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Low pH formulation of whole IgG antivenom: Impact on quality, safety, neutralizing potency and viral inactivation

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ABSTRACT

Low pH treatment improves the tolerance to intravenous infusion, the stability, and the viral safety of various therapeutic immunoglobulins G preparations, but has never been evaluated for horse plasma-derived antivenoms. We have studied the impact of low pH formulation on the quality, safety, stability, potency and viral inactivation of a whole IgG antivenom used to treat viperid snake bite envenoming. Horse plasma-derived whole immunoglobulins purified by caprylic acid were incubated for 24 h at low pH in the presence of 4% sorbitol, then sterile-filtered and stored liquid at 2–8 °C. Appearance, aggregates, purity, safety tests in mice, venom antibody titre, and neutralization potency tests were controlled. Low pH treatment did not affect the physico-chemical characteristics, safety and potency of antivenom for at least 6 months of storage, but a major increase in aggregates was observed. In vitro antibody titre and in vivo neutralizing potency were maintained. There were $\geq$ 5.5 log inactivation of Herpes Simplex Virus-1, an enveloped virus, but no significant inactivation of the non-enveloped Poliovirus type 3. Low pH treatment appears feasible to improve the viral safety of antivenoms without affecting the neutralization potency. The possibility to formulate antivenoms at low pH requires further investigations to avoid formation of aggregates.

1. Introduction

Immunoglobulins, extracted from human plasma or produced by recombinant technology, represent an important class of therapeutic products [1,2]. Depending upon origin and composition, they are used for passive prophylactic or curative passive immunotherapy against viral or bacterial infections, to prevent haemolytic anaemia of the newborn, or to treat autoimmune inflammatory or neurological disorders [3], as well as some cancers [4]. Another class of immunoglobulin-based biologicals, the animal-derived antisera, is fractionated from the plasma of animals that have been immunized against an antigen pathogenic or toxic to humans [1,5,6]. The hyperimmunization of the animals leads to an immune response, characterized by the synthesis of neutralizing antibodies against the specific antigenic components used for immunization. Antivenoms (AVs) are examples of such animal plasma-derived antisera. AV immunoglobulins are produced to neutralize the toxic effects occurring after the bites or stings of humans by venomous snakes, scorpions, or other living organisms [5–7]. They are essential products since up to 1,800,000 snake bite envenomings occur yearly in the world [8]. Snake bites affect severely sub-Saharan Africa and Asia, particularly India, where most of the 20,000 to 94,000 annual deaths occur [8]. The only way to treat envenomed patients is the administration of a specific AV by intravenous infusion [5,9]. These life-saving biologicals are immunoglobulin G (IgG) preparations, or fragments of IgG, obtained by the fractionation of plasma collected from animals, most often horses, hyperimmunized using well-defined venoms or venom mixtures [5–7,10]. AVs fractionation processes have been developed many years ago. Depending on whether or not an enzymatic treatment by pepsin or papain is applied, either $F(ab)'_2$ or Fab fragments or whole IgG AVs are prepared [9]. Recent WHO guidelines have reviewed the core manufacturing processes and highlighted the need to strengthen the production capabilities particularly to enhance viral safety and stability [9].

Apart from a few exceptions of pasteurized [11] or nanofiltered [12,13] products, none of the methods currently used to produce most AVs include a dedicated viral reduction step. The absence of records of transmission of infectious agents or other zoonotic diseases by AVs [9] may very well reflect that current manufacturing processes include steps efficiently inactivating or removing potential viruses present in animal plasma [9,14]. Species

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barrier may also limit risks of transmission of infectious agents to humans. Still, this apparent safety may also reflect the difficulty of conducting pharmacovigilance studies of pathogen transmissions in affected patients in remote rural areas [7]. Therefore, concerns about the potential risks of zoonosis from AVs remain among the scientific, medical, and regulatory communities [7]. Final product stability is another important issue to be addressed especially as AVs are formulated liquid and need to be deployed to remote rural areas in tropical countries. The possibility to introduce dedicated viral reduction treatments and enhance product stability should therefore continue to be evaluated, aimed at preserving the quality and efficacy of AVs, and impacting only marginally production yield and cost [5]. Having this concern in mind, we evaluated the possibility to formulate AVs at low pH. Indeed, experience from the human plasma fractionation industry and monoclonal antibodies show that low pH treatment and/or formulation are standard means to improve viral safety and stability in the liquid state [15]. We therefore studied the feasibility of treating and formulating at low pH a whole IgG AV used in Central America to treat envenomings from snakes of the Viperidae family. As part of this evaluation, we checked the impact of low pH on the biochemical characteristics, safety, stability, viral inactivation, and neutralizing potency of this AV.

2. Materials and methods

2.1. Experimental design

The experimental design is summarised in Fig. 1. Experiments were carried out in triplicates. One litre of hyperimmune plasma was obtained from the Industrial Division (Instituto Clodomiro Picado, San José, Costa Rica) following standard production procedures as described previously [16,17]. Plasma was subjected to 5.5% caprylic acid, pH 5.5 treatment for 2 h. The precipitate was removed by filtration through 8 μm pore filter paper. The resulting supernatant was dialysed. One aliquot was formulated at neutral pH with 0.9% NaCl (control). The remaining fraction was formulated at low pH and incubated at 37 °C during 24 h. Both aliquots were then sterile-filtered through a 0.22 μm pore membrane (Millipore Corp, Bedford, MA, USA), dispensed in 10 mL sterile glass vials, and stored in the dark at 5 ± 3 °C.

