Toxicon 53 (2009) 609–615

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

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

Álvaro Segura, María Herrera, Esteban González, Mariángela Vargas, Gabriela Solano, José María Gutiérrez, Guillermo León*

Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

ARTICLE INFO

Article history:
Received 3 October 2008
Received in revised form 15 January 2009
Accepted 19 January 2009
Available online 24 January 2009

Keywords:
Antivenom
IgG
$F(ab')_2$
Stability
Polyols
Climatic zone IV
Sorbitol
Caprylic acid

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

* Corresponding author. Tel.: +506 2229 3135; fax: +506 2292 0485.
E-mail address: gleon@icp.ucr.ac.cr (G. León).

0041-0101/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2009.01.012

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

concentration of 7.5% (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R-250.

2.7. Estimation of neutralizing potency

Mixtures containing a constant amount of *B. asper* venom, corresponding to 8 Median Lethal Doses (LD_{50}) per mL, and various dilutions of antivenom, in 0.12 M NaCl, 0.04 M phosphate, pH 7.2 (PBS), were prepared and incubated for 30 min at 37 °C. Controls included venom incubated with PBS instead of antivenom. Aliquots of 0.5 mL of the mixtures were injected, by the intraperitoneal route, to groups of eight CD-1 mice (16–18 g). Deaths were recorded during 48 h, and neutralizing activity, expressed as Effective Dose 50% (ED_{50}) was estimated by Spearman–Karber procedure (Gené and Robles, 1987). The 95% confidence limits were determined.

2.8. Biological safety test

Five CD-1 mice (19–20 g) were injected intraperitoneally with 0.5 mL of antivenom formulated with 2.0 M sorbitol. The appearance of signs of toxicity was followed during 7 days (USP/NF, 2008). In addition, a group of four rabbits (2.4–2.8 kg) was injected intravenously with antivenom formulated with 2.0 M sorbitol, at a dose of 3 mL/kg body weight. Animals were observed continuously during 2 h for any evidence of discomfort or toxicity, and then every day for 7 days. The weight of mice and rabbits was recorded before and 7 days after antivenom administration.

2.9. Statistical analyses

Analysis of the slope and elevation of the curves of turbidity was performed using *T*-test and *F*-test (Zar, 1999). Tukey's test was performed for post hoc analysis. Comparison of the ED_{50} values was performed using the 95% confidence limits. Values were considered significantly different when their 95% confidence limits did not overlap.

3. Results

3.1. Thermal stability of IgG and F(ab′)$_2$ antivenoms

F(ab′)$_2$ antivenom showed less turbidity than IgG antivenom immediately after preparation (Fig. 1). However, incubation of these antivenoms at 60 °C during 150 min induced a significantly higher increment in turbidity in F(ab′)$_2$ antivenom than in IgG antivenom (Fig. 1). The appearance of turbidity was not associated with the formation of visible particles in the preparations. Owing to the higher stability of IgG antivenom at 60 °C, the rest of the study was performed only with IgG antivenom preparations.

3.2. Effect of phenol concentration on turbidity

Different pilot batches of IgG antivenom were formulated to have a protein concentration of 6 g/dL and various concentrations of phenol (0, 0.1 g/dL, 0.2 g/dL and 0.3 g/dL), and incubated at 37 °C for 180 days. An increment in turbidity was observed in all antivenom formulations, even in the one that did not contain phenol. However, there was a significant increment in turbidity as the concentration of phenol increased (Fig. 2).

