Box 2
Example of immunization of horses using FCA, FIA and aluminium salts

The primary immunization could be made with venom(s) mixed with (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 an 18G needle as described above. Subcutaneous injections of 100–200 µl of immunogen are made at each site, up to as many as 8–12 sites, although some producers may use only 3–4 injection sites. 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 causing skin blisters. Thus, injections should be made to the upper (dorsal) part of the neck, close to the mane. About 4–6 injections can be made at each side of the neck. If injection in 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 aluminium 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 3–8 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.

11.8 Traceability of the immunization process

The traceability of the immunization process is critical for the quality control of the antivenoms produced and the steps to ensure it should be performed very accurately. Each immunized animal should be identified by its code number (see section 10). The details of each immunization step should be recorded precisely. The details to be recorded include:

- 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;
- names of the veterinary and supporting staff in charge of the immunization;
- eventual reaction and/or sickness.

The antivenom titre of the immunized animals should be followed throughout the immunization procedure either in vitro, using 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. a bar code), which should allow complete traceability to the donor animal. Information (such as the date of collection, the unique identification number of the immunized donor animal, and the reference number of the venom(s) used for immunization) should be recorded to allow traceability to all venoms. Computer-based databases are very useful for properly recording these data, which are crucial for the traceability of the antivenoms produced. 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 backup copies securely off-site.

11.9 Main recommendations

- **Venom solutions should be prepared in such a way as to minimize 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 on the basis of its effectiveness, side-effects, ease of preparation and cost.**
- **Primary immunization should be made by subcutaneous injections of small volumes at multiple sites close to the animal's lymphatic system to favour 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, at volumes and intervals depending on the type of adjuvant used, until the antivenom titre reaches a plateau or a pre-established minimum accepted titre.**
- **After collection of blood for antivenom production, animals should have a resting period of 3–8 weeks. After this, a new round of immunization can be performed as above without the use of Freund's complete adjuvant.**
- **All steps in the immunization of the immunized donor animal, as well as the collection of blood or plasma should be traceable.**

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.

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 enables 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 the apheresis procedure.

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

When an immunized animal has developed an antivenom antibody titre that meets the necessary specifications, it can be bled. Before bleeding is performed, the animals should be evaluated by a veterinarian or other qualified person and declared healthy. Animals showing evidence of clinical deterioration, such as weight loss, drop in haemoglobin or serum protein concentration below a critical predefined value, or evidence of infections, should not be bled.

12.2 Premises for blood or plasma collection

The bleeding of animals should be performed in designated rooms or areas dedicated to this activity and equipped with appropriate restraining devices. 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. The rooms or areas should be thoroughly washed and cleaned before and after each bleeding session and their design should facilitate such cleaning procedures, which should be clearly established. The room or area should be inspected before the confinement of the animal. Animals need to be made as safe and comfortable as possible, in a quiet environment, during bleeding to minimize the chance of injury to the animal or its handlers. Individual animals should be confined in circumstances that reduce the potential for stress as much as possible. It is recommended that these rooms allow the simultaneous bleeding of various horses to reduce the time required for this operation as well as the stress.

12.3 Blood or 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 disinfectant that has not reached the end of its recommended shelf-life, and, depending on the type of disinfectant, it should be allowed to dry. The disinfected area should not be touched or palpated before the needle has been inserted.

Before venipuncture all containers and tubing should be inspected for defects (for example, abnormal moisture or discoloration as these may suggest a defect). There should be means to determine the volume of blood or plasma collected (such as a weighing machine).

The clinical condition of the animals being bled should be closely monitored at the time of bleeding and during the days that follow, and bleeding should be suspended in the event of any adverse effect on the animal. If an animal shows signs of distress during the operation, the collection procedure should be terminated. In addition, animals should be kept under observation for at least 1 hour after the bleeding to allow any evidence of physical alterations to be detected.

12.4 Labelling and identification

The identity of the animal should be recorded immediately before venipuncture. Labels on all bottles or bags of blood or plasma should be marked with the animal's unique identification number. The label should contain the following information: specificity of antivenom, plasma unit number and date of collection.

12.4.1 Collection and storage of whole blood

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 13–15 ml of blood per kilogram body weight are collected in one bleeding session. For sheep, 0.5 l is a typical yield, whereas in the case of horses, the volume of blood may range between 3 and 6 l, depending on the size of the animal.

Blood is collected, ideally, in disposable plastic bags containing sterile citrate anticoagulant. Usually, the volume ratio of anticoagulant to blood is 1:9. 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.

While 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 a 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 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

The bags or bottles in which the whole blood has been 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 for not more than 24 hours before the reinfusion of the red cells.

Alternatively, aseptically-collected blood can be stored for a maximum of 7 hours at 20–25 °C to allow for sedimentation. Under such circumstances, great care should be taken to avoid bacterial contamination.

12.4.1.3 Separation of plasma from whole blood

Hyperimmune plasma should be separated from blood cells under aseptic conditions and should be transferred into sterile containers (plastic bags, bottles, or stainless steel containers). A designated room, designed to allow proper cleaning and sanitization, should be used for separation. When bottles are used, separation of plasma 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

Reinfusion of the erythrocytes after whole blood collection is recommended.

Blood cells, most specifically erythrocytes (red blood cells), should be separated from plasma by validated centrifugation or sedimentation procedures. Erythrocyte reinfusion should take place within 24 hours after blood collection, and after being suspended in sterile saline solution at 32–37 °C prior to infusion. This procedure in which 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 hypovolaemic and it reduces the risks of handling errors, in particular during re-infusion of the erythrocytes to the donor. Plasma from automatic apheresis tends to be less contaminated by blood cells (red blood cells, leukocytes and platelets) and in the experience of some laboratories is easier to fractionate, as the 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 the two. 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 anticoagulant citrate dextrose formula A (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 litres per session. The number of cycles ranges from 10 to 20 depending upon the haematocrit of the horses. The collection process lasts for 1–4 hours. The apheresis equipment and apheresis procedures should be validated, maintained and serviced. Machine plasmapheresis can take several hours and animals can be fed during the operation.

