11.4 Detoxification of venom

Some snake venoms can cause local and/or systemic toxicity when injected into naive horses at the beginning of an immunization course. Various physical or chemical means have been adapted in order to decrease venom toxicity, for example, treatment with aldehydes (formaldehyde or glutaraldehyde), hypochlorite, UV or gamma radiation, heat, etc. However, in most cases, not only the toxic sites but also the antigenic sites of the toxins are destroyed after these treatments (Moroz-Perlmutter et al., 1963). For example, when glutaraldehyde is used, the protein polymerization is often extensive and is difficult to control and reproduce. Thus, although the detoxified toxin (toxoid or venoid) induces vigorous antibody response, the antibodies usually fail to neutralize the native toxin. In fact, no detoxification is necessary if inoculation is made with small dose of venom well-emulsified in adjuvants like Freund's complete and incomplete adjuvants.

11.5 Immunological adjuvants

Various types of immunological adjuvants have been tested, for example, Freund's complete and incomplete adjuvants, aluminum salts (hydroxide and phosphate), bentonite, liposomes etc. (Freitas et al., 1991). The choice of adjuvant is determined by its effectiveness, side effects, ease of preparation, especially in large scale, and cost. It may vary depending upon the type of venoms and following manufacturers experience. Freund's complete adjuvant (FCA), which contains mineral oil and *Mycobacterium tuberculosis,* has been shown in experimental animals to be one of the most potent adjuvants known. However, horses are quite sensitive to FCA which tends to cause granuloma formation. For this reason, some producers prefer to use other adjuvants.

It has been noted that the granuloma caused by FCA is due to injection of a large volume (5-10 ml) of the emulsified immunogen at 1-2 sites. The large granuloma formed usually ruptures, resulting in large infected wound. If the emulsified immunogen is injected subcutaneously in small volumes (50-200 µl/site) at multiple sites of injection, granuloma formation may be avoided.

11.6 Preparation of immunogen in adjuvants

In order to minimize infection at the immunization sites, all manipulations should be carried out under aseptic conditions. Venom solutions are prepared in water or phosphate-buffered saline solution (PBS) and filtered through 0.22 µm membrane. The venom solution is then mixed and/or emulsified with adjuvant, according to the instruction of the supplier.

An example for the preparation of venom immunogen in FCA/FIA and aluminum salts is detailed in Box 1.

Box 1:

Example of preparation of venom immunogen in FCA,FIA and aluminum salts

Since FCA can cause severe irritation, precaution should be taken to avoid eye contact, and protective eyewear and gloves are recommended. The vial containing FCA is shaken to disperse the insoluble *Mycobacterium tuberculosis.* The venom solution is mixed in a stainless steel container with an equal volume of FCA at 4° C. The emulsification is achieved by vigorous blending in a high speed blender at a speed of approx. 3,000 rpm for 15 minutes. The container is put in ice water to dissipate the heat generated. The resultant emulsion should be quite thick and remains stable when dropped on the surface of cold water. The highly viscous emulsion is then transferred into a sterile 50 ml glass syringe with the puncher removed. The puncher is then put onto the syringe to expel any air pocket inside. By means of a 3-way stopcock, the emulsion is then transferred from the 50 ml syringe into a 1 or 2 ml glass syringe. With the use of an 18G-20G needle, the water-in-oil emulsion is ready for subcutaneous injection

Immunogen in FIA is prepared similarly as described above except that FIA is used in place of FCA. Both the FCA and FIA emulsified immunogens, if necessary, may be stored at 4° C for several weeks but re-emulsification is needed before their injection.

When the immunogen is prepared in $Al(OH)_3$ (aluminum hydroxide) or $Al(PO)_4$ (aluminum phosphate), a sterile venom solution and a suspension of aluminum salts are mixed in a ratio of 1:3 (v/v) and homogenized. When using other adjuvants, the preparation of solution or emulsion should follow the manufacturer's instructions for that type of adjuvant.

11.7 Immunization of animals

The areas to be immunized should be thoroughly scrubbed with a disinfectant such as soap, shaved and rubbed with 70% ethanol before venom immunogen injection.

In general, the sites of immunization (Figure 2) should be made in areas close to major lymph nodes, e.g., on the animal's neck and back, while the route of injection should be subcutaneous so as to recruit a large number of antigen presenting cells and consequently result in high antibody response. Some procedures call for small volume of injection at each site (50-200 µL) so that the total surface areas of the immunogen droplets is maximized, enhancing the interaction with the antigen presenting cells and the immuneresponse (Pratanaphon et al.,1997: Chotwiwatthanakun et al., 2001).

