Purification techniques for heterologous rabies antiserum

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In view of the high costs of rabies immunoglobulin of human origin (HRIG), heterologous (mainly equine) immunoglobulins are still frequently prescribed for the prevention of rabies in persons who have been severely exposed (category III) to the virus, even though they may cause sensitization and are eliminated more rapidly than HRIG.

Purification techniques can be used to reduce the risk of sensitization to ERIG. Their objective is to maximize the specific activity and to minimize the allergic substances in the product. When these techniques are implemented, it is advisable to adhere to the recommendations of the WHO Expert Committee on Biological Standardization.

The purification of immunoglobulins from human plasma is carried out according to the technique of Conklin et al. (2), based on the selective precipitation of proteins by chilled ethanol. This technique has been adapted for purifying heterologous immunoglobulins and was described in the previous edition (3). This chapter describes two other techniques for the purification of ERIG, which may be applied to clarified equine serum or plasma.

Preservation and storage of serum or plasma

After coagulation of the collected blood and retraction of the clot for 48 hours at 2–6°C, it is possible to obtain serum by spinning. Plasma can be obtained by decanting the collected blood into an appropriate volume of anticoagulant solution, e.g., citric acid buffer, pH 4.8 (see Annex; 1 litre per 10 litres of blood), and spinning after 12 and 24 hours at 2–6°C.

Plasma and serum are usually stored frozen at −20°C. Alternatively, an antiseptic may be added and the blood derivatives kept at 2–8°C until purification. The antiseptics most commonly used are phenol and methyl derivatives (creosol or metacresyl), e.g., 0.15–0.20% metacresyl acetate. The latter is preferable to phenol, which can yield coloured derivatives during purification. Phenol or its derivatives are initially diluted to 10 times the required concentration with distilled water and added very slowly, while shaking, to the serum or plasma to avoid precipitation of the protein.

All of these procedures should be carried out under conditions close to asepsis to avoid microbial contamination and proliferation, which could lead to pyrogens being present in the final product.

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During refrigerated storage, serum and plasma generate a sediment or coagulum which should be eliminated by continuous centrifugation at 5600 g and 15°C in a centrifuge equipped with a 50-litre retention bowl. Between 500 and 600 litres of serum or plasma may be centrifuged per hour. Starting from these clarified sera, two purification techniques may be applied.

Purification by enzyme treatment and heat denaturation

This technique (4–7) consists of:
- cleavage of the immunoglobulins by a proteolytic enzyme, pepsin, followed by separation of the F(ab’)2 fragments, responsible for their protective activity, from the Fc fragment;
- selective denaturation by heat;
- fractionated precipitation by neutral salts, such as ammonium sulfate.

Elimination of albumin and pigments

1. Collect the effluent of centrifugation from the retention bowl in a stainless steel or glass tank equipped with a stirrer. Dilute by half with distilled water.
2. Add pure crystalline ammonium sulfate slowly, while stirring, to give a final concentration of 1.75 mol/l. Check the pH and adjust to close to neutrality if necessary.
3. After at least 6 hours, collect the precipitate by filtering the mixture on a filter press equipped with nylon gauze (500 litres/hour per m² of gauze under 196 Pa). The filtrate, which must be clear, should be discarded. It contains most of the albumin, other non-precipitable proteins, pigments and free antiseptic, which could react with the immunoglobulins during subsequent procedures.
4. Resuspend the precipitate in a volume of distilled water equal to twice the volume of serum or plasma used.

Pepsin digestion

Place the protein suspension obtained in a thermoregulated double-walled reactor equipped with a stirrer. Adjust the pH to 3.2–3.3 with dilute hydrochloric acid (HCl) and add an appropriate amount of pepsin (about 1–2 g per litre of the serum or plasma to be purified). The temperature should be increased to 32°C, with stirring, and maintained for 30 minutes.

Pepsin digestion is essential and must be carefully controlled. The pepsin is tested to ensure its constant quality from one batch to the next and thereby the reproducibility of each digestion. The technique described by Arson (7), using proteolysis carried out with bovine haemoglobin, is suitable for this purpose.

Moreover, it is necessary to control the purity of pepsin and to check whether it is free of blood group A factor, which could contaminate the purified immunoglobulins. For this purpose, the absence of blood group A factor should be verified with appropriate tests, which should include an anti-A serum of human origin as control. If this is not possible, the pepsin can be purified by adsorption on calcium phosphate gel at pH 6.0–6.1.
Selective denaturation by heat

1. At the end of digestion, adjust the pH of the purified suspension to 4.3 with dilute ammonium solution (NH₄H₂).
2. Add an appropriate amount of ammonium sulfate (NH₄SO₄), while stirring, to achieve a final concentration of NH₄SO₄ of 0.75 mol/l.
3. Add 1 ml of toluene and 5 g of activated charcoal per litre of suspension.
4. Bring the temperature as rapidly as possible to 55 °C, and when this temperature has been reached, filter immediately on a filter press equipped with type K2 Seitz filter discs.
5. Rinse the filter with a 0.75 mol/l solution of ammonium sulfate and conserve the filtrate.

Adjustment of the pH of a concentrated suspension requires certain precautions. The suspension should be diluted to a lower concentration of 0.3 mol/l with distilled water before determining the pH.

Concentration of the protective fractions

1. After adjusting the pH of the filtrate to neutrality, increase the concentration of ammonium sulfate in the filtrate to 1.75 mol/l.
2. Leave the suspension to stand for several hours, and then filter on a filter press equipped with nylon gauze with a very fine mesh (500 litres/hour per m² of gauze under 196 Pa). Carefully collect the precipitate, which contains the fractionated immunoglobulins.