12.4.2.2 Plasma storage

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

12.5 Pooling

Plasma from individual animals should be pooled into sterile and sanitized containers before fractionation. For traceability purposes each plasma pool should be identified with a unique number. The number of plasma units collected from individual animals and used in the pool should be recorded.

Such pooling should be performed in an environment suitable to prevent microbial contamination, like classified areas (class D (*59*)) and pools should be adequately identified. The room should be designed to allow for appropriate cleaning and sanitization of all surfaces. Individual or pooled plasma should be stored at 2–8 °C in a room dedicated for this purpose. To ensure the prevention of microbial contamination of plasma, preservatives (phenol or cresols)[1] can be added at a dose of less than 3 g/l at this stage and kept during storage of plasma. Care should be taken to dilute the phenol or cresols with water or saline solution before they are added to plasma, to avoid denaturation of plasma proteins. The transportation of containers or bottles

[1] In these guidelines, cresol isomers are referred to as cresols.

containing pooled plasma within the production facility or between facilities should be performed in such a way that contamination is avoided and the cold chain is maintained.

To avoid the risk of contamination, it is recommended that individual or pooled plasma is not stored for too long before fractionation, i.e. the plasma should be fractionated as soon as possible after pooling. In the event that plasma is stored for prolonged periods of time (for instance 6 months), the storage time and conditions should be validated to ensure that there is no detrimental impact on the quality of the plasma material, on the fractionation process, or on the quality, efficacy and stability of the antivenoms.

It is also the experience of some manufacturers that plasma can be stored frozen at −20 °C, particularly if no preservative is added.

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 (bioburden assay). The neutralizing potency of the starting plasma should be ensured so that the resulting antivenoms will be within potency specifications. Additional checks may include, when relevant, a test for pyrogenic substances and total protein content.

Plasma pools should be discarded if the bioburden exceeds a defined limit stated in the marketing dossier or if the neutralizing potency is below a minimum limit established by the producer. Cloudy plasma, below this defined bioburden limit, may still be used for fractionation provided the fractionation process and product quality has been proven not to 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 which 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 with a controlled environment.**
- **Plasma containers should be thoroughly cleaned on their external surfaces, adequately identified and stored in refrigerated rooms for further fractionation.**
- **Plasma should be checked prior to fractionation to establish compliance with relevant acceptance criteria for fractionation, in particular the neutralizing potency.**
- **Special attention should be paid to ensuring traceability between individual animal donors and the plasma pool.**

- **A certificate from a veterinarian or other qualified person should be issued stating that the donor animals were checked periodically to ensure that they were in good health at the time of plasma collection and during the follow-up observation period.**

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

13.1 Good manufacturing practices

The purification of immunoglobulins and immunoglobulin fragments for the production of antivenoms should aim at obtaining products of consistent quality, safety and efficacy. The fractionation processes used should adhere to the GMP principles developed for medicinal products. All operations should therefore be carried out in accordance with an appropriate quality assurance system and GMP. This covers all stages leading to the finished antivenoms, including the production of water, the production of plasma (animal selection and health control, production of venoms and immunization protocols, containers used for blood and plasma collection, anticoagulant solutions and quality control methods) and the purification, storage, transport, processing, quality control and delivery of the finished product. Of particular relevance is the control of microbiological risks, contamination with particulates and pyrogens, and the existence of a documentation system that ensures the traceability of all production steps. To establish 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 done at established critical steps for in-process quality control tests.

WHO *Guidelines on good manufacturing practices for medicinal products* are available (*59*) and the main principles of GMP for the manufacture of blood plasma products of human origin have also been published (*60*, *61*). These Guidelines can serve as a general guide for manufacturing practices in the production of antivenoms. A useful reference in the field of antivenoms is also the *Note for guidance on production and quality control of animal immunoglobulins and immunosera for human use* (CPMP/BWP/3354/99) (*62*).

13.2 Purification of the active substance

Antivenoms are prepared from the starting plasma pool using diverse methods to obtain one of the following active substances:

— intact IgG molecules;

— $F(ab')_2$ fragments; or

— Fab fragments.

In general, fractionation procedures should not impair the neutralizing activity of antibodies; it should yield a product of acceptable physicochemical characteristics and purity with a low content of protein aggregates, which is non-pyrogenic and which 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 standard operating procedures. 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 these manufacturing procedures have often been developed by individual

fractionators and should be considered as acceptable when shown to yield consistently safe and effective preparations of antivenoms.

13.2.1 Purification of intact IgG antivenoms

13.2.1.1 Ammonium sulfate precipitation

In the past, most laboratories have used fractionation protocols based on salting-out procedures employing ammonium sulfate or sodium sulfate (*63*). 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 lead to a recovery of antibodies of between 40 and 50% and to the formation of protein aggregates. The final product of this procedure used to contain a relatively high proportion of contaminating proteins, such as albumin (*64*). This compromised the safety of the product, since a high incidence of early adverse reactions has been described in response to such intact IgG antivenoms (*65*).

13.2.1.2 Caprylic acid precipitation

The use of caprylic acid (octanoic acid) as an agent for precipitating proteins from animal plasma has been described in the literature (*66*). Several procedures for the purification of whole IgG antivenoms with a good physicochemical profile and purity using caprylic acid precipitation of non-immunoglobulin proteins have been developed (*64*, *67*, *68*) and are now used for the production of licensed antivenoms.

Figure 3 illustrates a particular process in which caprylic acid is added slowly to undiluted plasma, with 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 1 hour. The precipitated proteins are removed by filtration or centrifugation and discarded. The filtrate or the supernatant containing the immunoglobulins is then submitted to tangential flow filtration to remove residual caprylic acid and low-molecular-mass proteins, depending on the molecular cut-off of the ultrafiltration membranes, and to concentrate the proteins. The immunoglobulin solution is then formulated by adding sodium chloride solution (NaCl), an antimicrobial agent and any other excipient(s) needed, such as stabilizers. The pH is then adjusted to a neutral value and finally subjected to sterile filtration through a filter of pore size 0.22 µm, and dispensed into final containers (vials or ampoules). Variations of this procedure have been introduced by various manufacturers, and include dilution of plasma, changes in caprylic acid concentration, pH, and temperature among others.