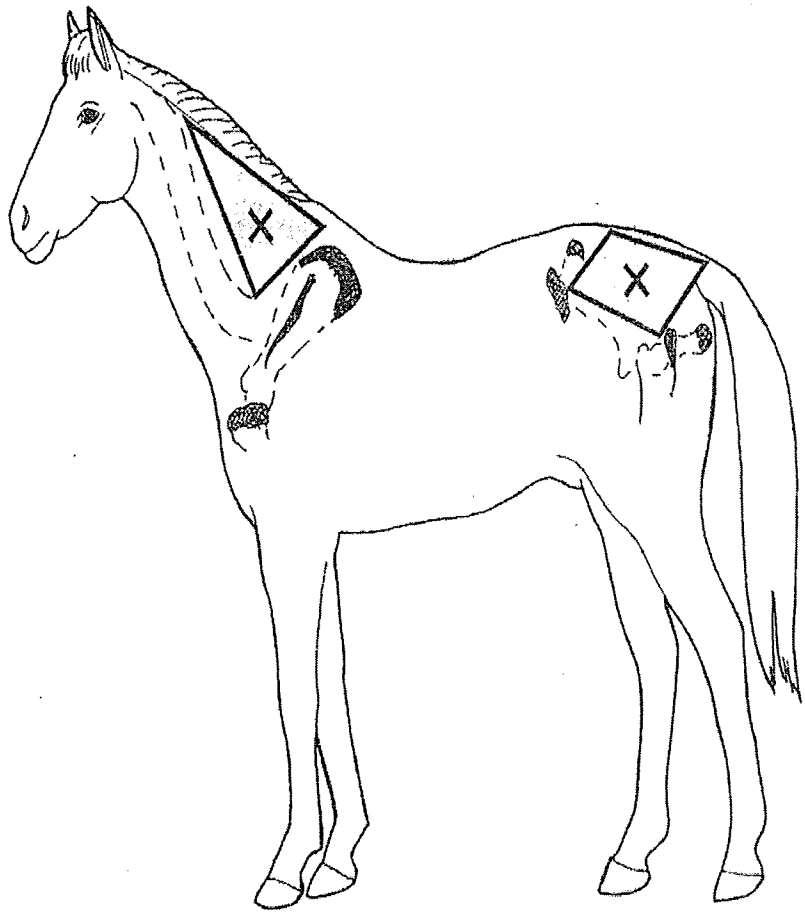

Figure 2: Recommended areas of immunization in horses

An example of immunization of horse using venom emulsified in FCA is described in the box 2.

Box 2

Example of immunization of horse using FCA adjuvant

The primary immunization could be made with venom(s) mixed with Freund's complete adjuvant (FCA) as described in Box 1. The initial dose of each venom could be as low as 1-4 mg/horse with a total combined volume of injection of about 2 ml. The immunogen is filled in a 1 ml glass syringe with 18G needle as described above. Subcutaneous injections of 100-200 µl of immunogen are made at each site, up to as many as 8 to 12 sites, although some producers may use 3 to 4 injection sites only. The neck of the horse, supplied with extensive lymphatic vessels and large lymph nodes, is a preferred area for immunization. If inoculation is made on the lateral sides of the neck, the animal tends to rub itself against the stable causing skin blisters. Thus, injections should be made to the upper part (dorsal) of the neck close to the mane. About 4 - 6 injections can be made at each side of the neck. If injection at the rump is possible, 1-2 injections can be made in the area between the outer hip bone and the top of the thigh bone. The scratching of injected sites by animals can be partially alleviated by massaging the injection site after venom injection to disperse the dose material.

Immunization using Freund's complete adjuvant is usually made only once; repeated use of this adjuvant may in most cases cause serious reactions which can affect the horse's health. After 2 weeks, the horses should receive a booster injection with the same venom(s) well emulsified in Freund's incomplete adjuvant. Similar volume and areas of injection to those described above can be made. Subsequent booster immunizations at 2 week intervals can be made with higher doses (5-10 mg) of venom(s) in saline or mixed with aluminum salts or any other adjuvant selected. In this case, subcutaneous injections of 1 ml of immunogen at each site in a total of 4 sites are recommended.

Blood (10-20 ml) should be drawn before each immunization. Serum or plasma is prepared and EIA (enzyme immunoassay) titres and/or lethality potency are determined. When the EIA titres reach a plateau, usually about 8-10 weeks after the primary immunization, an in vivo potency assay may be performed to confirm that the horse could be bled. After bleeding for antivenom production, the horses are allowed 4 to 8 weeks rest, depending on their physical condition. After the rest period, a new round of immunization can be made as described above but without the use of Freund's complete adjuvant.

Other immunization protocols, using larger amounts of venoms devoid of local tissue-damaging activity (such as those of some elapids) and/or adjuvants other than FCA may be used with satisfactory results, as long as the schedule does not compromise the health of the animals.

In situations where the m ain toxins of a given venom have a low molecular mass and would not induce a sufficient immune response if injected together with the other venom components, isolating such toxins using mild chromatographic procedures can be beneficial. Such isolated fractions can then be used for immunization.

11.8 Traceability of the immunization process

The traceability of the immunization process is very critical for the quality control of the produced antivenoms and it should be performed very accurately. Each immunized animal should be identified by their code number (see Section 10). The details of each immunization steps should be recorded: precisely: date of immunization, batch(es) of venom(s) used with its (their) reference number(s) (see Section 8), venom dose(s), adjuvant and/or salt used, name of the veterinary and supporting staff in charge of the immunization, eventual reaction and/or sickness, etc.

The antivenom titer of the immunized animals should be followed during all the immunization procedure either in vitro (enzyme immunoassays-EIA) during the immunization phase, or in vivo, by neutralization potency assays of lethality when the immunization plateau is reached or before each blood collection.

Each plasma batch should be assigned a unique reference number (e.g. "bar-code") which should allow identifying it unambiguously. Information such as the date of collection, the number of the immunized donor animal, the venom(s) and its (their) reference number(s) used for immunization, etc should be linked to each plasma batch. Computer-based databases are very useful to record properly these data which are of a major importance for the traceability of the produced antivenoms. Standard procedures should be used to protect the integrity of data stored on a computer, including regular, frequent backup, protection from unauthorized access, and storing back-up copies securely off-site.

11.9 Main recommendations

- **Venom solutions should be prepared in such a way that minimizes proteolytic digestion and denaturation of the venom proteins. Venom solution should be prepared under aseptic conditions to avoid infection at the injection sites.**
- **The type of adjuvant used is selected based on the effectiveness, side effects, ease of preparation and cost.**
- **Primary immunization should be made by subcutaneous injections in small volumes at multiple sites close to the animal's lymphatic system to favor the**

recruitment of antigen presenting cells and involving anatomically different groups of lymph nodes for antibody production

- **Subsequent booster injections can be made using venom immunogen doses, volumes and intervals depending on the type of adjuvants used, until the antivenom titer reaches a plateau or a pre-established minimum accepted titer.**
- **After collection of blood for antivenom production, animals should have a resting period of 4-8 weeks After this, a new round of immunization can be performed as above without the use of Freund's complete adjuvant.**
- **The traceability of each immunized animals, every step of the immunization procedure and every plasma batch collected is very important for the quality control of the produced antivenoms and is the responsibility of the antivenom manufacturer.**
-

12 COLLECTION AND CONTROL OF ANIMAL PLASMA FOR FRACTIONATION

Historically, serum separated from the blood of hyperimmunized horses was the basis of "antivenin serum-therapy", but today plasma is used, almost exclusively, as the starting material and undergoes a fractionation process for the separation of purified antivenoms. Thus "antivenom immunoglobulins" is the preferred term, rather than "anti-snakebite serum" or "antiserum" which are imprecise and confusing terms that refer to a crude therapeutic preparation.