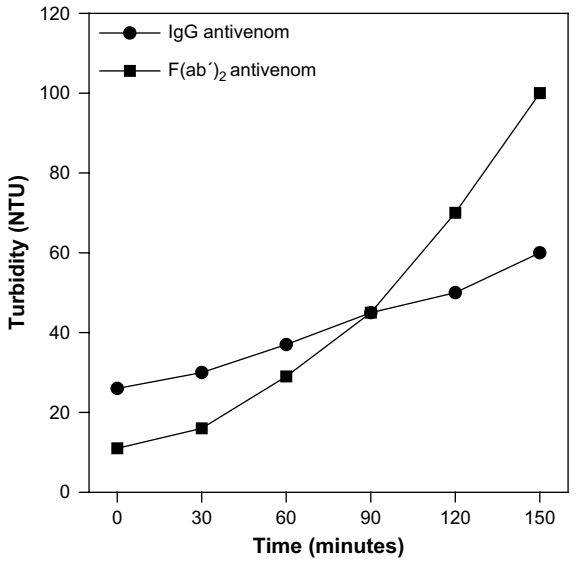

Fig. 1. Development of turbidity in IgG and F(ab′)$_2$ equine-derived antivenoms, fractionated by caprylic acid precipitation, under thermal stress. Antivenoms were incubated at 60 °C for 150 min, and turbidity was assessed before and at various times of incubation. Turbidity was expressed as nephelometric turbidity units (NTU). The slopes of IgG and F(ab′)$_2$ curves were significantly different ($P < 0.02$).

3.3. Effect of protein concentration on turbidity

Antivenom was formulated as to have a constant concentration of phenol (0.2 g/dL) and various concentrations of protein (2 g/dL, 4 g/dL, 6 g/dL and 8 g/dL) and incubated at 37 °C for 180 days. Before incubation, turbidity of antivenoms was directly proportional to the protein concentration. All antivenom preparations showed an increase in turbidity along the 180-day incubation period. Again, the increase in turbidity during incubation was proportional to the protein concentration (Fig. 3).

3.4. Effect of polyols on turbidity

Antivenom pilot batches were formulated as to have a constant concentration of phenol (0.2 g/dL) and protein (6 g/dL), and various concentrations of either mannitol (0.2 M or 0.7 M) or sorbitol (1.0 M or 2.0 M), and incubated at different temperatures for 180 days. Immediately after formulation, antivenoms containing 0.7 M mannitol or 1.0 M or 2.0 M sorbitol had a slightly lower turbidity than antivenoms formulated without these polyols (Fig. 4). None of the antivenoms stored at 4 °C or 25 °C showed increments in turbidity during this time interval (Fig. 4A and B). In contrast, when storage was performed at 37 °C, an increase in turbidity was observed. However, this increment was much lower in the case of antivenoms formulated with either 0.7 M mannitol or 1.0 and 2.0 M sorbitol

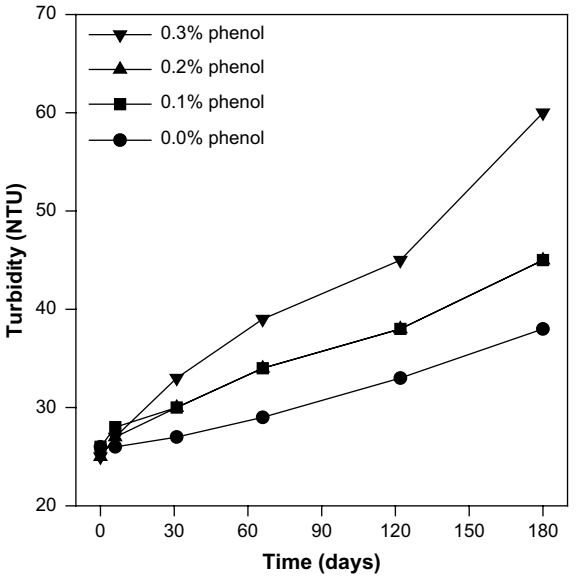

Fig. 2. Effect of phenol concentration on the development of turbidity in antivenoms. Caprylic acid-fractionated IgG antivenoms were formulated as to have 6 g/dL protein concentration and various concentrations of phenol. Turbidity was assessed at different times of storage at 37 °C during 180 days, as described in Materials and methods. Turbidity was expressed in NTU. All slopes are positive ($P < 0.0005$). Slopes of the formulations with 0.10 g/dL and 0.20 g/dL phenol are not different, but both are significantly different from slopes of formulations with no phenol and with 0.30 g/dL phenol ($P < 0.005$).