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 (*65*, *69*).

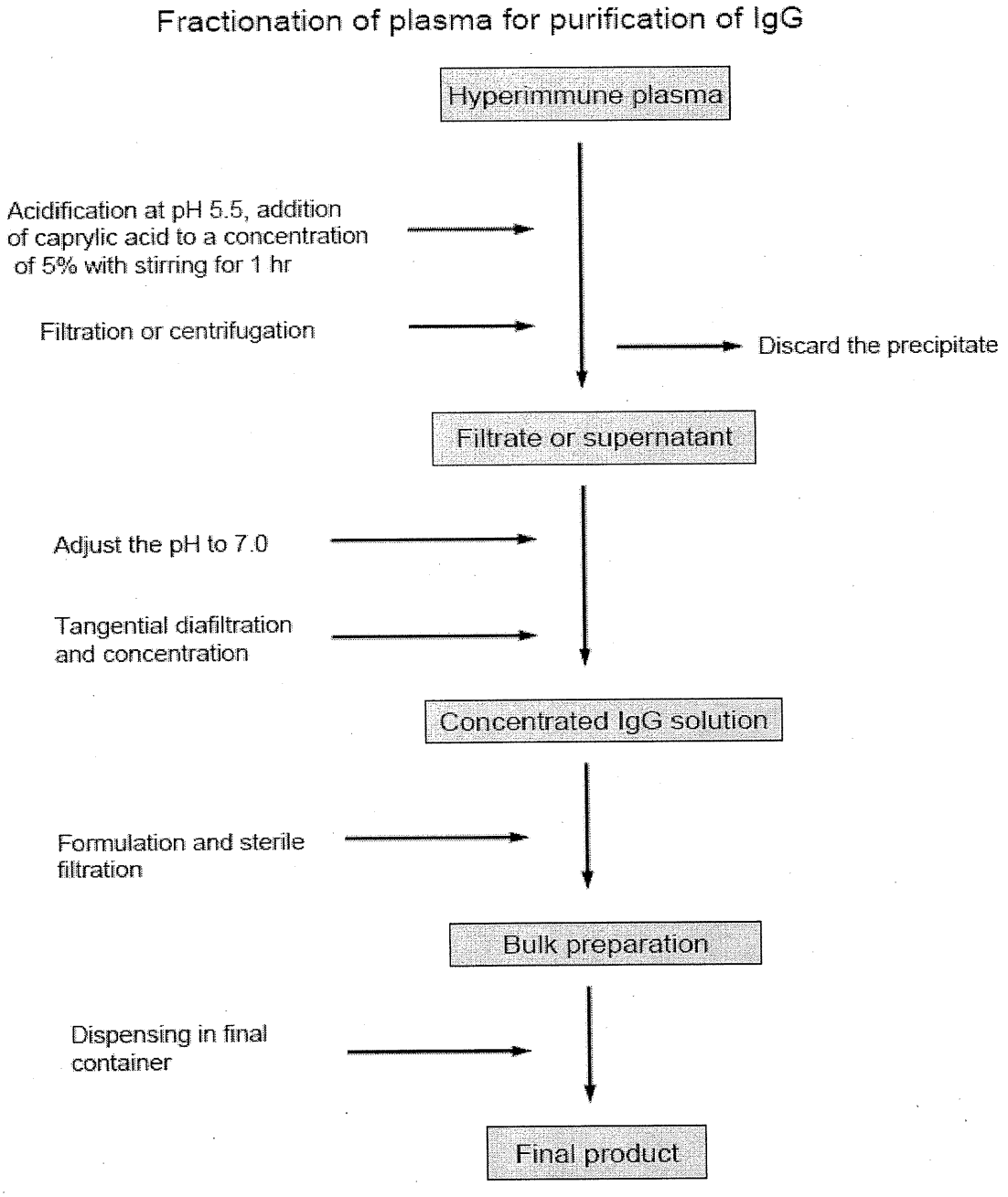

Figure 3
Example of a fractionation process in which intact IgG is prepared by caprylic acid precipitation of non-immunoglobulin proteins

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

Many manufacturers follow the classical protocol for F(ab')$_2$ antivenom production developed by Pope (*6*, *7*), with a number of recent modifications (*9*, *10*, *70*).

The method of pepsin digestion (see Figure 4) involves the digestion of horse plasma proteins by pepsin, leading to the degradation of many non-IgG proteins, and to the 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 sulfate are also key elements of this methodology. Some procedures involve performing the pepsin digestion step on a pre-purified IgG fraction that is obtained by treatment of plasma with ammonium sulfate to obtain an IgG-enriched precipitate, whereas albumin is not precipitated.

Pepsin digestion is accomplished at a pH of 3.0–3.5. A typical protocol is based on incubation at pH 3.3 for 1 hour, at 30–37 °C in a jacketed tank, with a pepsin concentration of 1 g/l. Other procedures can be used which give similar results. Each manufacturer should adjust the pepsin concentration to achieve the required enzymatic activity.

13.2.2.1 Downstream processing using ammonium sulfate

After pepsin digestion, the pH is adjusted to 4.5–5.0, by adding NaOH or a weak alkaline buffer; then ammonium sulfate is added with 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 1 hour; this is known as "thermocoagulation"). After thermocoagulation, the preparation is cooled down to less than 30 °C, e.g. by passing cold water through a jacketed vessel. The resulting fraction is filtered or centrifuged to remove the precipitate. The pH is then adjusted to 7.0–7.2 with NaOH, and a solution of ammonium sulfate is added with 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 desalted (to remove the ammonium sulfate) 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, the 23% (w:v) step is bypassed by some manufacturers and, directly after the heating step, the filtrate obtained is subjected to ultrafiltration. Additional precipitation could also be applied on the starting material at a low ionic strength and acid pH to remove "euglobulins" (*10*).

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 a neutral value. Finally, the preparation is sterilized by filtration through 0.22-µm filters, and dispensed into final containers (vials or ampoules). Such a process, or similar ones developed by other manufacturers, using pepsin digestion, ammonium sulfate 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%.

13.2.2.2 Downstream processing using caprylic acid

Purification of F(ab')$_2$ has also been shown, on an experimental scale, to be achievable by caprylic acid precipitation of non-F(ab')$_2$ proteins after pepsin digestion, with an improved yield (~60%) (*71*). However, the yield obtained on a large scale has not been reported. Figure 5 shows a fractionation scheme of F(ab')$_2$ using caprylic acid. F(ab')$_2$ is 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.

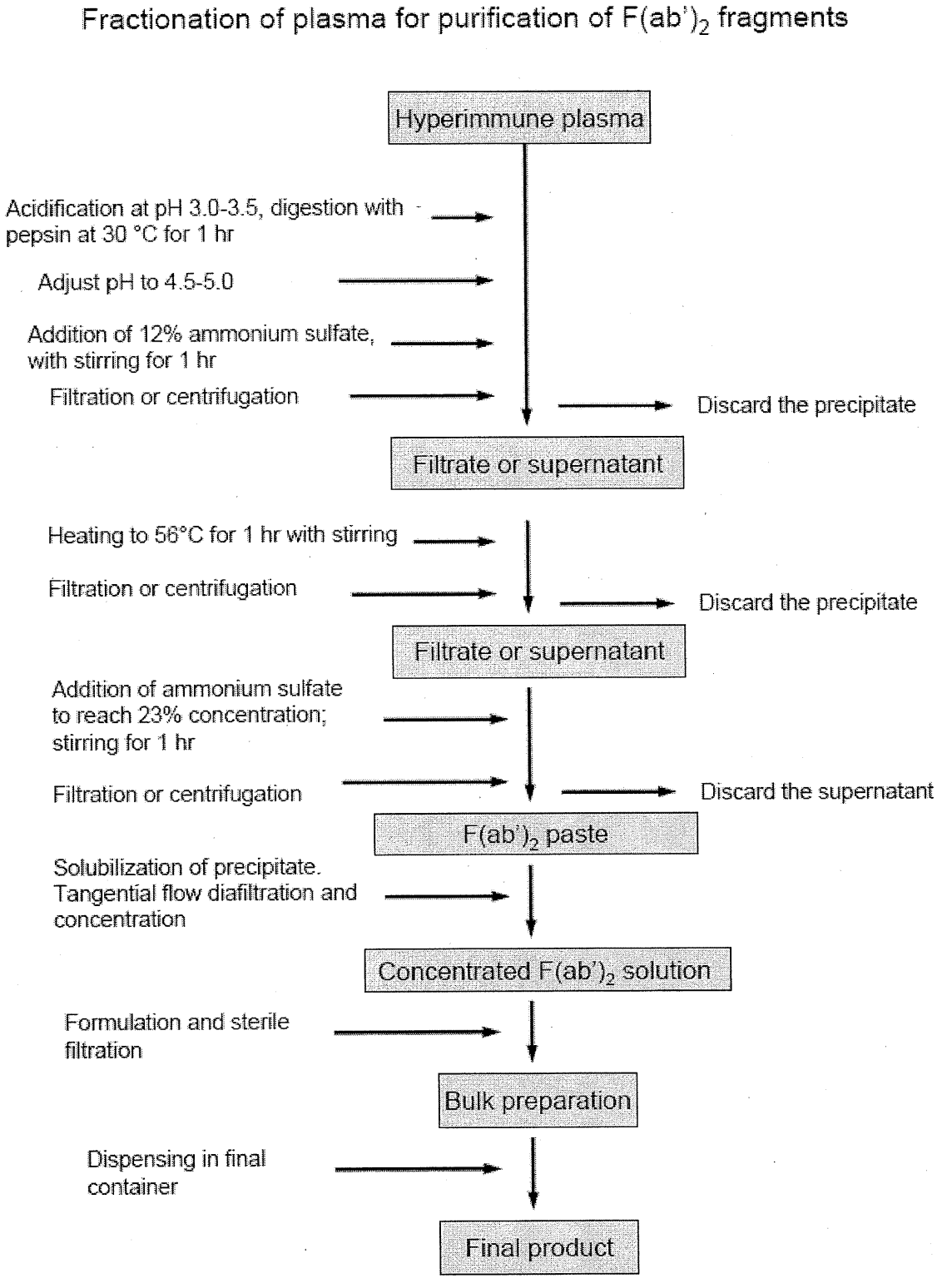

Figure 4
Example of a fractionation process in which $F(ab')_2$ fragments are prepared by pepsin digestion and ammonium sulfate precipitation

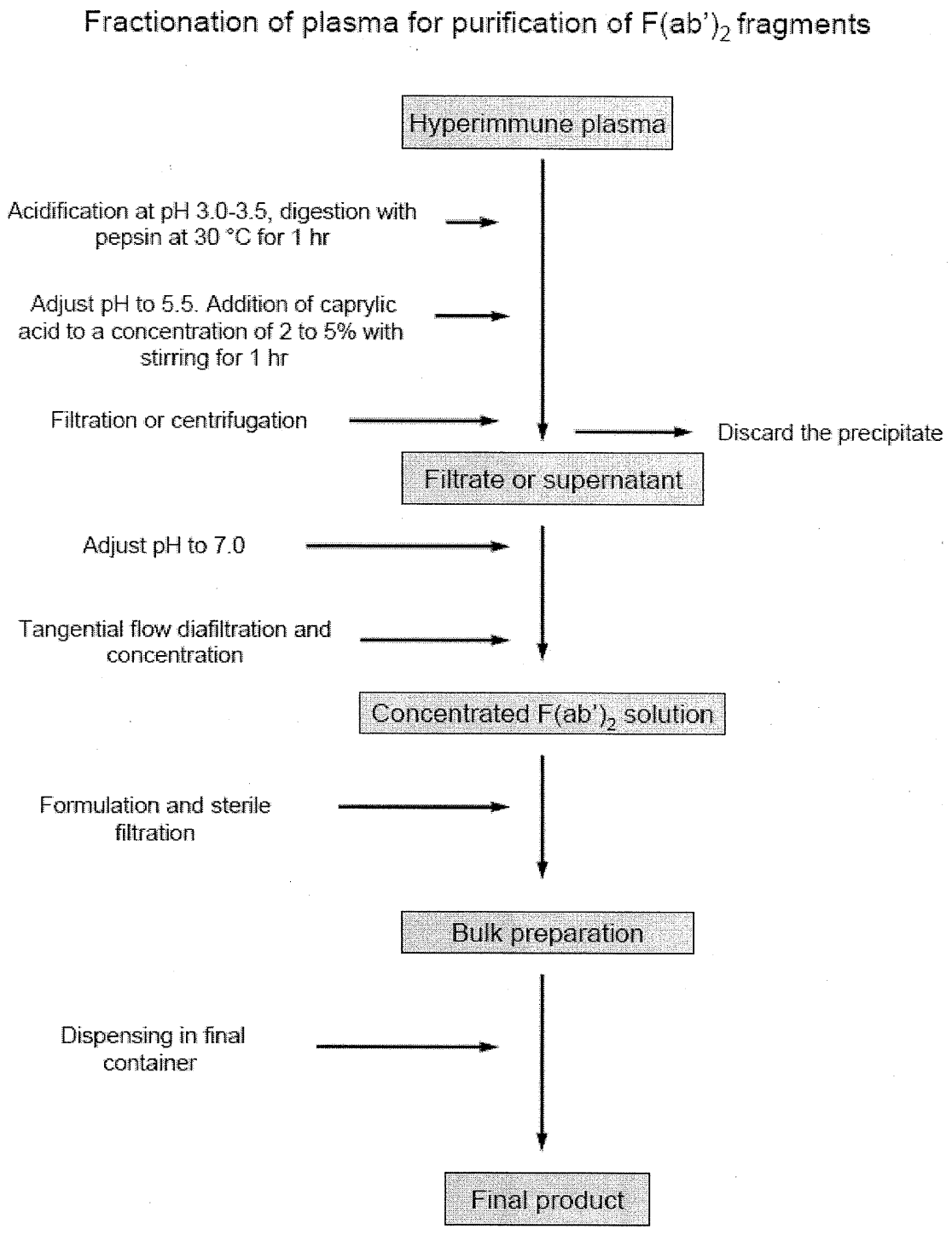

Figure 5
Example of a fractionation process in which F(ab')$_2$ fragments are prepared by pepsin digestion and caprylic acid precipitation

13.2.3 Purification of Fab antivenoms

Production of monovalent Fab fragments is performed by some manufacturers (*72*), currently using hyperimmunized sheep plasma. Papain is used to carry out the enzymatic digestion, and the process of preparation of the fragment may use ammonium sulfate, sodium sulfate or caprylic acid.

Figure 6 shows a process in which immunoglobulins are precipitated from plasma by adding ammonium sulfate or sodium sulfate to a concentration of 23%. After filtration the filtrate is discarded and the immunoglobulin precipitate is dissolved in a sodium chloride solution at pH 7.4. Papain is added and digestion performed at 37 °C for 18–20 hours in a jacketed tank. Reaction is stopped by adding iodoacetamide. The product is then applied to a diafiltration system to remove iodoacetamide, salts and low-molecular-mass peptides and equilibrated with a buffered isotonic NaCl solution. The preparation is then chromatographed on an anion-exchanger (usually in quaternary aminoethyl (QAE)-based or diethylaminoethyl (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 and any other excipients needed, and the pH is adjusted. Finally, the preparation is sterile-filtered and dispensed into the final containers.

13.2.4 Optional additional steps used by some manufacturers

When performed following GMP 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 those described below.

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 DEAE or QAE gels or membranes, such as quaternary ammonium cellulose microporous membranes, can be used at neutral pH to adsorb protein contaminants (*10*, *70*, *73*). Alternatively, cation-exchange columns, e.g. carboxymethyl or sulfopropyl gels, have been used for purification of IgG or $F(ab')_2$ fragments (*71*). The column is equilibrated at acid pH, e.g. pH 4.5, to bind the antivenom, whereas protein contaminants are eluted in the break-through.

Chromatographic procedures should be applied following GMP. Columns should be adequately regenerated, sanitized, and stored to prolong their useful lifetime. The reproducibility of columns over cycles should be validated. Measures to avoid batch to batch contamination should be in place. Specific standard operating procedures 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 (*74*). However, columns usually deteriorate rather rapidly, and meticulous care should be taken to wash, sanitize and store them under appropriate conditions. Procedures should be followed to ensure that any substances leaching from the columns do not affect the quality and safety of the product or else are completely removed during downstream processing; this is especially critical in affinity chromatography using immobilized venom. Affinity processes may affect recovery and high-affinity antibodies may be lost and/or denatured owing to the harsh elution conditions needed to elute them from the chromatographic material.