2.2. In vitro quality control assays

Total protein, chloride and phenol concentrations, as well as turbidity and pH, were determined as in our previous study [18], and osmolality was measured using Advanced MicroOsmometer (model 3300, Advanced Instruments, Inc., MA, USA). Sodium dodecyl sulphate polyacrylamide gradient gel electrophoresis (SDS-PAGE) was run under non-reducing and reducing conditions in 4–20% polyacrylamide gels [18]. Fast protein liquid chromatography (FPLC) was performed using a gel filtration column (Superdex 10/300 GL, GE Healthcare, Piscataway, NJ, USA) at 0.5 mL/min and 1.5 MPa pressure. Protein elution was monitored at 280 nm. The running buffer (20 mM Tris HCl − 0.15 M NaCl) at different pH (pH 4, 5, 6, or 7) was filtered through 0.22 μm membrane (Millipore).

2.3. General safety test

General safety was assessed in groups of five mice, as described in the USP XXX, NF25. Briefly, mice were injected with 0.5 mL of AV by the intraperitoneal route. A group of mice received 0.5 mL of PBS as control. The mice were weighed daily over a week. Any evidence of toxicity was recorded. Batches evaluated at T0 were the control AV control and the AV formulated with 4% sorbitol and pH 4.3. Batches evaluated at T6 were the control AV and the AV formulated with 4% sorbitol at pH 4.3 (real time stability and accelerated stability).

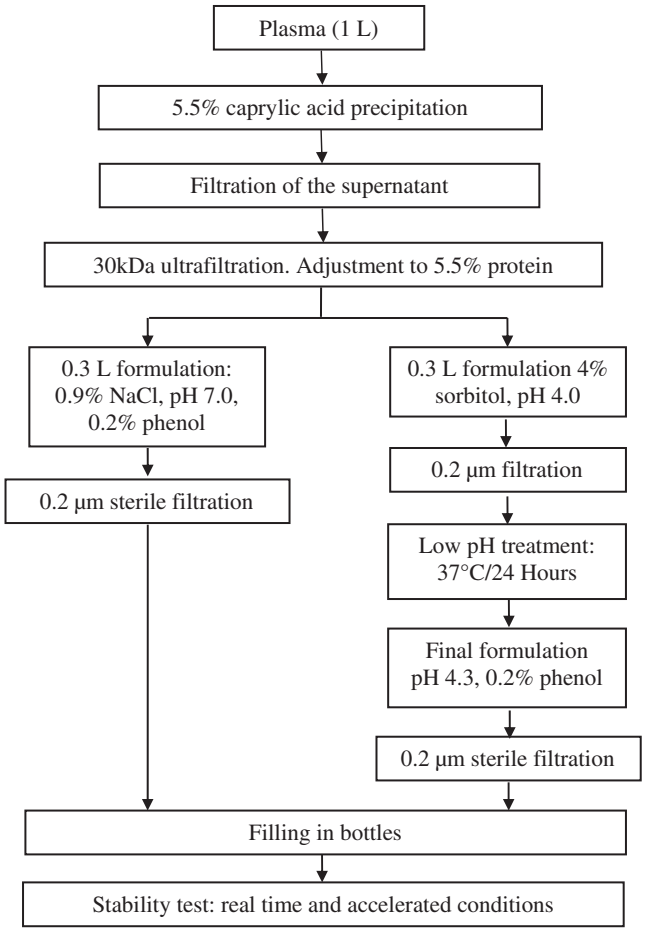

Fig. 1. Protocol design. Low pH treatment was applied on the final purified AV using formulations containing different sorbitol concentrations and pH. The control AV was formulated with 0.9% NaCl and neutral pH.

2.4. Potency and antibody titre

The neutralizing efficacy against the lethal effect of *Bothrops asper* venom was determined by the median effective dose (ED_{50}) assay, as described previously [19]. Controls included venom incubated with PBS only. 0.5 mL aliquots, containing an amount of venom corresponding to 4 median lethal doses (LD_{50}s) and various dilutions of antivenom, were injected into groups of five CD-1 mice (16–18 g body weight) by the intraperitoneal route. Deaths were recorded over a period of 48 h. ED_{50} and the 95% confidence limits were estimated using probit analysis. Antibody titre against *B. asper* venom was determined by ELISA, as previously described [18].

2.5. Stability study

A stability test was performed according to WHO Guidelines [9]. Accelerated stability were performed at 30 °C and 70% relative humidity and real time stability at 2–8 °C. Testing was done after 30 and 60 days.

2.6. Viral inactivation tests

Herpes simplex virus-1 (HSV-1) (F Strain, ATCC-VR-733) and Poliovirus type 3 (Sabin) (Fox [Wy3] strain, ATCC-VR-193) were