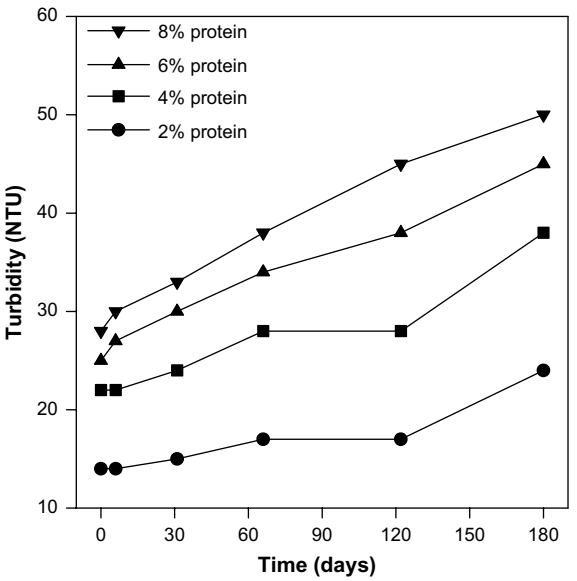

Fig. 3. Effect of protein concentration on the development of turbidity in antivenoms. Caprylic acid-fractionated IgG antivenoms were formulated as to have a phenol concentration of 0.2 g/dL, and various concentrations of protein. Turbidity was assessed at different times of storage at 37 °C during 180 days, as described in Materials and methods. Turbidity was expressed in NTU. All slopes are positive ($P < 0.0001$). Differences in the slopes of all curves and in turbidity of the various samples are significant ($P < 0.0001$).

