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Stability of equine IgG antivenoms obtained by caprylic acid precipitation: Towards a liquid formulation stable at tropical room temperature

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ABSTRACT

Liquid formulations of antivenom require a cold chain for their distribution and storage, especially in tropical countries characterized by high temperature and humidity (climatic zone IV). Since cold chain is often deficient in many regions, there is a need to develop novel formulations of liquid antivenoms of higher stability at room temperatures. The effect of addition of the polyols mannitol and sorbitol on the thermal stability of caprylic acid-fractionated equine whole IgG antivenoms was assessed in preparations having different concentrations of protein and phenol. Results evidenced that: (1) turbidity increases proportionally to phenol and protein concentration. (2) After one year of storage at 25 °C, caprylic acid-purified antivenoms, formulated with or without polyols, did not show evidences of instability. (3) Formulation of antivenoms with 2.0 M sorbitol prevents the appearance of turbidity after one year storage at 37 °C; however, there was a partial loss in neutralizing potency in these conditions. Results suggest that formulation based on sorbitol is an option to obtain liquid whole IgG antivenoms of higher stability at tropical room temperatures.

1. Introduction

Antivenoms are heterologous preparations of purified immunoglobulins, or immunoglobulin fragments, used in the treatment of snakebite envenomation (Lalloo and Theakston, 2003). Traditionally, ammonium sulfate 'salting-out' precipitation has been the most common procedure for immunoglobulin purification in antivenom-manufacturing laboratories, either to generate whole IgG preparations or for the purification of $F(ab')_2$ fragments obtained by pepsin digestion (Theakston et al., 2003). Alternatively, caprylic acid precipitation of non-IgG plasma proteins constitutes a simple and cost-effective method for IgG purification, which has high yield (60%, Rojas et al., 1994). It has been successfully adapted for the industrial production of IgG-based antivenoms (Rojas et al., 1994; Gutiérrez et al., 2005) and for the preparation of $F(ab')_2$ antivenoms at the experimental scale (dos Santos et al., 1989; Raweerith and Ratanabanangkoon, 2003). In addition, clinical trials have shown that IgG antivenoms produced by caprylic acid fractionation present good efficacy and safety profiles (Otero-Patiño et al., 1998; Otero et al., 1999).

There is a current crisis in the accessibility of antivenoms in many regions of Africa, Asia and Latin America (Theakston et al., 2003; Gutiérrez et al., 2006; WHO, 2007). This serious and complex issue is due to reduced levels of production, in some regions, but also to economic constrains in the public health systems, lack of adequate distribution networks, and poor health infrastructure, among other causes (WHO, 2007). An additional complication is that liquid antivenom preparations have to be

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stored at 2–8 °C (WHO, 1981; Gené et al., 1986; Theakston et al., 2003). Storage of liquid antivenoms at room temperature generates turbidity, a signal of physical instability in liquid protein pharmaceuticals (Wang, 2005), which may result in loss of biological activity (Christensen, 1975; Rojas et al., 1990), thus precluding their use even when the expiry date has not been reached. Most snakebite cases occur in rural locations of regions in climatic zone IV, characterized by temperatures around 30 °C and relative humidity of 70% (ICH, 2003). This fact, together with difficulties in the maintenance of the cold chain for transportation and storage of antivenoms in these locations, hampers the proper deployment and use of these immunobiologicals where they are mostly needed (WHO, 2007).

In conditions where the cold chain cannot be ensured, antivenoms have to be formulated as freeze-dried preparations. However, the process of freeze-drying introduces an additional step in the manufacturing process which increases the costs of production and, consequently, the price of the product. Thus, the possibility of formulating liquid antivenoms that could be stable at room temperature would represent a very important step forward in the accessibility of antivenoms in circumstances where the cold chain cannot be guaranteed.