The use of plasma as a starting material is preferred to serum largely because erythrocytes can be returned to the animal, thus preventing anaemia and hypovolaemia in the donor animal and allowing more frequent bleeding. Some laboratories have found that using plasma provides higher recovery of antibodies per donation and it is less contaminated with haemoglobin. Separation of plasma from anticoagulated blood is much more rapid than separation of serum from clotted blood. Plasma for fractionation can be obtained either from the collection of whole blood or by apheresis procedure.

12.1 Health control of the animal prior to and during bleeding sessions

When an immunized animal has reached an acceptable antivenom antibody response, it can be bled. Before bleeding is performed, the animals should be evaluated by a veterinary and declared healthy. Animals having evidence of clinical deterioration, such as low hemoglobin concentration, low protein content, or evidence of infections, should not be bled. Moreover, as already indicated in Section 10, if the animals have received antibiotics or any other therapeutic drugs or live vaccines during the last four weeks, they should not be bled either. It is very

important to assure, as much as may be ascertained, that the animal is free of pathogens and that any drugs the animal may have received for treatment.has been cleared from the blood.

12.2 Blood/plasma collection premises

The bleeding of animals should be performed in designated rooms dedicated to this activity. Some producers may design the bleeding rooms so that they can be closed, if needed, during the bleeding sessions, but this is not general practice. Rooms should be extensively washed and cleaned before and after each bleeding session and their design should facilitate such cleaning procedures, which should be clearly established. Animals need to be made as safe and comfortable as possible during bleeding to minimise the chance of injury to the animal or handlers. Individual animals should be confined in circumstances that reduce the stress as much as possible. It is recommended that these rooms allow the simultaneous bleeding of various horses in order to reduce the time required for this operation as well as the stress.

12.3 Blood/plasma collection session

Animals are bled by venipuncture from the external jugular vein. The area surrounding the venipuncture site should be shaved before bleeding and thoroughly cleaned and disinfected, using a non-expired disinfectant, and, depending on the type of disinfectant, allowed, to dry. The prepared area should not be touched before needle has been inserted.

Prior to venipuncture the containers –either plastic bags, plastic bottles or glass bottles, should be pre-weighed and inspected for defects. Any abnormal discolouration suggests a defect and the container should not be used.

The clinical condition of the animals being bled should be closely scrutinized during the time of bleeding and the days afterwards, and bleedings should be suspended in the event of any adverse effect to the animal. Animals showing signs of distress during the operation should have the collection operation terminated

12.4 Labelling and identification

The identity of the animal should be recorded immediately before venipuncture. Each bottle/bag of plasma should be labelled with details of animals from which plasma comes from, specificity of antivenom, blood/plasma weight or volume, donation number, and date of collection.

12.4.1 Whole blood collection and storage

12.4.1.1 Collection

The volume of blood to be obtained depends on the species and size of the immunized animal. It is recommended that around 13 to 15 mL of blood per kilogram body weight are collected in one bleeding session. In the case of sheep, 0.5 L is a typical yield, whereas in the case of horses, the volume of blood may range between 3 to 6 L, depending on the size of the animal.

Blood can be collected, ideally, in disposable plastic bags containing sterile citrate anticoagulant. Usually, the volume ratio of anticoagulant to blood is of 1 to 9. Use of double plastic bags containing anticoagulant is recommended to avoid bacterial contamination and for ease of use. When plastic bags are not available, disposable polypropylene plastic bottles, or sterilized glass bottles containing anticoagulant may be considered.

When the bleeding is taking place, a constant flow of blood should be ensured. Blood should be gently and continuously mixed with the anticoagulant solution to ensure an homogeneous distribution of the anticoagulant, to avoid the risks of activation of the coagulation cascade and, therefore, avoid the formation of clots. The duration of a bleeding session is usually comprised between 30 and 45 minutes depending upon the weight of the animal and the total volume collected. Care should be taken to avoid contamination of the blood by exposing the needle to contaminated surfaces.

12.4.1.2 Storage

Whole blood: bags or bottles in which whole blood is collected should be appropriately cleaned and sanitized on their external surfaces. They should be put into a refrigerated room (2-8 °C) for the plasma and blood cells separation procedure. They should be stored up to 24 hours until the reinfusion of the red cells.

12.4.1.3 Separation of plasma from whole blood

Hyperimmune plasma should be separated from blood cells under aseptic conditions and it should be transferred into sterile containers (plastic bags or bottles). A designated room, designed to allow a proper cleaning and sanitization, should be used for separation. When bottles are used, plasma separation from blood cells should be performed in a laminar flow cabinet located in a room separated from the plasma fractionation area.

12.4.1.4 Reinfusion of the erythrocytes

Blood cells, most specifically erythrocytes (red blood cells), should be separated from plasma by validated centrifugation or sedimentation procedures. It is highly recommended that erythrocyte suspensions should be returned to the horse. Erythrocyte reinfusion should take place within 24 hours after blood collection, after being suspended in sterile saline solution and warmed up to 35-37°C prior to infusion. Such procedure where whole blood is collected and erythrocytes are reinfused to the animal is commonly referred to as "manual apheresis".

12.4.2 Plasma collection by automatic apheresis and storage

12.4.2.1 Plasma collection

In some laboratories, plasmapheresis machines are used to perform automatic plasma collection. This has proved a useful investment in some facilities; it ensures that the animal does not become hypovolemic and it reduces the risks of handling errors, in particular during re-infusion of the erythrocytes to the donor. Automatic apheresis plasma tends to be less contaminated by blood cells (red blood cells, leucocytes and platelets) and in the experience of some laboratories is easier to fractionate as filtration steps, in particular, are more readily performed, resulting in higher yields.