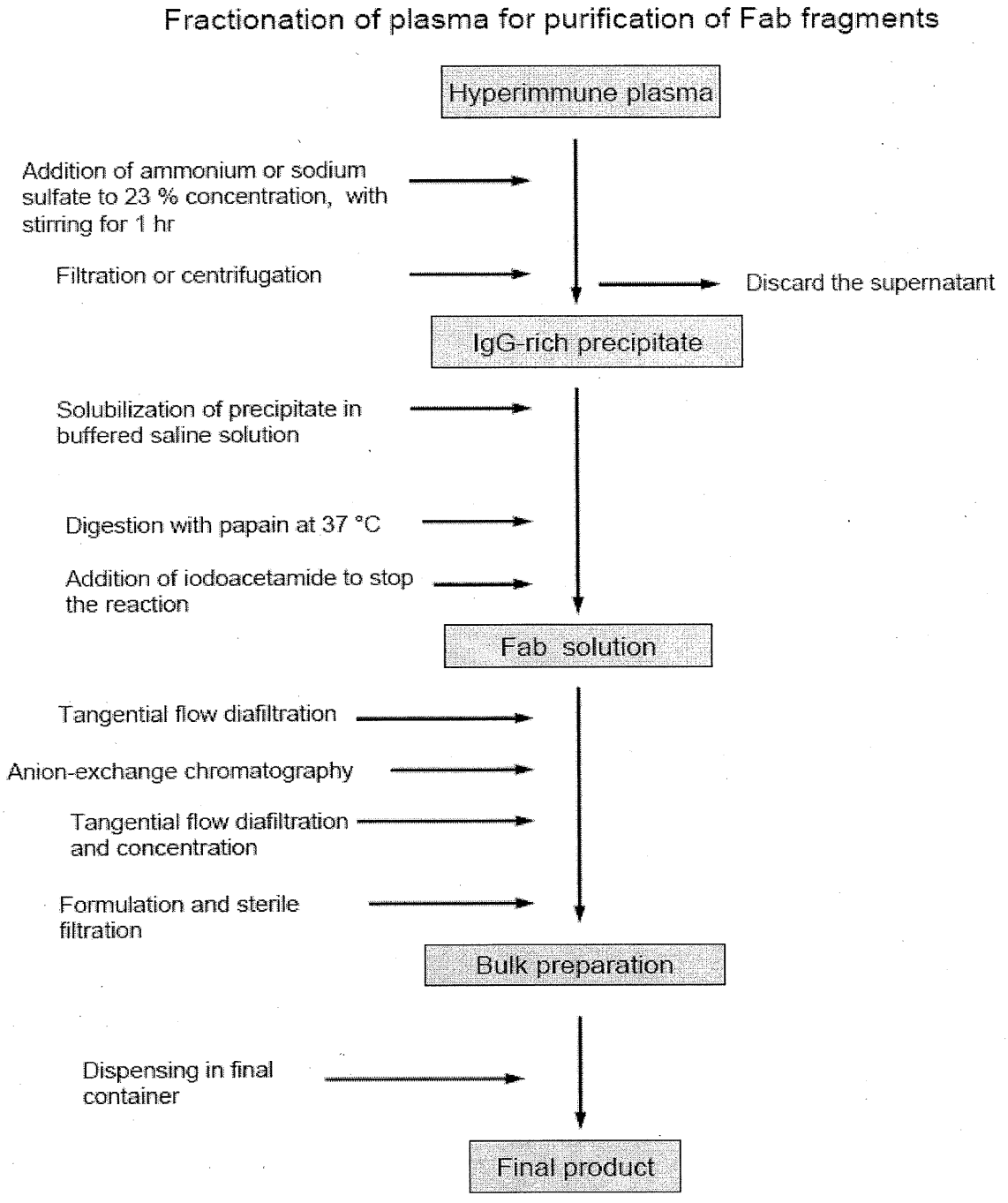

Figure 6

Example of a fractionation process in which Fab fragments are prepared by papain digestion and ammonium sulfate precipitation

13.2.4.3 Process improvement

Some manufacturers have introduced process improvements to enhance the quality or the yield of antivenoms. These include the use of a depth filtration system combined with filter-aids to facilitate filtration steps and improve antivenom recovery. In addition, other manufacturing steps may be introduced to ensure inactivation or removal of infectious agents (see section 14).

13.2.5 Formulation

During formulation of antivenoms after diafiltration steps one should consider the addition of salts to adjust the osmolality, addition of preservatives, other excipients, if needed for protein stability, and the adjustment of pH.

In general, antivenoms are formulated at neutral pH (pH 7.0 ± 0.5) although some manufacturers are exploring the feasibility of formulation at more acidic pH to improve stability and/or to reduce aggregate formation.

Formulation at pH higher than 7.5 is not recommended, since the stability of immunoglobulins and their fragments at alkaline pH may be poor, and the formation of aggregates may be favoured.

13.2.6 Analysis of bulk product before dispensing

The biological, physical and chemical characteristics of the final bulk product should meet pre-established specifications before dispensing. Analysis may include tests required to demonstrate:

- the purity and potency of the product;
- the sterility;
- the compliance with the specifications for the aggregate content;
- the pyrogen limit and/or the bacterial endotoxin content; and
- the formulation.

When the product is formulated in liquid form, some of these tests (such as the potency assay) may not need to be duplicated on the final container if the processing after the bulk preparation has been validated and shown not to alter this activity.

The sterilization equipment and the integrity of the membrane should be guaranteed before sterilization; moreover, the aseptic filling should be validated.

13.2.7 Dispensing and labelling of final product

Once compliance of the final bulk product with the quality control specifications is established, the final product is bottled. For this, final glass containers (vials or ampoules) should be used. General principles prevailing for the dispensing of parenteral medicinal products should be applied. The dispensing should be performed in class A (*59*) clean room conditions, usually under a laminar flow hood. The equipment used for dispensing should be calibrated beforehand to ensure that the correct volume is delivered.

In the case of ampoules, the dispensing system should ensure an aseptic closure and the sealing of the ampoule should prevent risk of protein denaturation due to heat. For vials, insertion of rubber stoppers should be done inside this clean dispensing area. The quality of the rubber stoppers should be such as to guarantee inertness and to prevent leaching. Thereafter, aluminium seals should be placed on each vial in a clean area outside the class A area. Ampoules or vials containing the final product should then be properly identified and stored in a quarantine area

maintained under proper storage conditions. Samples of the antivenoms should be sent to the quality control laboratory for analysis.

When an antivenom complies with all the quality control tests established for the final product, it should be properly labelled and identified. The vial or ampoule should be labelled with, at least, the following information:

- name of the product and of the producer;
- animal species used to produce the antivenom;
- batch number;
- pharmaceutical presentation (liquid or freeze-dried);
- volume content;
- administration route;
- specificity (venoms neutralized by the antivenom, including both the common and the scientific name of the snake(s)[1]);
- neutralizing potency;
- storage conditions; and
- expiry date.