The addition of excipients is a well-known alternative to stabilize proteins in solution (Wang, 1999). A previous study described the use of sorbitol for the stabilization of equine IgG and $F(ab')_2$ preparations obtained by affinity chromatography (Rodrigues-Silva et al., 1997), as well as of an $F(ab')_2$ antivenom generated by pepsin digestion and ammonium sulfate precipitation (Rodrigues-Silva et al., 1999). In contrast, addition of sorbitol or mannitol to Fab ovine-derived antivenom did not increase its thermal stability (Al-Abdulla et al., 2003). This suggests that the effect of polyols may vary depending on the active substance of particular antivenoms, i.e. IgG, $F(ab')_2$ or Fab, or on the profile of contaminating proteins in various antivenom preparations. Thus, there is a need to investigate the effect of polyols in caprylic acid-fractionated whole IgG antivenoms.

The stability of caprylic acid-fractionated equine IgG antivenom at various temperatures was investigated in the present study. The effects of the concentration of proteins and phenol, as well as the stabilizing potential of the polyols mannitol and sorbitol, were also assessed. Our results show that novel formulations of equine IgG antivenoms, incorporating polyols, show significantly improved stability under room temperature conditions in tropical countries.

2. Materials and methods

2.1. Antivenoms

Whole immunoglobulins were purified from the plasma of horses immunized with a mixture of the venoms of *Bothrops asper*, *Crotalus simus* (formerly classified as *Crotalus durissus durissus*) and *Lachesis stenophrys*, following a standard immunization protocol used at Instituto Clodomiro Picado. Pools of 100 L of plasma were fractionated. Non-IgG proteins were precipitated by the addition of caprylic acid (5% final concentration). After filtration, the resulting immunoglobulin solution was diafiltered using a membrane of 30 kDa cut-off value, and formulated with 0.15 M NaCl, pH 7.0, and various phenol and protein concentrations (Rojas et al., 1994). Finally, the product was sterilized by filtration in 0.22 μm pore membranes and aseptically filled in 10 mL glass vials. Some samples were formulated additionally with 0.2 M or 0.7 M mannitol, or with 1.0 M or 2.0 M sorbitol. This was performed by addition of solid reagents to the final IgG preparations. All reagents used in the fractionation and formulation were from Sigma–Aldrich (St. Louis, MO). For comparative purposes, an $F(ab')_2$ antivenom was also prepared by digestion of equine plasma with 1% (w:v) pepsin, at pH 3.0 and 25 °C, for 40 min. Then, $F(ab)_2$ fragments were purified by caprylic acid precipitation as previously described (León et al., 1997), and antivenom was formulated as described above, without the addition of polyols.

2.2. Thermal treatment

To compare the relative thermal stability of IgG and $F(ab')_2$ antivenoms, these formulations were incubated at 60 °C for 150 min. To study the effect of phenol, protein and polyol concentration on antivenom stability, different formulations were incubated at either 4 °C, 25 °C or 37 °C during 180 days. This incubation time is the minimum recommended by the International Conference on Harmonization for Biological Products in pharmaceutical preparations having a shelf-life higher than six months (ICH, 1995). Since antivenom formulation with 2.0 M sorbitol showed the highest thermal stability at this time interval, it was analyzed for six additional months. All incubations were carried out at a relative humidity of 70%.

2.3. Determination of protein concentration

Protein concentration in antivenom preparations was determined by the Biuret reaction (Schosinsky et al., 1983).

2.4. Quantification of turbidity

Turbidity of the preparations was quantified using a turbidimeter (La Motte, model 2020, Chestertown, MD). Turbidity was expressed as nephelometric turbidity units (NTU). Turbidity, which is a consequence of protein aggregation and/or denaturation, is an important parameter in the assessment of the physicochemical quality of antivenoms, since it is associated with the appearance of early adverse reactions to antivenom administration (Otero et al., 1999).

2.5. Determination of osmolarity

Osmolarity was determined using a micro-osmometer (Advanced™ MicroOsmometer, model 3300, Advanced Instruments, Inc., Norwood, MA).

2.6. Electrophoretic analysis

Electrophoretic analysis was performed by SDS-PAGE, under non-reducing conditions, using an acrylamide