In such procedures, whole blood is collected from the animal, mixed with anticoagulant, and passed through an automated cell separator. The plasma is separated from the cellular components of the blood, which are returned to the animal in a series of collection/separation and return cycles. The plasma is separated from the red blood cells by centrifugation or filtration, or a combination of both. The operational parameters of the plasmapheresis equipment are provided by the manufacturers of the equipment. In general, the anticoagulant is delivered at a rate yielding a specified ratio of anticoagulant to blood. The anticoagulant solutions used include AB16 (35.6 g sodium citrate; 12.6 g citric acid monohydrate; 51.0 g glucose monohydrate per 1 litre using water for injection) and ACDA (22.0 g sodium citrate; 8.0 g citric acid; 24.5 g dextrose monohydrate, per 1 litre using water for injection). The number of collection/separation and return cycles for each donor animal depends on the total volume of plasma that is to be harvested. For horses, the average volume of plasma collected may be about 6 liters per session. The number of cycles ranges from 10 to 20 depending upon the hematocrit of the horses. The collection process lasts for 1 to 4 hours. The apheresis equipment and apheresis procedures should be validated, maintained and serviced. Machine plasmapheresis can take hours and so animals can be fed during the operation

12.4.2.2 Plasma storage

Apheresis plasma: bags should be stored in a refrigerated room (2-8 °C) under the dark until the fractionation process starts. This storage room should be designed to allow a proper cleaning and sanitization.

12.5 Pooling

Plasma from individual animals should be mixed in sterile and sanitized bottles or plastic containers to prepare pools before fractionation. For traceability purpose, each plasma pool should be identified with a unique number (e.g. "bar-code") and the numbers of plasmas from individual animals used in the pool should be recorded precisely.

Such pooling should be performed under aseptic conditions and pools should be adequately identified. Individual or pooled plasma should be stored at 2-8°C in a room devoted for this purpose. In order to assure the prevention of microbial contamination of plasma, preservatives (phenol or metacresol) can be added at a dose of less than 3g/l at this stage and kept during storage of plasma.

In order to avoid the risk of contamination, it is recommended that individual or pooled plasma are not stored for long time intervals before fractionation, i.e. they should be fractionated as soon as possible after pooling. In the event that plasma should be stored for prolonged periods of time (for instance 6 months), the storage time and conditions should be validated to ensure that this does not have a detrimental impact on the quality of the plasma material, on the fractionation process, and on the quality, efficacy and stability of the antivenoms.

12.6 Control of plasma prior to fractionation

Before fractionation, pools of plasma should be checked for macroscopically-evident precipitates, gross haemolysis and bacterial contamination (sterility test or bioburden assay). The neutralizing potency of the starting plasma should be ensured so that the resulting antivenoms will be within potency specifications. Additional controls may include, when relevant, pyrogen test, endotoxins determination, and total protein content.

Plasma pools should be discarded in case of bacterial contamination or if the neutralizing potency is below a minimum limit established by the producer. Cloudy plasma proved free of bacterial contamination may still be used for fractionation provided the fractionation process and product quality has been proven not be impaired. Grossly haemolysed plasma should not be used for fractionation.

12.7 Main recommendations

- **When animals have developed an adequate immune response against venoms, and if they are in good health, they can be bled for antivenom production. Bleeding should be performed in enclosed rooms that should be kept scrupulously clean. Traceability of the donations should be ensured.**
- **Plasma is preferred to serum as a source material. Animals should be bled from the external jugular vein. Plasma can be obtained either from whole blood or by automated plasmapheresis and using approved anticoagulants. Blood or plasma should ideally be collected into closed plastic bags. When this is not possible, glass or plastic bottles can be used, if they can be readily cleaned and sterilized.**
- **Plasmapheresis is recommended using either automatic or manual procedures. When manual apheresis is used, blood cells should be sedimented, separated from the plasma, resuspended in saline solution and returned to the animals within 24 hours. Plasma separation should be performed in a designated room under aseptic conditions.**
- **Bags or bottles containing plasma should be thoroughly cleaned on their external surfaces, adequately identified and stored in refrigerated rooms for further fractionation.**
- **Plasma should be controlled prior to fractionation to establish compliance with relevant acceptance criteria for fractionation, in particular the neutralizing potency.**
- **A special attention should be directed to ensuring traceability between individual animal donors and the plasma pool.**

13 PURIFICATION OF IMMUNOGLOBULINS AND IMMUNOGLOBULIN FRAGMENTS IN THE MANUFACTURE OF ANTIVENOMS

The purification of immunoglobulins and immunoglobulin fragments for the manufacture of antivenoms should aim at obtaining products of consistent safety and efficacy, manufactured following GMP principles. These preparations should have the following characteristics:

- Effective in the neutralization of the targeted venoms,
- High purity of the active substance,
- Low content of protein aggregates,
- Free of microbial contaminants, and
- Low in endotoxins.

13.1 Good Manufacturing Practices

The quality and safety of antivenoms rely largely on the manufacturing procedures that have an impact on the biological characteristics of the end-products. The fractionation process used to manufacture antivenoms should adhere to the general GMP recommendations developed for medicinal products. All operations should therefore be carried out in accordance with an appropriate and relevant system of Quality Assurance and GMP. This covers all stages leading to the finished antivenoms, from production of plasma (including animal selection and health control, production of venoms and immunization protocols, containers used for blood and plasma collection, and anticoagulant solutions, and quality controls methods) to purification, storage, transport, processing, quality control and delivery of the finished product. Of particular relevance is the control of microbiological risks, particulate and pyrogen contamination, and the existence of a documentation system that ensures the traceability of all production steps. In order to establish a satisfactory traceability of the antivenom produced, all the steps of the purification procedure used for the preparation of the antivenom batch should be recorded carefully in pre-established and approved batch record documents, and sampling should be made at established critical steps for in-process quality control tests.

WHO Guidelines on Good Manufacturing Practices for medicinal products are available (2007d) and main principles on GMP for the manufacture of blood plasma products of human origin have also been published (WHO 2003; 2007e). These Guidelines can serve as a general guide for manufacturing practices used for preparing antivenoms derived from animal plasma. Useful specific reference in the field of antivenoms includes also the Note for Guidance on production and quality control of animal immunoglobulins and immunosera for human use (CPMP/BWP/3354/99) (CPMP, 2002).

13.2 Purification of the active substance

Antivenoms are prepared from the starting plasma pool using diverse methodologies to obtain one of the following active substances: (1) Intact IgG molecules, (2) $F(ab')_2$ fragments, or (3) Fab fragments.

