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# Long-term physicochemical and immunological stability of a liquid formulated intact ovine immunoglobulin-based antivenom

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ABSTRACT

An antivenom should be stable under the conditions that it will be both transferred and stored. Thus instability may lead to a loss of efficacy and an increased incidence and severity of adverse effects. Stability is a particular problem in countries where the temperatures and humidity are high. Here we investigate the stability of a liquid-formulated, intact ovine immunoglobulin-based antivenom, EchiTAbG™, which is used extensively in Nigeria to treat envenoming by the West African saw-scaled viper, *Echis ocellatus*. Ampoules of antivenom were assessed as to their specific antibody content by small scale affinity chromatography and their purity by size exclusion gel filtration and turbidity. Three different batches of the antivenom revealed no significant changes, using these assessment techniques, during 42 months storage at 4 °C or at ambient temperature, followed by one month at 37 °C. These real-time studies indicate that the antivenom remains stable for a minimum of 3.5 years and that it can be exposed to tropical temperatures without any loss in immunoglobulin binding activity. This further highlights the clinical utility of liquid formulated ovine IgG antivenoms by demonstrating their retention of potency in the event of a short term failing in the cold chain.



## 1. Introduction

Antivenoms should meet a number of strict requirements. First and foremost they must be effective at neutralising all the toxic components in the relevant venom(s) and their pharmacokinetics should be such that they persist in the circulation for as long as venom continues to be released from the bite site. They must also be safe and cause minimal or no side-effects. Other important requirements are that they are consistent from batch to batch and from vial to vial and are affordable by those at risk. Finally, they must be stable under the conditions that they will be transferred and stored since instability leads to both a loss of efficacy and an increase in adverse effects due to protein aggregation. Stability is a particular problem in parts of Africa where the temperature and humidity are high, and where it is difficult to ensure a cold chain and refrigerated storage is not always possible.

One approach to ensure stability is to freeze dry the individual vials of antivenom. Thus when we first produced an ovine Fab-based antivenom for the treatment of *Echis ocellatus* (the West African saw-scaled viper) envenomation it was supplied in a freeze dried format. While it proved highly effective in preclinical (Laing et al., 1995) and clinical (Meyer et al., 1997) studies, freeze drying is relatively complex and expensive; suitable vials of diluent must be provided which adds further to cost; and treatment may be delayed while the antivenom is being reconstituted. By

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changing to an acetate buffer and reducing the pH to 4.0, it proved possible to provide a liquid formulated ovine Fab-based antivenom that was stable at 4 °C and at 25 °C for more than a year (Al-Abdulla et al., 2003). Indeed accelerated stability studies indicated a stability of 3–4 years (Silver et al., 1986; Al-Abdulla et al., 2003). Subsequently we moved to a liquid formulated ovine intact immunoglobulin-based antivenom, EchiTAbG™. This decision was taken to avoid the risk of recrudescence which can be a problem with Fab-based antivenoms (Meyer et al., 1997; Gold et al., 2004). Thus they, unlike their intact IgG and $F(ab)_2$-based counterparts, are cleared rapidly via the kidneys and insufficient may remain in the circulation to neutralise the final amounts of venom released from the bite site. Furthermore, avoidance of an enzyme cleavage step with papain increases both the yield and potency of EchiTAbG™ while reducing production costs (Unpublished observations). This antivenom has proved highly effective in both preclinical (Abubakar et al., 2010a; Casewell et al., 2010) and clinical studies (Abubakar et al., 2010b).