Dialysis

All of the above steps are conducted in the presence of a concentration of ammonium sulfate sufficiently high to be bacteriostatic. From this stage of dialysis onwards, precautions should be taken to avoid bacterial contamination as the medium is very favourable to microbial proliferation.

Dialysis, to eliminate all traces of ammonium sulfate, is controlled with the aid of Nessler's reagent on the dialysate, and ultimately on the retained material. Dialysis is carried out by diafiltration using filters with a relative molecular mass cut-off of 10,000. However, if this type of equipment is not available, a haemodialysate or dialysis tubes sterilized with a 0.5% phenol solution can be used.

If necessary, a preliminary ultrafiltration of the dialysate may be carried out to ensure that no pyrogenic substances are present.

Clarification

The technique most commonly used consists of adsorption onto aluminium hydroxide gel. The gel can be activated with glutamic acid (A. Hansen, personal communication). The required quantity of gel is approximately 3–5 g (expressed in aluminium oxide) per litre of solution. The protein concentration of the solution is adjusted to 8–10%. After several hours, the suspension is centrifuged at 5600 g, then filtered on a filter press equipped with type EK Seitz filter discs. Approximately 150 litres of suspension can be centrifuged per hour.
LABORATORY TECHNIQUES IN RABIES

Purification by precipitation using ethacridine lactate and ethanol

*Ethacridine lactate precipitation*

This technique was introduced in 1966 for the purification of HRIG, and has since been adapted to the purification of FRIG. Ethacridine lactate is added to the serum or plasma to give a final concentration of 5.2 g per litre. Approximately 90% of the added amount is eliminated with the precipitated proteins, while the remainder, which ensures the complete precipitation of unwanted proteins, is removed by adsorption on charcoal.

1. Add the calculated volume of aqueous 1.2% ethacridine lactate solution to the clarified serum or plasma during 15-30 minutes, stirring well.
2. Stir the mixture for a further 30-40 minutes at 20°C and then hold at the same temperature for 1-2 hours without stirring.
3. Carefully decant the supernatant and centrifuge the precipitate at 5600 g and 20°C for 30 minutes.
4. Determine the volume of the resulting supernatant combined with that obtained in step 2. For each litre of this solution, add 6 g of activated charcoal to adsorb the dissolved ethacridine lactate. Stir the mixture for at least 2 hours at room temperature.
5. Filter the solution on a 40-V Seitz Orion-type filter press equipped with filter sheets at a pressure of 70-100 kPa. Rinse the filter with 40 litres of distilled water and add the rinsing water to the filtrate.

*Ethanol precipitation*

1. Add the calculated volume of ice-cold 95% ethanol to the filtrate during 4-6 hours, to give a final concentration of 27%, stirring well. During this step, the temperature should be gradually lowered from 0°C to −4°C.
2. When all the alcohol has been added, lower the temperature to −10°C and hold the solution at this temperature for at least 16 hours (or overnight), with stirring.
3. Filter the solution on a 40-V filter press, measuring 40 x 40 cm, containing carrier sheets covered on the smooth side with filter paper of the same size. During this step, the pressure should be gradually increased from 100 kPa to 200 kPa, while maintaining a constant temperature. The filtration takes about 3.0-3.5 hours. The filtrate is collected for recuperation of the ethanol.
4. Remove the filter cake containing the precipitated immunoglobulins (IgG) by pumping compressed air through the filter for 10 minutes. Collect the filter cake in a suitable stainless steel vessel equipped with a stirrer and resuspend in about 6 litres of phosphate-buffered saline-sodium chloride buffer (PBS-NaCl), pH 7.4 (see Annex), at room temperature.
5. Clarify the suspension by filtering on a 20 x 20-cm filter containing filter sheets of the same size, at a pressure of 50-100 kPa. Rinse the filter with 4 litres of PBS-NaCl pumped through the filter for 5-10 minutes.
6. Combine the filtrate and rinsing solvent. Freeze-dry the solution containing the precipitated IgG in a freeze-drier reserved for the lyophilization of animal proteins.
Purification Techniques for Heterologous Antiserum

Stabilization and preservation of purified HRIG

Regardless of the purification technique used, the purified HRIG suspension is stabilized using a concentrated buffer containing glycine (10 g/l) and sodium chloride (5 g/l) to be isotonic (pH 6.1–7.1), and an antiseptic such as thiomersal (1.5–2.0 g/l) or m-cresyl acetate (0.1%) is added as preservative.

Standardization of the final product

After sterile filtration, the activity of the preparation is determined by appropriate tests (see Chapter 47) to permit standardization of the final bulk product. The standard dose of ERIG for post-exposure treatment of humans is 40 IU per kg of body weight.

References


1 Also known as mercurothiolate and thiomersal.
Annex

Preparation of buffers

**Citric acid buffer, pH 4.8**
- Citric acid, monohydrate: 20 g
- Dextrose: 22.3 g
- Trisodium citrate, dihydrate: 7.3 g
- Distilled water to make: 1000 ml

**PBS–sodium chloride buffer, pH 7.4**
- Sodium phosphate, dibasic, dihydrate (Na₂HPO₄·2H₂O): 7.6 g
- Sodium chloride (NaCl): 4.8 g
- Potassium phosphate, monobasic (KH₂PO₄): 1.45 g
- Distilled water to make: 1000 ml