In general, fractionation procedures should not impair the neutralizing activity of antibodies, should yield a product of acceptable physicochemical characteristics and purity, with low content of protein aggregates, free of endotoxins, and should provide good recovery of antibody activity.

The characteristics of a batch of plasma to be fractionated should be clearly established, and the methods used to purify the active substance and the in-process controls should be described in detail in SOPs.In the following sections, examples of basic protocols used for the production of IgG, $F(ab')_2$ and Fab antivenoms are described. Some additional methodologies introduced to further purify the active substance of antivenoms are also discussed. Variations in those manufacturing procedures have often been developed by individual fractionators and should be considered as acceptable when shown yield safe and effective for antivenoms preparations.

13.2.1 Purification of intact IgG antivenoms

13.2.1.1 Ammonium sulphate precipitation

In the past, some laboratories have utilized fractionation protocols based on salting-out procedures employing ammonium sulphate or sodium sulphate (Bolaños and Cerdas, 1980). Two precipitation steps are included using two different salt concentrations, in addition to the elimination of 'euglobulins' by precipitation in a diluted acidic solution.

Such fractionation protocols generally leads to a low recovery of antibodies (40-50 %) and to the formation of protein aggregates. The final product contains a relatively high content (>15 %) of contaminating proteins, such as albumin (Rojas et al., 1994). This impairs the safety of the product, since a high incidence of early adverse reactions has been described with such intact IgG antivenoms (Otero-Patiño et al., 1998; Otero et al., 1999).

13.2.1.2 Caprylic acid precipitation

The use of caprylic acid (octanoic acid) as a precipitating agent of proteins from animal sera has been described in the literature (Steinbuch and Audran, 1969). Several procedures for the purification of whole IgG antivenoms of good physicochemical profile and purity using caprylic acid precipitation of non-immunoglobulin proteins have been developed and are now used at production scale (Dos Santos et al., 1989; Rojas et al., 1994; Gutiérrez et al., 2005).

Fig 3 describes a particular process where caprylic acid is added slowly to undiluted plasma, under constant stirring, to reach a concentration of 5 % (v/v) and pH 5.5. The mixture is stirred at 22-25 °C for a minimum of one hour. Precipitated proteins are then removed by filtration or centrifugation. The precipitate is discarded and the filtrate (when using filtration) or the supernatant (centrifugation) containing the immunoglobulins is then submitted to tangential flow diafiltration, to eliminate the residual caprylic acid and to concentrate the filtrate. Alternatively, elimination of residual caprylic acid can be achieved by dialysis. The immunoglobulin solution is then formulated by adding NaCl, an antimicrobial agent and any other excipient needed for formulation, such as protein stabilizers, and the pH is adjusted, generally to neutral values. Finally, the preparation is sterilized by filtration on 0.22 µm filters, and dispensed into final containers (vials or ampoules). Variations in this procedure have been introduced by various manufacturers, and include dilution of plasma, changes in caprylic acid concentration, pH, temperature, etc.

Caprylic acid fractionation allows the production of antivenoms of relatively high purity and with a low protein aggregate content because the immunoglobulins are not precipitated. The yield may reach up to ~ 60-75% of the activity in the starting plasma, depending upon the details of the procedure and/or the equipment used. The efficacy and safety profiles of caprylic acid-fractionated antivenom immunoglobulins have been demonstrated in clinical trials (Otero-Patiño et al., 1998; Otero et al., 1999).

