and annealed at −10 °C for 4 h. The primary drying was conducted at −20 °C for 64 h, and the secondary drying at 30 °C for 4 h and 200 mTorr.

2.6. Residual moisture

Residual moisture was measured by the Karl Fisher 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®

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