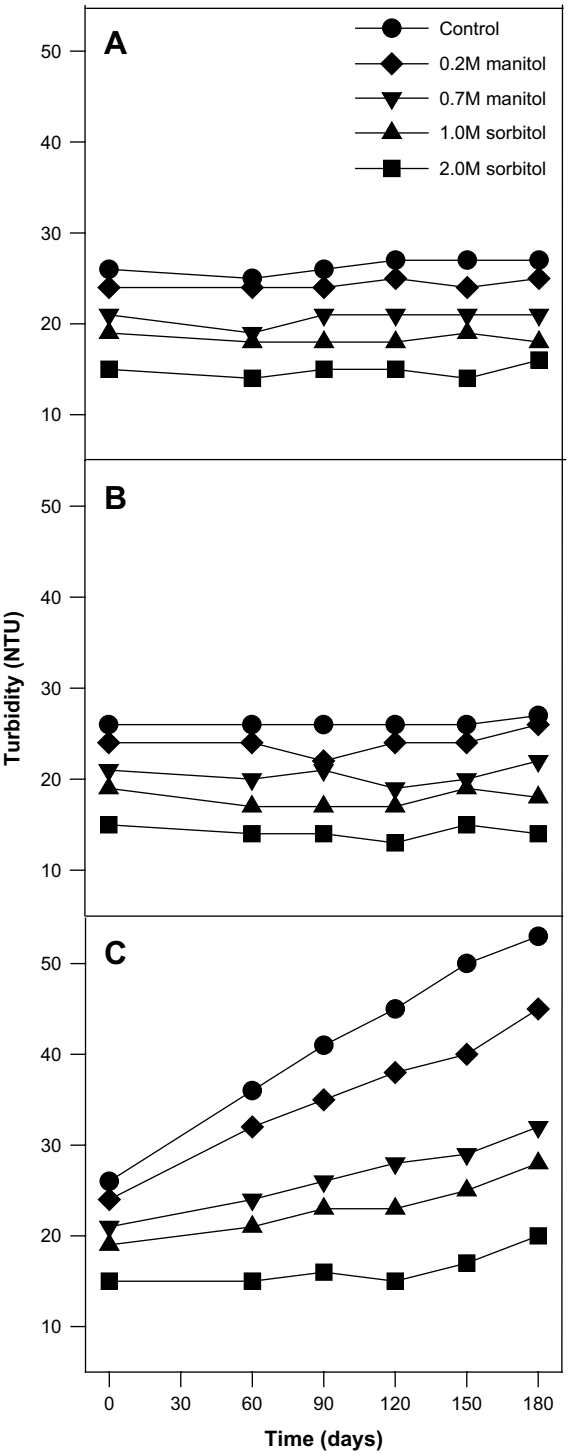

Fig. 4. Effect of mannitol and sorbitol on the development of turbidity in antivenoms. Caprylic acid-fractionated IgG antivenoms were formulated as to have 6 g/dL protein, 0.2 g/dL phenol, and either no polyols or various concentrations of mannitol or sorbitol. Antivenoms were stored at 4 °C (A), 25 °C (B) or 37 °C (C), and turbidity was assessed at various times during 180 days. Turbidity was expressed in NTU. At 4 °C and 25 °C curves did not have a significant slope ($P > 0.2$), and turbidity was different among the various samples ($P < 0.001$). In contrast, at 37 °C all curves, except that of 2.0 M sorbitol, showed significant slopes ($P < 0.005$), which were significantly different among the groups ($P < 0.0001$).

than in antivenoms formulated without polyols or with 0.2 M mannitol (Fig. 4C). Electrophoretic analysis evidenced a predominant band of 150 kDa in the three antivenom samples incubated at 4 °C, 25 °C and 37 °C, corresponding to IgG (Fig. 5). On the other hand, samples of antivenom incubated at 25 °C and 37 °C showed a faint, but visible band in the upper region of the gel, probably corresponding to high molecular mass aggregates (Fig. 5).

3.5. Effect of sorbitol in antivenom neutralizing potency

Two pilot batches of antivenom were formulated with 6 g/dL protein, 0.2 g/dL phenol and either 2.0 M sorbitol or no sorbitol. Osmolarity of the formulations was 2471 ± 16 mOsm/L and 316 ± 4 mOsm/L, respectively. Both antivenoms were stored for 180 days at either 4 °C, 25 °C or 37 °C. No loss in neutralizing potency was observed at any of these temperatures during this time interval (Table 1). When storage was extended to one year, no drop in potency was observed in both antivenoms stored at 4 °C and 25 °C. A partial drop in the value of ED_{50} was observed in antivenom formulated with 2.0 M sorbitol after storage at 37 °C (Table 1).

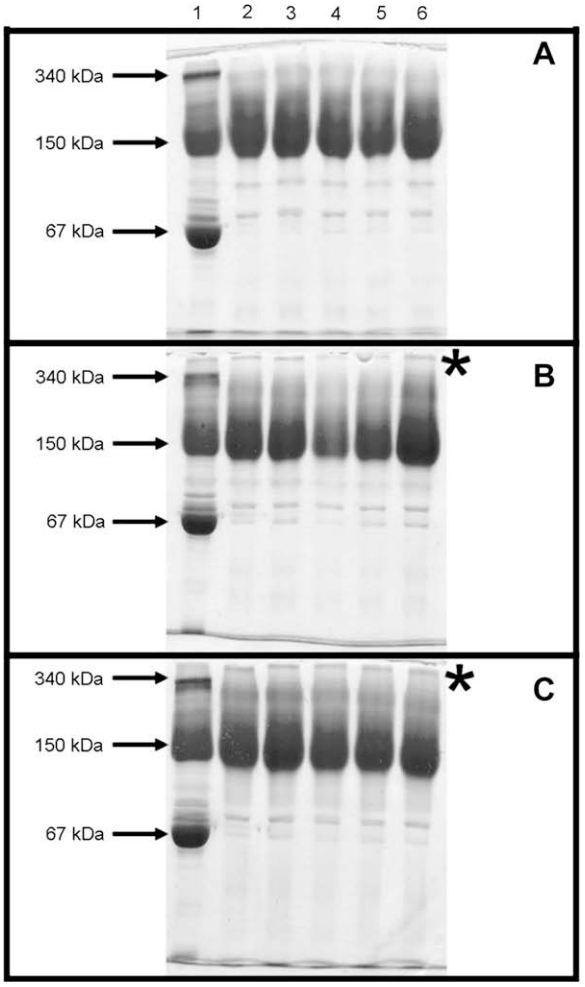

Fig. 5. SDS-PAGE of antivenoms stored at 4 °C (A), 25 °C (B) or 37 °C (C). Non-reduced samples were loaded in a 7.5% polyacrylamide gel in the presence of SDS. Proteins were stained with Coomassie Brilliant Blue R-250. Lane 1 corresponds to equine plasma; lane 2 corresponds to antivenom formulated without polyol. Lanes 3, 4, 5 and 6 correspond to antivenom formulated with 2 M sorbitol, 1 M sorbitol, 0.7 M mannitol and 0.2 M mannitol, respectively. Notice a faint band at the upper part of the gel in (B) and (C), probably corresponding to high molecular mass protein aggregates (*).