Additional information may be requested by the national regulatory authorities. The package, which is usually a cardboard box, in which the vials or ampoules are packed, should include the same information as is given on the primary container. The package insert should include all the information relating to the product, as established by national regulatory agencies, including:

- the neutralizing potency;
- the recommended dosage;
- reconstitution procedure, if lyophilized;
- the mode of administration (e.g. the dilution of antivenom in a carrier fluid such as saline);
- the rate of administration;
- details on the symptoms and treatment of early and delayed adverse reactions;
- snake species against which the antivenom is effective;
- recommended storage conditions, and
- an indication that the product is for single use.

13.2.8 Use of preservatives

The addition of preservatives to prevent bacterial and fungal contamination should be kept to a minimum during plasma storage and during fractionation. Their inclusion during the manufacturing process should be clearly justified, and should never substitute for any aspect of GMP. Preservatives can be considered in the final product, especially if it is manufactured in liquid form, and most specifically preservatives are required for multiple-dose presentations. Antimicrobial agents currently used in antivenom formulation include phenol and cresols. In

[1] Special care should be taken considering the frequent changes in snake species taxonomy.

general, phenol concentration is adjusted to 2.5 g/l, and concentration of cresols should be less than 3.5 g/l. The concentration of preservatives should be validated by each production laboratory on the basis of assays to test their efficacy and keeping in mind that they may degrade with time and cease to be effective. It is necessary to ascertain that any agent used has no potential detrimental interaction with the active substance and excipients of antivenoms. Any change in the formulation involving preservatives, or the elimination of preservatives from the final product, requires a very careful risk–benefit assessment on various microbial safety aspects, as well as a detailed validation procedure. Mercury-containing preservatives are not recommended in antivenom manufacture. The volume of antivenom required for the treatment of envenoming (in excess of 50 ml) might lead to an exposure to mercury far higher than the amounts currently used for other biological preparations and levels at which they are toxic, especially in young children, are not known (*75*, *76*).

13.2.9 Freeze-drying

Antivenoms are available either as liquid or as freeze-dried preparations. Freeze-dried antivenoms, which may usually be stored at a temperature not exceeding 25 °C, are generally distributed to markets where the cold chain cannot be guaranteed, such as in many tropical regions of the world. The absence of guarantee of a cold chain during distribution highlights the need for manufacturers to demonstrate the stability of the antivenoms under the high temperatures found in tropical climates.

Freeze-drying is a critical operation. Careful attention should be given to the rate of freezing as well as to the protocol used for the primary and secondary drying cycles (*77*). The details of the freeze-drying protocols are product-specific and should be adjusted according to the particular formulation of each antivenom. Inadequate freeze-drying protocols may affect the physicochemical quality of the product, inducing protein precipitation and denaturation, as well as aggregate formation, and altering stability and reconstitution. Specific stabilizers, such as sugars or polyols, aimed at protecting proteins from denaturation and aggregation, may be added to the final formulation of the antivenom. Bulking agents, frequently used for some biological products, are generally not required in the case of antivenoms owing to their relatively high protein concentration; however they are sometimes used for high-titre monospecific antivenoms.

13.2.10 Inspection of final container

All the vials or ampoules of each batch of liquid antivenoms should be inspected, either visually, or using a mechanical device. Any vial or ampoule presenting turbidity, abnormal coloration, presence of particulate matter, or defects of the vial, stopper, or capsule should be discarded. In the case of freeze-dried products, a representative sample of the whole batch should be dissolved in the solvent and inspected as described. Turbidity can be assessed quantitatively by using a turbidimeter.

13.2.11 Archive samples of antivenoms

In compliance with GMP, manufacturing laboratories should archive a number of vials of each antivenom batch, under the recommended storage conditions, in an amount that would enable the repetition of all quality control tests, when required.

13.3 Pharmacokinetic and pharmacodynamic properties of IgG, $F(ab')_2$ and Fab

Owing to their different molecular mass, the pharmacokinetics of heterologous IgG molecules (approximately 150 kDa) and $F(ab')_2$ (approximately 100 kDa) and Fab (approximately 50 kDa) fragments differ significantly. In envenomed patients, Fab fragments have the largest volume of distribution and readily reach extravascular compartments. Fab fragments are, however, rapidly eliminated, mainly by renal excretion, thus having a short elimination half-life (from 4–24 hours) (*78*, *79*). In contrast, $F(ab')_2$ fragments and intact IgG molecules are not eliminated by the renal route and therefore have a more prolonged elimination half-life (between 2 and 4 days) (*11*, *80*, *81*). Such different pharmacokinetic profiles have important pharmacodynamic implications, and the selection of the ideal type of active substance in an antivenom should rely on a careful analysis of the venom toxicokinetics and antivenom pharmacokinetics.

Another difference between low-molecular-mass fragments, such as Fab and those with a higher molecular mass, such as $F(ab')_2$ and IgG, is the number of paratopes of each molecule: Fab has one antigen binding site while IgG and $F(ab')_2$ each have two binding sites. Thus they will be able to form large and stable complexes or precipitates with antigens carrying several epitopes, while the former will form small, reversible non-precipitable complexes.

Ideally, the volume of distribution of an antivenom should be as similar as possible to the volume of distribution of the main toxins in a particular venom; however, this is rarely the case. In venoms composed of low-molecular-mass toxins, such as some elapid snake venoms, low-molecular-mass neurotoxins are rapidly absorbed into the bloodstream and are rapidly distributed to the extravascular spaces where toxin targets are located. Furthermore, low-molecular-mass toxins are eliminated from the body in a few hours. In these cases, an antivenom of high distribution volume that readily reaches extravascular spaces, such as as Fab, might be convenient, although its action is then eliminated within a few hours. It should be noted, however, that a number of elapid venoms contain some high-molecular-mass toxins of great clinical significance, such as procoagulants and pre-synaptic neurotoxins.

In contrast, in the case of viperid snake venoms and other venoms made up of toxins of larger molecular mass, including a number of elapid venoms, many of which act intravascularly to provoke bleeding and coagulopathy, the situation is different. The time required for toxins to distribute to extravascular spaces is longer than in the case of low-molecular-mass neurotoxins, and the targets of some of these toxins are present in the vascular compartment. In addition, the toxins of viperid venoms have a long half-life in vivo and can remain in the body for several days (*82*, *83*). In this case, an antivenom made by Fab fragments neutralizes the toxins that reach the circulation but, after a certain time has elapsed, the Fab fragments are eliminated and non-neutralized toxins reach the circulation, thus giving rise to the well-known phenomenon of recurrent envenoming, i.e. the reappearance of signs and symptoms of envenoming at later time intervals after an initial control of envenoming. This situation demands repeated administration of antivenom to maintain therapeutic levels of Fab in the circulation (*84*). Therefore, in such envenomings, antivenoms made of IgG or $F(ab')_2$ may be more appropriate because of their longer elimination half-lives. Moreover, it has been proposed that formation of venom–antivenom complexes in the circulation results in the redistribution of venom components from the extravascular space to the blood compartment, where they are bound and neutralized by circulating antivenom, provided that the dose of antivenom is sufficient (*85*, *86*). Consequently, the maintenance of a high concentration of specific antivenom antibodies in the circulation for many hours is required for complete neutralization of toxins reaching the bloodstream during both early and late phases of envenoming (redistribution of toxins) present in the extravascular space. In conclusion, IgG and $F(ab')_2$ antivenoms have a pharmacokinetic profile that makes them more effective in many types of snakebite envenoming.

13.4 Main recommendations

- **Antivenoms should be manufactured using fractionation procedures that are well established, validated, and shown to yield products with proven safety and efficacy. Fractionation processes used for the manufacture of antivenoms should adhere to the principles of GMP for parenteral medicinal products.**
- **Antivenoms can be comprised of intact IgG molecules, $F(ab')_2$ fragments or Fab fragments. Intact IgG antivenoms are mainly produced by caprylic acid precipitation of non-IgG plasma proteins, leaving a highly purified IgG preparation in the supernatant or filtrate.**
- **$F(ab')_2$ fragment antivenoms are produced by pepsin digestion of plasma proteins, at acidic pH, usually followed by $F(ab')_2$ purification by salting out with ammonium sulfate solutions or by caprylic acid precipitation. Fab monovalent fragments are obtained by papain digestion of IgG at neutral pH.**
- **Further to ultrafiltration to remove low-molecular-mass contaminants, preparations are formulated, sterilized by filtration and dispensed in the final containers. Formulations of antivenoms may include preservative agents.**
- **Antivenoms can be presented as liquid or freeze-dried preparations. Freeze-drying of antivenoms should be performed in conditions that ensure no denaturation of proteins and no formation of protein aggregates.**
- **IgG, $F(ab')_2$ and Fab antivenoms exhibit different pharmacokinetic profiles: Fab fragments have a larger distribution volume and a much shorter elimination half-life. Thus, for viperid envenomings, IgG or $F(ab')_2$ antivenoms have a more suitable pharmacokinetic profile, whereas Fab fragments may be useful for the neutralization of venoms rich in low-molecular-mass neurotoxins which are rapidly distributed to the tissues. However, in general terms, IgG and $F(ab')_2$ antivenoms have shown a better pharmacokinetic profile than Fab antivenoms.**

14 CONTROL OF INFECTIOUS RISKS

14.1 Background

The viral safety of any biological product results from a combination of measures to ensure a minimal risk of viral contamination in the starting material (e.g. plasma), together with steps to inactivate or remove potential contaminating viruses during processing.

There are currently several recognized complementary approaches used for virus risk reduction for biological products. These are:

- minimizing the potential initial virus content by implementing a quality system for the production of the starting material;
- contribution of the manufacturing processes to inactivating and/or removing residual viruses during manufacture of the biological product; such a contribution can be inherent to the existing production technology or may result from the introduction of dedicated viral reduction steps;
- adhesion to GMP at all steps of the manufacturing process;
- appropriate and timely response to any infectious events recognized during the clinical use of the product.

Production steps to inactivate and/or remove viruses have long been shown to play a powerful role in ensuring safety of biologicals (*60*). Similarly, keeping to a minimum the potential viral load at the stage of the plasma pool, through appropriate epidemiological surveillance and health control of the donor animals, is also an important safety measure (see section 10).

Based on experience with human plasma products, a production process for antivenoms that includes two robust steps for viral reduction (comprising preferably at least one viral inactivation step) should provide a satisfactory level of viral safety. However, it should be kept in mind that non-enveloped viruses are more difficult to inactivate or remove than lipid-enveloped viruses.

14.2 Risk of viral contamination of the starting plasma

The main structural characteristics of viruses reported to possibly infect horses, sheep and goats are presented in Tables 7 and 8. They include viruses with a DNA or RNA genome, with and without a lipid envelope, and varying widely in size (22 to 300 nm).

A few of these viruses have been identified as possibly present, at least at some stages of the infection cycle, in the blood, or are considered as being pathogenic to humans. Special attention should be paid to these viruses.

14.3 Viral validation of manufacturing processes

Understanding how much a manufacturing process may contribute to the viral safety of antivenoms is fundamental to both manufacturers and regulators. Such an understanding can only be achieved by viral validation studies. These studies are complex and require well-established virology laboratory infrastructure and cell culture methodologies. They are usually carried out by specialized laboratories, outside the manufacturing facilities.

The principles guiding such studies have been described in WHO Guidelines (*60*) and are summarized below.

14.3.1 Down-scale experiments

The contribution of manufacturing processes towards inactivation and/or removal of potential viral contamination should be demonstrated. For this purpose, viral validation studies should be performed using at least three viruses exhibiting different structural characteristics. The antivenom manufacturer should first identify the steps that, based on the existing literature, are likely to remove or inactivate viruses and, then, provide evidence and quantitative assessment of the extent of virus reduction achieved for the specific process evaluated.

Validation should be done by down-scale experiments. The accuracy of the down-scale process should be assessed by comparing the characteristics of the starting intermediate and the fraction resulting from that step, for both the laboratory and the production scales. Selected physical factors (e.g. temperature, stirring or filtration conditions) and chemical factors (e.g. pH or concentration of precipitating agents such as caprylic acid) should be as close as possible to those used at manufacturing scale.