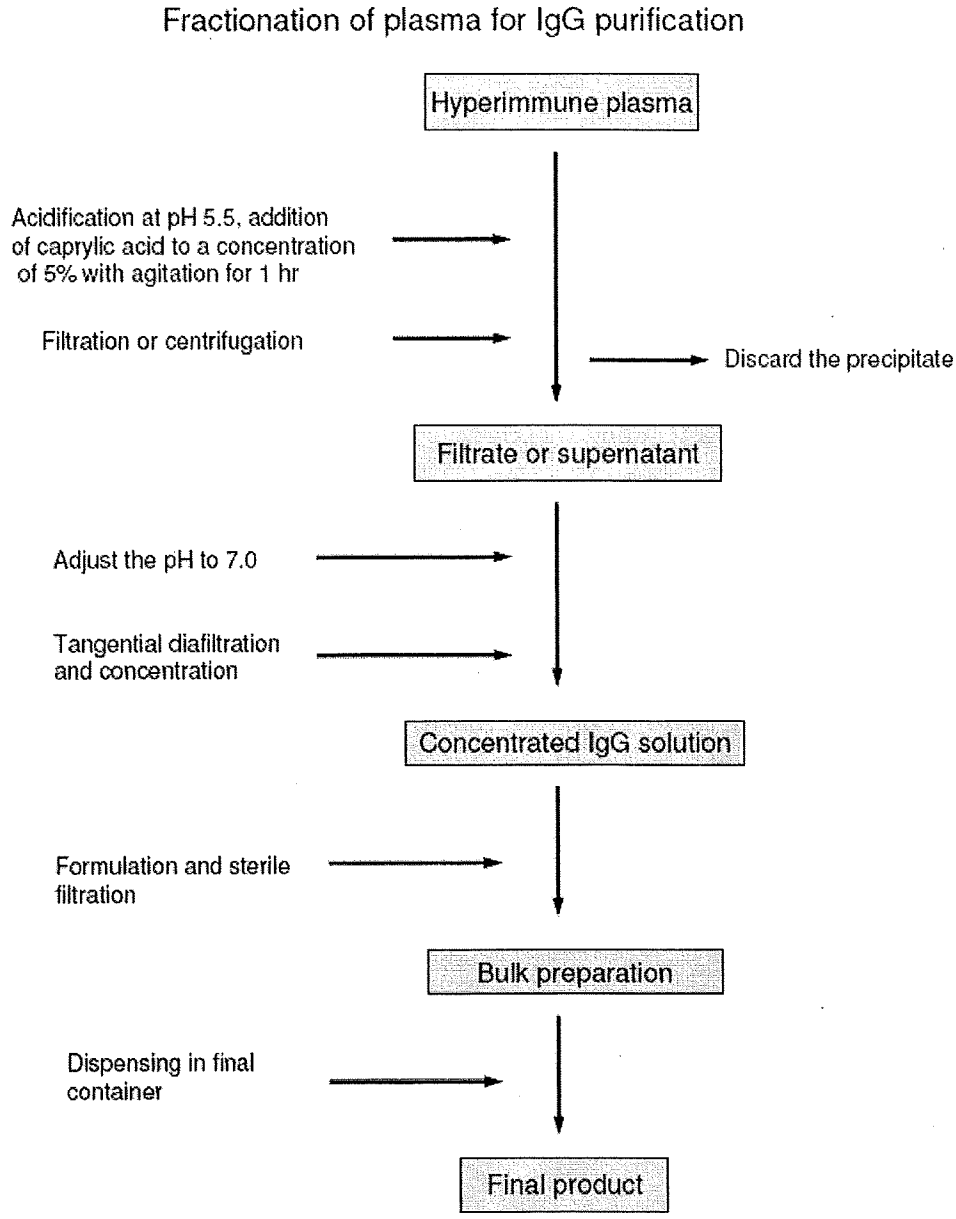

Fig 3: Example of one fractionation process of intact IgG antivenoms prepared by caprylic acid precipitation of non-immunoglobulin proteins

13.2.2 Purification of $F(ab')_2$ antivenoms

Many manufacturers follow the classical protocol for $F(ab')_2$ antivenom production of Pope (1939a, 1939b), with a number of recent modifications (Raw et al., 1991; Grandgeorge et al., 1996; Jones and Landon, 2003).

Pepsin digestion: The method (Fig 4) first involves the digestion of horse plasma proteins by pepsin, leading to a degradation of many non-IgG proteins, and to a cleavage of IgG into bivalent $F(ab')_2$ fragments by removal and digestion of the Fc fragment into small peptides. A heating step and the purification of $F(ab')_2$ fragments by salting-out using ammonium sulphate are also key elements of this methodology. Some procedures perform the peptic digestion step on a pre-purified IgG fraction that is obtained by ammonium sulphate treatment of plasma to obtain an IgG-enriched precipitate, whereas albumin is not precipitated.

Pepsin digestion is accomplished at a pH of 3.0-3.5. The incubation period ranges from 1 to 24 hours, at 30-37 °C in a jacketed tank. A typical protocol is based on incubation at pH 3.3 for one hour with a pepsin concentration of 1 g/L, although other procedures can be used with similar results. Each manufacturer should adjust the pepsin concentration in order to achieve the required enzymatic activity.

Downstream processing using ammonium sulphate: after pepsin digestion, the pH is adjusted to 4.5-5.0, by adding NaOH, to stop pepsin digestion; then, a solution of ammonium sulphate is added under stirring to a final concentration usually close to 12% (w:v). The precipitate is eliminated by filtration or centrifugation, and the filtrate, or supernatant, is heat-treated (usually at 56 °C for one hour; this is known as 'thermocoagulation'). The resulting fraction is filtered or centrifuged to eliminate the precipitate.The pH is then adjusted to 7.0-7.2 with NaOH, and a solution of ammonium sulphate is added under stirring to a final concentration high enough to precipitate the $F(ab')_2$ fragments (usually 23% (w:v) or higher). After an additional filtration step, or following centrifugation, the $F(ab')_2$ precipitate is dissolved, and then it is desalted (to eliminate the ammonium sulphate) and concentrated preferentially by tangential flow diafiltration. Care should be taken to avoid aggregate formation by ensuring gentle mixing and rapid dissolving of the precipitate. Alternatively, desalting can be achieved by dialysis initially against water for injection (WFI) and then against an isotonic saline solution. The $F(ab')_2$ solution is then formulated by adding NaCl, an antimicrobial agent and any other excipient needed for formulation, such as protein stabilizers, and the pH is adjusted, generally to neutral values. Finally, the preparation is sterilized by filtration on 0.22 µm filters, and dispensed into final containers (vials or ampoules). Such process, or similar ones developed by other manufacturers, using pepsin digestion, ammonium sulphate precipitation and tangential diafiltration is the most often used for the manufacture of $F(ab')_2$ fragments. The yield of this fractionation protocol usually ranges between 30 % and 40 %.

Downstream processing using caprylic acid: Purification of $F(ab')_2$ has also been shown, but only at an experimental scale, to be achievable by caprylic acid precipitation of non-$F(ab')_2$ proteins after pepsin digestion, with an improved yield (~60 %) (Raweerith and Ratanabanangkoon, 2003). However, the yield at large-scale is not known. Fig 5 shows a fractionation scheme of F(ab')2 using caprylic acid. $F(ab')_2$ are not precipitated, therefore

reducing the formation of aggregates. Some manufacturers have introduced additional processing steps such as ion-exchange chromatography or ultrafiltration to eliminate low molecular mass contaminants (see below).