3.6. Biological safety test

None of the mice or rabbits injected with antivenom formulated with 2.0 M sorbitol showed any sign of toxicity during the 7 days of observation. In the case of rabbits, continuous observation for 2 h after antivenom administration did not evidence any discomfort or sign of toxicity, and both animal species presented a normal increase in weight at the seventh day. Therefore, these formulations were non-reactive.

4. Discussion

$F(ab')_2$ and IgG liquid antivenom formulations show adequate stability when stored at 2–8 °C for a period of time that varies with each particular preparation; in general terms, liquid preparations have a shelf-life of ~3 years at 2–8 °C (Theakston et al., 2003). The possibility of developing novel antivenom liquid formulations stable at room temperature in tropical countries represents an excellent alternative aimed at reducing production costs and overcoming the limitations of the cold chain in many developing countries. In this study, the effects of various formulations in antivenom stability were assessed only with IgG preparations since incubation of antivenoms at 60 °C showed a higher increment in turbidity of $F(ab')_2$ antivenom than of IgG antivenom, supporting previous observations evidencing the lower stability of $F(ab')_2$ antivenoms to thermal stress (Rodrigues-Silva et al., 1997). It is noteworthy that this conclusion cannot be extrapolated to

Table 1
Effect of sorbitol and storage temperature on the neutralizing potency of IgG antivenoms.

Storage time (months)	Storage temperature	Neutralizing potency (ED_{50}) (mg venom per mL antivenom)	
		Antivenom without sorbitol	Antivenom with 2.0 M sorbitol
0		2.80 (2.05–3.83)	3.12 (2.37–4.11)
6	4 °C	4.32 (3.05–6.12)	2.44 (1.77–3.35)
	25 °C	3.15 (1.92–6.23)	2.65 (1.87–3.75)
	37 °C	3.13 (2.16–4.53)	2.26 (1.65–3.10)
12	4 °C	2.48 (1.88–3.14)	2.32 (1.67–3.00)
	25 °C	3.26 (2.64–4.06)	2.44 (1.86–3.47)
	37 °C	2.06 (1.49–2.57)	1.67 (1.32–2.06)*

Antivenoms were formulated to have 6 g/dL protein, 0.2 g/dL phenol, and either no sorbitol or 2.0 M sorbitol. After storage for 6 and 12 months at various temperatures, the neutralizing potency was determined. Results are expressed as Effective Dose 50% (ED_{50}), corresponding to mg of venom neutralized per mL of antivenom. 95% confidence limits are presented in parentheses. *A significant variation in ED_{50} was observed in antivenom formulated with 2.0 M sorbitol, stored at 37 °C for 12 months, when compared with the potency at day 0.

other IgG and $F(ab')_2$ preparations obtained by using different fractionation protocols.

Although antivenoms are sterile-filtered and dispensed under sterile conditions, most manufacturers add preservatives in their formulation, in order to ensure the control of potential microbial contaminants. Phenol and cresol are commonly utilized in antivenoms as bacteriostatic agents. Our results evidenced that phenol promoted the development of turbidity secondary to protein aggregation induced by thermal stress in antivenoms, in agreement with previous studies (Rojas et al., 1993; Rodrigues-Silva et al., 1999; García et al., 2002). Since the elimination of phenol from antivenom formulations is not currently feasible for some manufacturers, studies should be carried out in order to establish the minimum bacteriostatic concentration of phenol, as a step to reduce its concentration in antivenoms.

The protein concentration of antivenoms varies depending on several factors. Since antivenoms have to be formulated as to have an established neutralizing potency, the protein concentration has to be adjusted as to achieve such potency. In some products this may imply a high protein concentration. Our results evidenced that protein concentration was directly proportional to the appearance of turbidity due to thermal stress in antivenoms, in agreement with findings in other liquid protein-based pharmaceuticals (Wang, 1999). Thus, the reduction in the protein content of antivenoms would result in an increase in their thermal stability. The best way to achieve this goal is to increase the antibody titers in immunized animals, so that a similar neutralizing potency could be reached with a lower protein concentration. Therefore, efforts should be carried out in order to reduce the concentration of phenol/cresol and protein in the final formulation of antivenoms.

The various formulations of IgG antivenom stored at 4 °C and 25 °C showed no increase in turbidity during 180 days, evidencing that caprylic acid-fractionated antivenom, without the addition of polyols, is stable at 25 °C during this time interval. In contrast, when antivenom lacking polyols was incubated at 37 °C, there was an evident increase in turbidity after 180 days. The formulation of IgG antivenoms with either 0.7 M mannitol or 1.0 or 2.0 M sorbitol resulted in a significant reduction in turbidity, when incubated at 37 °C, as compared with antivenoms formulated without these polyols. The best results were obtained with 2.0 M sorbitol. When samples were analyzed by SDS-PAGE, a faint band of high molecular mass was observed in antivenoms stored at 25 °C and 37 °C. However, samples stored at 25 °C did not show an increment in turbidity, thus suggesting that these are soluble aggregates that do not contribute to turbidity.

Since accelerated stability studies may not be appropriate for biological products, results of these analyses should be confirmed by stability studies carried out at real storage conditions (ICH, 1995). In our case, storage of an antivenom formulated with 2.0 M sorbitol at 25 °C or 37 °C for one year did not show an increment in turbidity, in contrast with antivenom formulated with no polyols, whose turbidity increased drastically at 37 °C, thus reinforcing the concept that 2.0 M sorbitol has a significant stabilizing effect on this product at 37 °C. However, a partial reduction in the neutralizing potency was observed after storage for one year at 37 °C. When storage was carried out at 25 °C, no such partial drop in neutralizing potency was observed in these antivenoms.

Sorbitol is used for the stabilization of pharmaceutical protein preparations, such as human immunoglobulins for intravenous administration (IVIG), some of which have sorbitol concentrations of 5 g/dL (Matamoros et al., 2005). In an IVIG preparation having a protein concentration of 5 g/dL, a dose of 200–400 mg protein per kg (Chapel et al., 2000; Matamoros et al., 2005) would require the infusion of 280–560 mL in a 70 kg patient, thus corresponding to 14–28 g of sorbitol. In the case of antivenom, administration of five vials of 10 mL (a common dosage for these products, Smalligan et al., 2004; Otero et al., 1999) of a formulation containing 2.0 M sorbitol (corresponding to 36 g/dL) would result in the administration of 18 g sorbitol to the patient. Therefore, this amount of sorbitol administered is similar to that used in the infusion of some IVIG preparations. This analysis, together with our animal experiments showing lack of toxicity, strongly suggests that such sorbitol concentration in antivenoms has a good safety profile. This hypothesis needs to be further corroborated in preclinical tests and then validated in clinical trials.

In conclusion, a new formulation of caprylic acid-fractionated horse IgG antivenom, containing 0.15 M NaCl, 0.20 g/dL phenol and 2.0 M sorbitol, at pH 7.0, showed a higher stability, during one year of storage at 37 °C, than the current formulation of the antivenom without polyols. It is necessary to extend these studies to more prolonged storage times, in order to determine the expiry date of this novel formulation at various temperatures. Moreover, since room temperatures in many regions are lower than 25 °C, our observations suggest that liquid IgG antivenoms formulated with 2.0 M sorbitol are likely to be stable at room temperature in these regions for at least one year. Despite the positive results obtained with this novel formulation, further investigations are required with caprylic acid-fractionated IgG antivenoms in order to increase their stability and to achieve the goal of finding novel formulations that would allow their storage at room temperature, thus avoiding the problems that arise when the cold chain cannot be properly maintained.

Acknowledgements

The collaboration of Mahmood Sasa in the statistical analysis is acknowledged. This study was supported by Vicerrectoría de Investigación, Universidad de Costa Rica (project 741-A9-003) and CYTED (project 206AC0281). The authors thank Eduardo Valerio (Escuela de Química, Universidad de Costa Rica), Isela Bolaños (Hospital Calderón Guardia, Costa Rica) and Rafael Otero (Universidad de Antioquia, Colombia) for their collaboration.

Conflict of interest

The authors declare that there are no conflicts of interest regarding this manuscript.

References

Al-Abdulla, I., Arnvwa, J.M., Rawat, S., Smith, D.S., Landon, J., Nasidi, A., 2003. Formulation of a liquid ovine Fab-based antivenom for the treatment of envenomation by the Nigerian carpet viper (*Echis ocellatus*). Toxicon 42, 399–404.

Chapel, H.M., Spickett, G.P., Ericson, D., Engl, W., Eibl, M.M., Bjorkander, J., 2000. The comparison of the efficacy and safety of intravenous versus subcutaneous immunoglobulin replacement therapy. J. Clin. Immunol. 20, 94–100.

Christensen, P.A., 1975. The stability of refined antivenin. Toxicon 13, 75–77.

dos Santos, M.C., D'Imperio Lima, M.R., Furtado, G.C., Colletto, G.M.D., Kipnis, T.L., Dias da Silva, W., 1989. Purification of F(ab′)$_2$ anti-snake venom by caprylic acid: a fast method for obtaining IgG fragments with high neutralization activity, purity and yield. Toxicon 27, 297–303.

García, M., Monge, M., León, G., Lizano, S., Segura, E., Solano, G., Rojas, G., Gutiérrez, J.M., 2002. Effect of preservatives on IgG aggregation, complement-activating effect and hypotensive activity of horse polyvalent antivenom used in snakebite envenomation. Biologicals 30, 143–151.

Gené, J.A., Robles, A., 1987. Determinación de la dosis letal 50% por el método de Spearman-Karber. Revista Médica Hospital Nacional de Niños (Costa Rica) 22, 25–40.

Gené, J.A., Gómez, M., Cerdas, L., 1986. Estudio sobre la estabilidad de la actividad neutralizante del suero antiofídico contra veneno de tercipelo (*Bothrops asper*). Rev. Cost. Cienc. Méd. 7, 3–5.

Gutiérrez, J.M., Rojas, E., Quesada, L., León, G., Núñez, J., Laing, G.D., Sasa, M., Renjifo, J.M., Nasidi, A., Warrell, D.A., Theakston, R.D.G., Rojas, G., 2005. Pan-African polyspecific antivenom produced by caprylic acid purification of horse IgG: an alternative to the antivenom crisis in Africa. Trans. R. Soc. Trop. Med. Hyg. 99, 468–475.

Gutiérrez, J.M., Theakston, R.D.G., Warrell, D.A., 2006. Confronting the neglected problem of snake bite envenoming: the need for a global partnership. PLoS Med. 3, e150, doi:10.1371/journal.pmed.0030150.

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, 1995. Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products Q5C. www.ich.org/cache/compo/276-254-1.html.

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2003. Stability Testing of New Drug Substances and Products. Harmonized Tripartite Guideline Q1A (R2). www.ich.org/cache/compo/276-254-1.html.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lalloo, D.G., Theakston, R.D.G., 2003. Snake antivenoms. J. Toxicol. – Clin. Toxicol. 41, 277–290.

León, G., Rojas, G., Lomonte, B., Gutiérrez, J.M., 1997. Immunoglobulin G and F(ab′)$_2$ polyvalent antivenoms do not differ in their ability to neutralize hemorrhage, edema and myonecrosis induced by *Bothrops asper* (Terciopelo) snake venom. Toxicon 35, 1627–1637.

Matamoros, N., De Gracia, J., Hernández, F., Pons, J., Alvarez, A., Jiménez, V. , 2005. A prospective controlled crossover trial of a new presentation (10% vs. 5%) of a heat-treated intravenous immunoglobulin. Int. Immunopharmacol. 5, 619–626.