Here we assess the long-term stability of EchiTAbG™ formulated in citrate buffer pH 6.0 without the addition of stabilisers or preservatives. Ampoules of the antivenom have been stored at 4 °C and at ~25 °C for 42 months followed by one month at 37 °C and assessed for their purity by turbidity and size exclusion chromatography and for their specific antibody content by small scale affinity chromatography. Recently, in a number of elegant studies, Segura et al. (2009) have demonstrated the stability at tropical temperatures of a formulated intact equine-based antivenom, also prepared by caprylic acid precipitation. Here we demonstrate that ovine immunoglobulin-based antivenoms appear to be equally stable.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Antivenom

EchiTAbG™ is provided in 10 ml liquid filled ampoules containing 250 mg of highly purified ovine intact IgG. The IgG fraction is extracted from ovine sera by the addition of caprylic acid (octanoic acid) at a concentration of 6% (volume/volume) of the antisera followed by dilution with 1.5–2.0 parts saline and mixed vigorously to precipitate non-IgG proteins. The product is formulated at a concentration of 25 g/L in 20 mM citrate buffer, containing 153 mM NaCl (pH 6.0 ± 0.2), prior to filling. Unlike many similar products, the EchiTAbG™ antivenom contained no preservatives or stabilisers. Each batch of EchiTAbG™ is subjected to rigorous quality assurance, which includes testing for its sterility and lack of contamination by pyrogens. Three batches of antivenom (EOG00063, EOG00065 and EOG00068) manufactured in November 2005 and January 2006 were selected for the stability testing regimen outlined in Table 1. Two batches were stored at 4 °C for 28 months followed by 14 months at ambient temperature (AT) (~25 °C), whilst the remaining batch was stored for 42 months at 4 °C. All three batches were then stored finally at 37 °C for one month.

### 2.2. Assessments of stability

#### 2.2.1. Turbidity and physical appearance

The turbidity of selected samples was assessed spectrophotometrically by measuring their absorbance at 580 nm (1 cm path length). Samples were compared with a control turbid solution prepared by addition of 0.1 ml of concentrated HCl to the contents of one antivenom ampoule (10 ml). The physical appearance of all samples was also assessed to monitor changes in visible particles, colour and clarity.

#### 2.2.2. Purity

The purity of selected batches were assessed by size-exclusion gel filtration chromatography using a Pharmacia AKTA/FPLC chromatography system with a Pharmacia Superose 12 HR 10/30 column, equilibrated and eluted with 20 mM sodium citrate saline, pH 6.0, containing 153 mM sodium chloride (SCS) at a flow rate of 0.5 ml/min (cf. Al-Abdulla et al., 2003). The areas of the principal immunoglobulin peak(s) were measured as a percentage of the total peak area of the chromatogram for comparison. The position (retention volume) of IgM and IgG peaks were known from size exclusion chromatography of normal ovine sera.

#### 2.2.3. Specific antibody concentration

A small-scale affinity chromatography assay was utilised to determine the specific antibody content (i.e. those cross-reacting with *E. ocellatus* venom) of the selected batches of EchiTAbG™ (cf. Smith et al., 1992). The small scale affinity column was prepared as described previously (Casewell et al., 2010). Briefly, 5 mg of *E. ocellatus* venom (1 mg/ml in 0.1M sodium carbonate, pH 8.3, solution) was coupled to cyanogen bromide-activated Sepharose Fast Flow 4B (GE Healthcare, UK) by end-over-end mixing overnight at AT and packed into small glass columns (BioRad, UK). Active groups were blocked with 1M ethanolamine-Cl and the columns washed with washing buffer (0.1M sodium phosphate, pH 7.5, containing 0.5M NaCl) followed by elution buffer (0.1M glycine, pH 2.5, containing 0.1M HCl) and stored until used. Columns were washed immediately prior to use with washing buffer and EchiTAbG™ (1 ml) was added to the column and mixed end-over-end for 2 h at AT.

**Table 1**
The stability testing regimen of three manufactured batches of EchiTAbG™ IgG antivenom.

| Batch # | Manufacturing date | Storage at 4 °C (months) | Storage at AT (months) | Storage at 37 °C (months) | Total testing time (months) |
|---|---|---|---|---|---|
| EOG00063 | November 2005 | 28 | 14 | 1 | 43 |
| EOG00065 | January 2006 | 28 | 14 | 1 | 43 |
| EOG00068 | January 2006 | 42 | – | 1 | 43 |