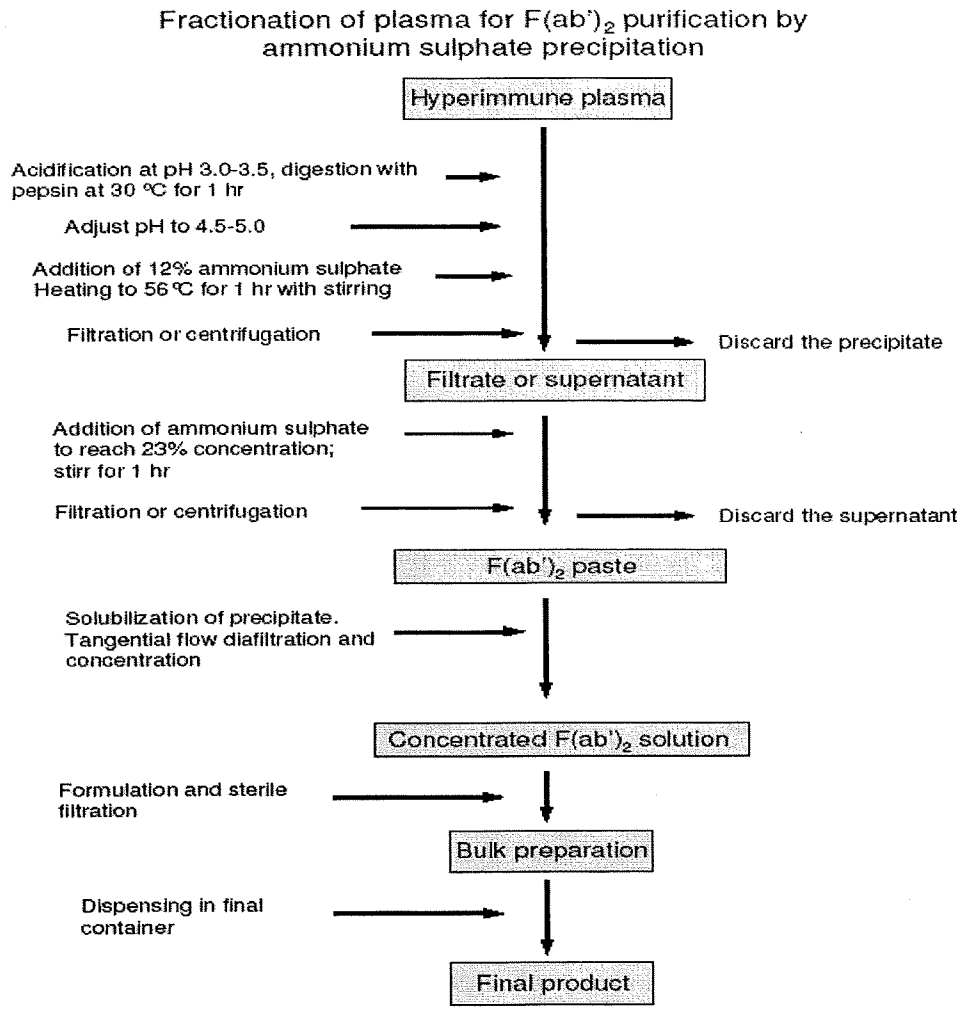

Fig 4: Typical fractionation process of $F(ab')_2$ fragments by ammonium sulphate precipitation

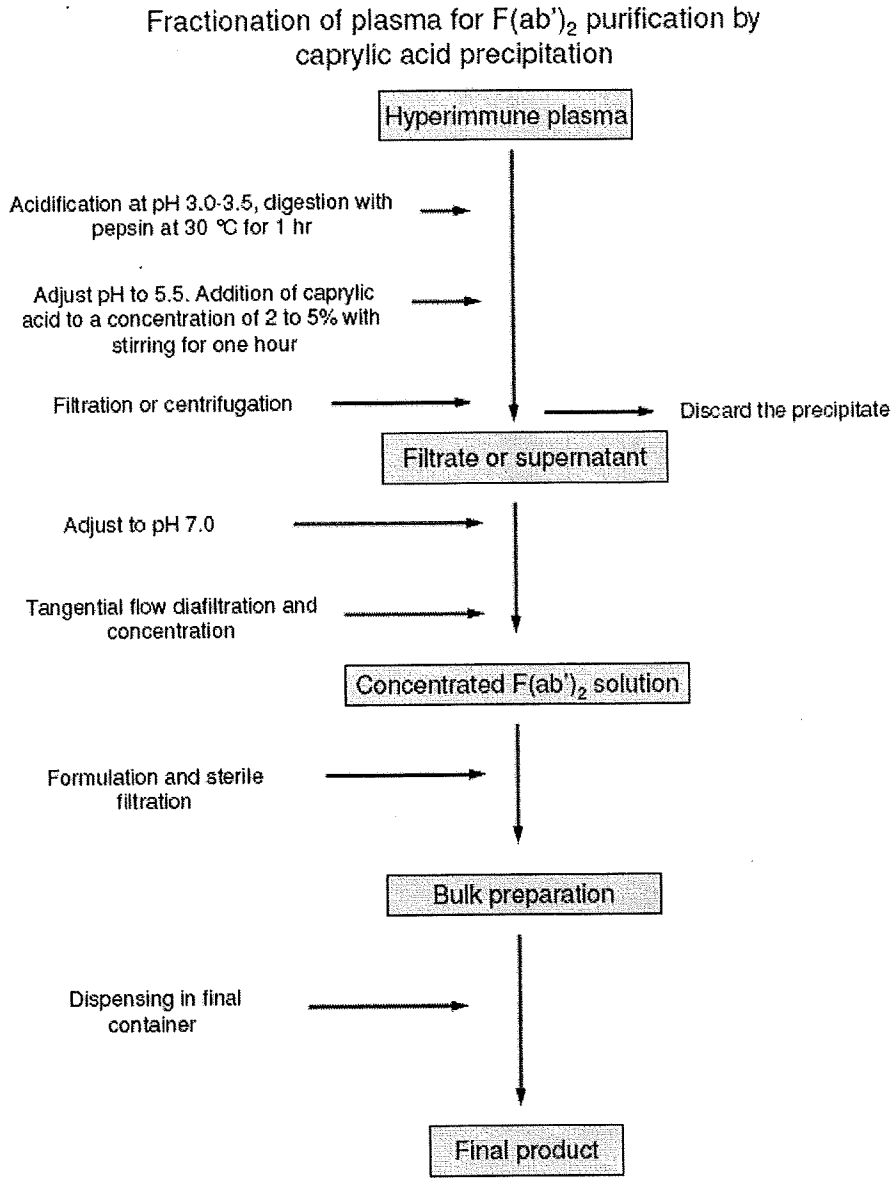

Fig 5: Typical fractionation process of $F(ab')_2$ fragment by caprylic acid precipitation

13.2.3 Purification of Fab antivenoms

Production of monovalent Fab fragments is performed by some manufacturers (Al-Abdulla et al., 2003), currently using sheep plasma. Papain is used in place of pepsin to the enzymatic digestion step, and the process of preparation of the fragment may use ammonium sulphate, sodium sulphate or caprylic acid.