Otero, R., Gutiérrez, J.M., Rojas, G., Nuñez, V., Díaz, A., Miranda, E., Uribe, A. F., Silva, J.F., Ospina, J.G., Medina, Y., Toro, M.F., García, M.E., León, G., García, M., Lizano, S., de la Torre, J., Márquez, J., Mena, Y., González, N., Arenas, L.C., Puzón, A., Blanco, N., Sierra, A., Espinal, M.E., Arboleda, M., Jiménez, J.C., Ramírez, P., Díaz, M., Guzmán, M.C., Barros, J., Henao, S., Ramírez, A., Macea, U., Lozano, R., 1999. A randomized blind clinical trial of two antivenoms, prepared by caprylic acid or ammonium sulphate fractionation of IgG, in *Bothrops* snake bites in Colombia. Correlation between safety and biochemical characteristics of antivenoms. Toxicon 37, 895–908.

Otero-Patiño, R., Cardoso, J.L.C., Dias Da Silva, W., Higashi, H.G., Nuñez, V., Díaz, A., Toro, M.F., García, M.E., Sierra, A., García, L.F., Moreno, A.M., Castañeda, N., Silva-Díaz, J.F., Murcia, M., Cardenas, S.Y., 1998. A randomized blinded comparative trial of one pepsin-digested and two whole IgG antivenoms in *Bothrops* snake bites in Urabá, Colombia. Am. J. Trop. Med. Hyg. 58, 183–189.

Raweerith, R., Ratanabanangkoon, K., 2003. Fractionation of equine antivenom using caprylic acid precipitation in combination with cationic ion-exchange chromatography. J. Immunol. Methods 282, 63–72.

Rodrigues-Silva, R., Martins, M.S., Magalhães, A., Santoro, M.M., 1997. Purification and stability studies of immunoglobulins from *Lachesis muta muta* antivenom. Toxicon 35, 1229–1238.

Rodrigues-Silva, R., Antunes, G.F.C., Velarde, D.T., Santoro, M.M., 1999. Thermal stability studies of hyperimmune horse antivenoms. Toxicon 37, 33–45.

Rojas, G., Espinoza, M., Lomonte, B., Gutiérrez, J.M., 1990. Effect of storage temperature on the stability of the liquid polyvalent antivenom produced in Costa Rica. Toxicon 28, 101–105.

Rojas, G., Vargas, M., Robles, A., Gutiérrez, J.M., 1993. Turbidity of hyperimmune equine antivenom: the role of phenol and serum lipoproteins. Toxicon 31, 61–66.

Rojas, G., Jiménez, J.M., Gutiérrez, J.M., 1994. Caprylic acid fractionation of hyperimmune horse plasma: description of a simple procedure for antivenom production. Toxicon 32, 351–363.

Schosinsky, K., Vargas, M., Vinocour, G., González, O.M., Brilla, E., Gutiérrez, A., 1983. Manual de Técnicas de Laboratorio. Química Clínica, Universidad de Costa Rica, San José.

Smalligan, R., Cole, J., Brito, N., Laing, G.D., Mertz, B.L., Manock, S., Maudlin, J., Quist, B., Holland, G., Nelson, S., Lalloo, D.G., Rivadeneira, G., Barragan, M.E., Dolley, D., Eddleston, M., Warrell, D.A., Theakston, R.D.G., 2004. Crotaline snake bite in the Ecuadorian Amazon: randomized double blind comparative trial of three South American polyspecific antivenoms. Br. Med. J. 329, 1129–1133.

Theakston, R.D., Warrell, D.A., Griffiths, E., 2003. Report of a WHO workshop on the standardization and control of antivenoms. Toxicon 41, 541–557.

United States Pharmacopeia, 2008. In vivo Biologic Reactivity Test. USP31/NF26 2008. United States Pharmaciopeia/Natl. Formulary (Chapter 88).

Wang, W., 1999. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int. J. Pharm. 185, 129–188.

Wang, W., 2005. Protein aggregation and its inhibition in biopharmaceutics. Int. J. Pharm. 289, 1–30.

World Health Organization, 1981. Progress in the Characterization of Venoms and Standardization of Antivenoms. WHO Offset Publication No. 58. World Health Organization, Geneva.

World Health Organization, 2007. Rabies and Envenomings: A Neglected Public Health Issue. World Health Organization, Geneva.

Zar, J.H., 1999. Biostatistical Analysis. Prentice-Hall Inc., New Jersey.