Fig 6 shows a process where immunoglobulins are precipitated from plasma by ammonium or sodium sulphate. The IgG precipitate is dissolved in a sodium chloride solution at pH 7.4. Papain is added and digestion performed at 37 °C for 18-20 hr in a jacketed tank. Reaction is stopped by adding iodoacetamide. The product is then applied to a diafiltration system and equilibrated with a buffered isotonic sodium chloride solution. Afterwards, the preparation is chromatographed on an anion-exchanger (usually in QAE- or DEAE-based media). Fc fragments and other impurities are bound on the column, whereas Fab fragments pass through. After an additional diafiltration/dialysis step, the product is formulated by adding NaCl, antimicrobial agents (when used), any other excipients needed and the pH is adjusted. Finally, the preparation is sterile-filtered and dispensed into the final containers.

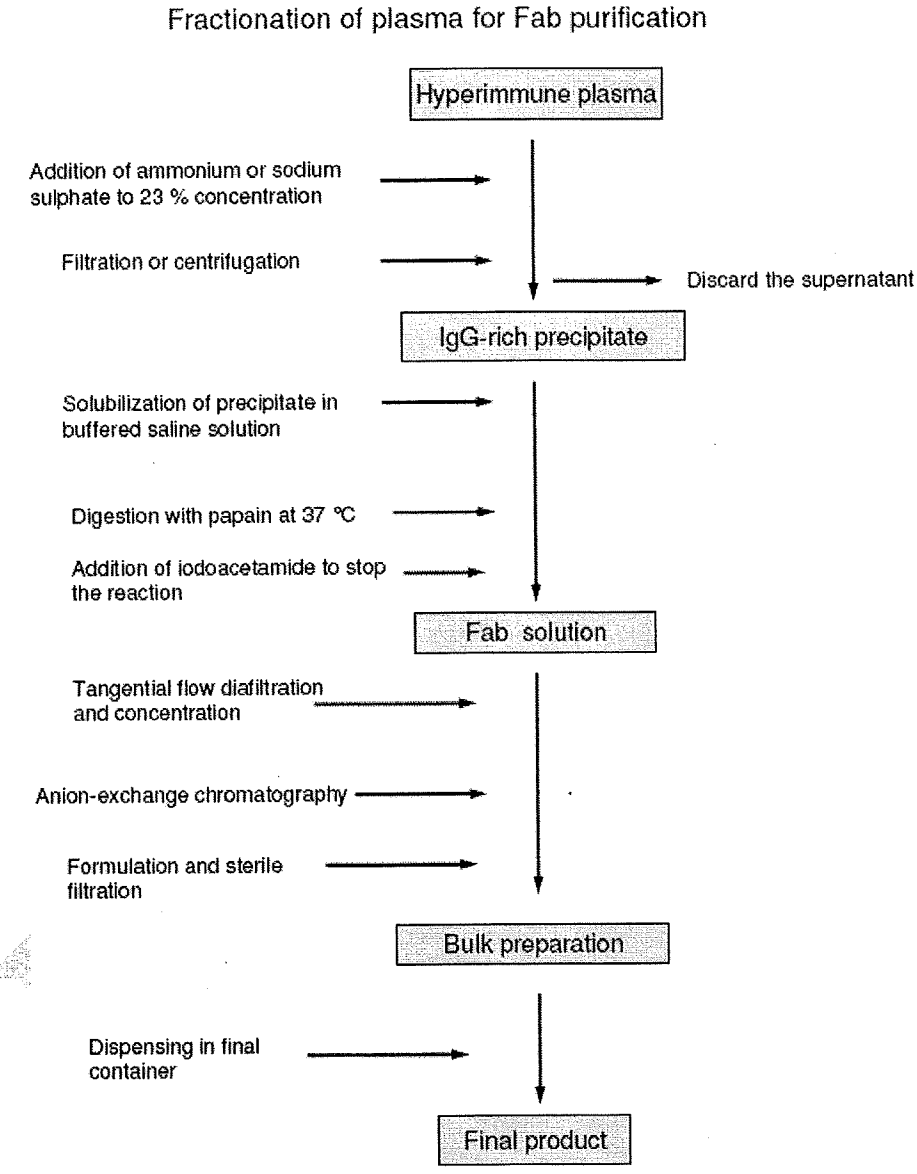

Fig 6: Typical fractionation process of Fab fragments

13.2.4 Optional additional steps used by some manufacturers

When performed following GMPs and using validated fractionation protocols, the basic methodologies described above for the manufacture of IgG, $F(ab')_2$ and Fab antivenoms allow the production of antivenoms of adequate purity, safety and efficacy. Nevertheless, some manufacturers include additional steps to enhance product purity. The methodologies include:

13.2.4.1 Ion-exchange chromatography

Ion-exchange chromatography can be successfully used for antivenom purification based on charge differential with the contaminants. Anion-exchange columns of diethyl amino ethyl (DEAE) or quaternary ammonium (QAE) gels or membranes such as quaternary ammonium cellulose microporous membranes can be used at neutral pH to adsorb protein contaminants (Grandgeorge et al., 1996; Saetang et al., 1997; Jones and Landon, 2003). Alternatively, cation-exchange columns, e.g. carboxymethyl (CM) or sulphopropyl (SP) gels, have been used for IgG or $F(ab')_2$ fragment purification (Raweerith and Ratanabanangkon, 2003). The column is equilibrated at acid pH, e.g. 4.5, to bind the antivenom, whereas proteins contaminants are eluted in the break-through.

Chromatographic procedures should be applied following GMP. Columns should be adequately regenerated, sanitized, and stored in order to prolong their life-time. Measures to avoid batch to batch contamination should be in place. Specific SOPs should be developed and followed.

13.2.4.2 Affinity chromatography

Affinity chromatography using either immobilized venom or other ligands can be designed to bind immunoglobulins or their fragments (Sullivan and Russell, 1982). However, columns usually deteriorate rather rapidly, and meticulous care should be taken to wash, sanitize and store them under adequate conditions. Procedures should assure that any leaching substances from the columns do not affect the quality and safety of the product or are thoroughly eliminated during downstream processing; this is especially critical in affinity chromatography using immobilized venom. Affinity processes may impact recovery and high affinity antibodies may be lost and/or denatured due to the harsh elution conditions needed to elute them from the chromatographic material.

13.2.4.3 Process improvement

Some manufacturers have introduced process improvements to enhance the quality or the yield of antivenoms. Those include the use of: