

5. Preparation of and Clinical Experience with Antihemophilic Factor Concentrates

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Because of increasing awareness of the clinical need for a potent, high-purity antihemophilic factor (AHF) concentrate, the American National Red Cross Blood Program has produced progressively more potent AHF preparations over the past six years (1).

The first procedure used to prepare AHF for the ANRC was that of the Blombäck (4) (Table 1). Since there is a tendency toward decreased yield and purity

Table 1. AHF purification by ANRC using various fractionation methods.

AHF for clinical use	Volume containing 250 units AHF	Times purified	Size of batch
1. Fresh frozen plasma (11 P [*])	310 ml	1	1 unit
2. Blombäck low-purity (1961—1965)	50 ml	7—20	Small, TP only [†]
3. ANRC low-purity (EACA-glucose, 1962—1965)	80—100 ml	7—15	Medium, 11 P
4. ANRC intermediate-purity (ethanol-cryoprecipitated, 1966—)	18—25 ml	20—40	Large, FFP
5. ANRC high-purity (precipitated with PLG, 1967—)	2.5 ml	300	Large, FFP
6. ANRC very-high purity (precipitated with PhC, 1967—)	< 1.0 ml	1000	Large, FFP
7. ANRC ultra-high purity (experimental use only, 1967—)		10,000	

* FFP contains 0.8 units AHF/ml.

** Fresh plasma.

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when large amounts of plasma are fractionated by this method, it was modified by: 1. use of fresh frozen plasma; 2. filtration through Millipore, instead of maintaining sterility by a closed system; 3. addition of epsilon aminocaproic acid (EACA) and glycine to inhibit plasmin and increase product stability, and to

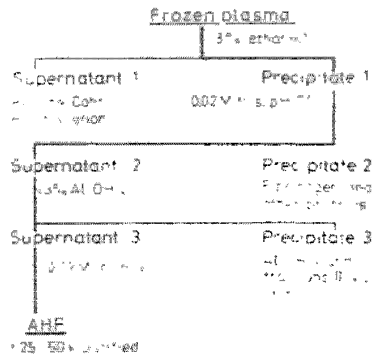


Fig. 1 Flow diagram for fractionation of intermediate-purity AHF.

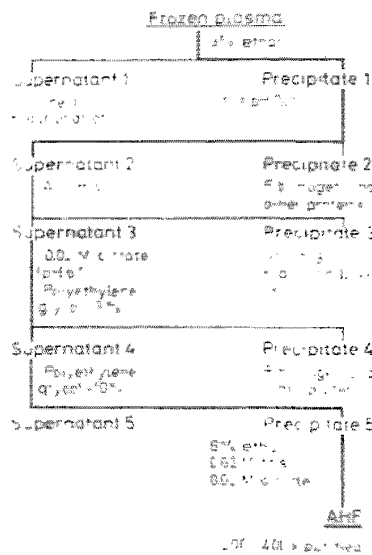


Fig. 2 Flow diagram for fractionation of high-purity AHF.

precipitate the AHF without prothrombin. By this method, large amounts of a clinically useful AHF preparation were made which also contained von Willebrand factor, but it was only 5 × concentrated and about 15 × purified (9). Moreover, it was still necessary to inject large fluid volumes *in vivo* to achieve adequate hemostasis. Further purification and concentration were considered essential; the objective was to make available an AHF concentrate sufficiently potent to ensure surgical hemostasis by injection of the contents of a single syringe.

The next method resulted in an intermediate-purity AHF (11), a clinically effective preparation that is about 12 × concentrated and 30 × purified (Table 1). Further purification of this material yielded the clinically effective high-purity preparation (Table 1, #5) which is approximately 100 × concentrated and 300 × purified (10, 11, 12). About 1000 units (200 liters) of plasma are now being fractionated weekly, yielding over 200 bottles of this concentrate, each containing about 400 AHF units.

The present report is concerned primarily with preparative methods and clinical experience with both the intermediate- (Fig. 1) and high-purity concentrates (Fig. 2).

Fractionation methods

Immediately after the blood is collected, it is centrifuged for 15 min at approximately 5000 × g at 4 °C; the platelet-poor plasma is separated and frozen on dry ice. The frozen plasma, shipped to a central point for fractionation, can be kept in this state for at least two months without appreciable loss of activity.

Although the batch size is unlimited, plasma from about 200 donors (40 l) is routinely pooled and fractionated in a single batch.

The frozen plasma is crushed in the bag, and the slush is pooled in a container kept at about 2° C (Fig. 1). Ice-cold ethanol is added slowly to a final concentration of 30% as described by Magnusson (16), with very gentle mixing; foam formation is prevented by adding a few drops of capryl alcohol. The plasma is allowed to melt; this process is facilitated by immersing in the plasma a stainless-steel coil with fluid circulating through it at 4–6° C. As melting proceeds, the position of the coil is changed to keep it in constant contact with the unmelted portion of the plasma. When melting is complete, the precipitate remaining is collected by centrifugation at 5000 × g for 10 min at 1–2° C. It contains at least 60% of the AHF. The protein fractions remaining in the supernatant may be removed subsequently by conventional Cohn fractionation (6) and other procedures (Fig. 3). Coagulation factors II, VII, IX and X are removed from the supernatant with DEAE cellulose (17) by a batch procedure.

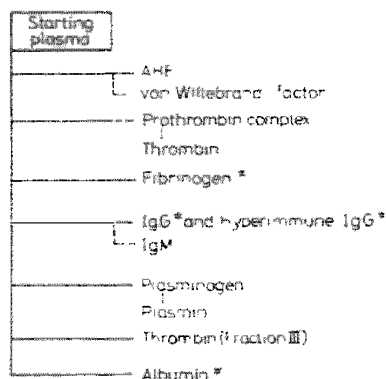


Fig. 2. Sequence of protein removal during routine fractionation of plasma. Broken line indicates proteins under investigation but not yet in production. Available as NIH licensed product to A.R.C., some Community Blood Banks and the Department of Defense.

Tris [(hydroxymethyl) aminomethane] buffer is added to the precipitate (125 ml of 0.02 M tris, pH 7.0, per 1 l of starting plasma), and the mixture is stirred vigorously with a Vibromixer for 30 min. This and all subsequent steps are carried out at room temperature. The AHF is extracted by the tris and is separated from the insoluble protein residue by centrifugation at 5000 \times g for 10 min. The small amounts of contaminating factors II, VII, IX and X are removed from the AHF-containing supernatant by adsorption for 5 min with 30 ml of albumin hydroxide gel per liter of tris extract. The gel, with adsorbed proteins, is removed by centrifugation and filtration (Millipore pre-filter). Sodium citrate is added to the AHF supernatant to a final concentration of 0.02 M; it is then sterilized by Millipore filtration and freeze-dried for clinical use, or further processed to produce the high-purity concentrate. The yield at this stage averages 50%.

To prepare high-purity AHF, the pH of the intermediate material is adjusted to 6.1 by adding citric acid (Fig. 2). When polyethylene glycol with a molecular weight of 6000 (PEG-6000) or 4000 (PLG-4000) is added, either in the form of dry flakes or in solution to a concentration of 3% or 6%, respectively, most of the fibrinogen is precipitated, and the AHF remains in the supernatant. After centrifugation and removal of the precipitate, the PEG level is raised to 10% (PEG-6000) or 12% (PEG-4000) to precipitate the remaining protein including the AHF. After centrifugation, the precipitate is dissolved in buffer (0.02 M tris + 0.02 M citrate, pH 7.0), sterile filtered through Millipore, and lyophilized. Since little AHF is lost in this procedure, the yield is still approximately 50%.

Recovery following intravenous administration in man

To determine whether the intermediate and high-purity concentrates were suitable for therapeutic use, they were infused in nonbleeding hemophilic volunteers with a severe form of the disease ($< 1\%$ AHF) to determine recovery and half-life. Blood samples drawn at frequent intervals were assayed for AHF level by a two-stage method based on the thromboplastin generation test (TGT) (2).

Recovery studies were carried out with intermediate-purity material in 7 subjects and with high-purity material in 11 subjects. A typical die-away curve for the high-purity material is shown in Fig. 4. Mean recovery was 68%, and the biological half-life ranged from 8—13 h. Results with the intermediate material were similar; the mean recovery was 73% and the half-life 11—18 h.

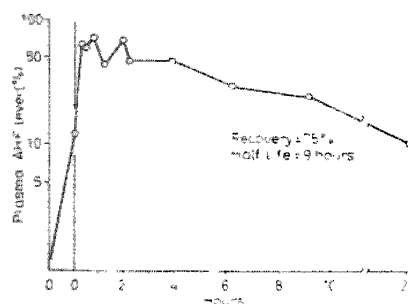


Fig. 4. Die-away curve and half-life of high-purity AHF in nonbleeding hemophiliac ($< 1\%$ AHF). Primary dose of 15 AHF units/kg was infused at 0 time, followed by dose of 41 units/kg 60 min later (0' on abscissa).

High-purity AHF was administered intramuscularly to 2 hemophilic volunteers ($< 1\%$ AHF). A subject weighing 79 kg received 4800 units of AHF in four divided doses of 2.0 ml each, within a few moments. (To infuse this many AHF units in the form of plasma would require an amount equivalent to 120% of his total plasma volume.) No AHF activity could be detected on assay of the blood from either subject although their clotting times decreased sharply toward normal over a period of 4 h, then gradually returned to pre-injection levels.

Therapeutic results

Thirty-six patients with a variety of conditions were treated with intermediate-purity AHF. About half were hospitalized for major surgery or treatment of

Table 2. Representative data on clinical use of intermediate-purity AHF

Patient	Indication	Days of treatment	Units AHF administered	Highest plasma AHF level recorded (%)
W. C. ^a	Evacuation of subdural hemorrhage (50-70 ml); tracheostomy	24	48,860	> 200
D. M. ^a	Craniotomy and evacuation of subdural hemorrhage	14	14,984	unknown
G. C. (AHF inhibitor)	Correction of patent ductus arteriosus	23	4,924	80
N. M. (12% AHF)	Thoracotomy and biopsy of mediastinal mass and pericardium	12	30,350	unknown
G. W. ^a	Through-knee amputation	20	71,974	67
G. W. ^a	Wound debridement and resuture; split skin graft	14	21,685	53
P. Y. ^a	Appendectomy	9	11,500	35
J. C. ^a	Repair of strangulated inguinal hernia	8	13,800	50
T. D. ^a	Removal of medial meniscus from knee	14	49,160	100
J. R.	Spontaneous intra-abdominal and intra-thoracic hemorrhage, epistaxis, GI bleeding	14	34,118	unknown

^a Less than 1% AHF.

severe trauma: representative data are shown in Table 2. It was thought necessary to maintain their AHF blood level above a minimum of 30% for at least 1 week after surgery (7, 14) and above 15% for approximately 1 more week, to ensure completely normal hemostasis. AHF levels, usually varying from a maximum of 75% to a minimum of 30%, were maintained by giving the concentrate every

Table 3. Schedule for management of surgery or severe trauma with AHF concentrate

Treatment day	Frequency of injection	Approx. units AHF for 70 kg man	Minimum plasma AHF level (%)
1-2	8 hourly (including pre-surgery)	1500 (22 u/kg)	25-30
3-7	12 hourly	1500 (22 u/kg)	25-30
8-14	12 hourly	750 (11 u/kg)	15

12 h except for the day of surgery when it was given every 8 h. The dosage was calculated on the basis of body weight, as indicated in Table 3. The regimen proved effective for all patients except those with inhibitors to AHF. They required a larger dose to achieve hemostasis, and the desired blood levels were frequently not attained.

High-purity AHF was administered to 7 patients with severe hemophilia during and after various surgical procedures according to the suggested dosage schedule (Table 4). Each 12-h dose of approximately 30 units/kg was given in a single injection of about 20 ml.

Hemostasis appeared to be normal in all the hemophiliacs treated with either the intermediate- or high-purity concentrates.

Table 4. Summary of data on clinical use of high-purity AHF in patients with severe hemophilia

Patient	Diagnosis	Duration of treatment	Units AHF administered	Highest plasma AHF level recorded
D. W.	dental surgery	6 days (10 days total)	11,280 (3,000 ^a)	90% ^a
S. R.	dental surgery	5 days (9 days total)	24,000 (11,000)	56% ^a
P. B.	dental surgery	7 days	43,250	89% ^b
J. M.	dental surgery	14 days	35,608	172% ^a
J. D.	dental surgery, repair of hernia	7 days (21 days total)	48,960 (49,160)	91% ^a
G. N.	excision of tonsillar sinus	7 days (15 days total)	78,000 (18,380)	150% ^b
R. P.	repair of inguinal hernia	10 days	25,812 (2,160)	99% ^a

^a Therapy initiated with high-purity AHF, was completed in all but two patients (P. B. and J. M.) with intermediate-purity AHF (number of units indicated in parentheses).

Discussion

Two methods have been presented for the large-scale preparation of AHF for clinical use. Intermediate-purity concentrate is the starting material for the high-purity material. Fresh-frozen ACD plasma is the starting material for the intermediate-purity. The frozen plasma, which is routinely separated from platelets and packed red cells, can be collected nationally, accumulated in the frozen state for 1—8 weeks and shipped to one or more points for fractionation. Collection

of large amounts of plasma nationally, to be fractionated into concentrates, usually makes it possible to meet local needs for large amounts of AHF.

AHF is the first protein obtained during the routine fractionation of plasma. The plasma remaining may be fractionated by the routine Cohn procedure (6). A large pool of plasma may be processed at one time for the sake of economy; the amount is limited only by the risk of hepatitis. Both intermediate- and high-purity fractionation methods may be performed at room temperature after initial thawing of the plasma; both are "open" methods with sterilization as the final step; both are relatively simple and the fractionation volume is not excessive after initial thawing of the plasma.

Since the concentrates are relatively stable at room temperature following lyophilization, they may be shipped without special precautions, or even stored for a few weeks without refrigeration.

Like all concentrates made on a large scale, the ANRC materials may be assayed prior to distribution, making it possible to maintain a precise dosage schedule throughout therapy. During the treatment period the dosage is ideally based on results of frequent assays of the patient's plasma. When hemophiliacs are treated in institutions where accurate assays are not available, the dosage schedule in Table 3 may be used in conjunction with concentrates of known potency to maintain AHF levels above 30% in severely affected patients, thus ensuring normal hemostasis (7, 14).

Neither of the ANRC concentrates has caused significant side effects even when administered to patients who habitually have severe allergic reactions to fresh-frozen plasma. None of the patients given the high-purity AHF had any allergic reaction, despite rapid injection. Two patients receiving the intermediate-purity material complained of circumoral tingling and prickling of the back of the neck during rapid infusion, and one showed shortened red cell survival during two weeks of treatment, possibly due to red cell antibodies in the concentrate. Hemolytic reactions have been reported with other AHF concentrates (15, 19). No difficulty is anticipated from the trace amounts of PEG in the final product; acute and chronic toxicity studies in various animals have revealed no deleterious effects although the PEG was administered in 10—1000 times the amount remaining in the AHF.

Many advantages of the intermediate-purity ANRC concentrates are shared by other human AHF concentrates for clinical use (3, 5, 22), but the high-purity material is unique. Hemostasis can be maintained during and after the most formidable surgical procedures by means of two relatively small intravenous injections daily. The patient can be as mobile as other persons undergoing similar operations since restricting intravenous infusions are not necessary; this is a decided advantage to the medical and nursing staff as well as the person being

treated. Moreover, the concentrate is readily soluble and can be reconstituted in less than 5 min. Because of its potency, the material is lyophilized in small containers which are easily shipped, stored in the patient's refrigerator, or carried on his person.

The usefulness of this material in the management of hemophiliacs during surgery has been demonstrated. It may prove even more beneficial in the treatment of spontaneous bleeding such as joint and soft-tissue hemorrhage as well as the relatively rare internal bleeding which may be a severe threat to life. Repeated spontaneous joint hemorrhages are the most frequent and disabling manifestations of severe hemophilia. These situations might be prevented by the rapid injection of large doses of a very potent preparation as soon as symptoms appeared. With availability of such a material, normal hemostasis could be achieved in minutes, preventing permanent joint pathology and obviating hospitalization.

Clinically useful amounts of high-purity AHF would also raise the possibility of maintenance therapy in hemophiliacs. Self-administration is not feasible because of the failure of intramuscular adsorption (18, 21), but intravenous injection every few days or weekly might maintain a minimum plasma level of AHF of 2% or more in severely affected patients. Persons with moderate hemophilia, who naturally have such levels, have fewer serious bleeding problems (7). Maintenance therapy for the country's hemophiliacs would, of course, require enormous amounts of concentrate.

Very-high-purity AHF, for laboratory investigation, has been produced by further purification of the high-purity AHF: reprecipitation with PEG and sucrose gradient ultracentrifugation, or chromatography on agarose gel. Fibrinogen-free AHF with a specific activity of 166 units/mg protein was obtained by these methods. On preliminary examination, this material shows two components on acrylamide gel electrophoresis. Ultra-violet spectroanalysis revealed prominent peaks at 210 and 230 m μ , unlike other proteins such as fibrinogen, albumin and gamma globulin. Analysis for amino acids showed that they account for only 20% of the sample weight. Biophysical studies indicate that the material has a very high molecular weight, confirming the views of others (1, 8, 13, 20). It is hoped that fundamental work of this nature will lead to a greater understanding of the defect in classical hemophilia.

Summary

Two AHF concentrates for clinical use are described: one of intermediate-purity (30%) containing an average of 12 units AHF activity/ml, and the other of high-purity (more than 300%) containing an average of 100 units/ml. Methods for their preparation are described, and clinical data are reported. The recovery

and biological half-life of the AHF in both concentrates, when injected in severely affected hemophiliacs, are similar to those found with transfusion of normal plasma in these individuals. Completely normal hemostasis was achieved in hemophiliacs undergoing surgery; plasma AHF levels exceeding 30% were easily maintained. With the high-purity material, hemostatic doses could be given in a relatively small fluid volume by syringe injection. No serious side effects were encountered. Possible future use of the high-purity concentrate in the management of severe hemophilia, including prophylaxis, is discussed. Preliminary biochemical and biophysical characteristics of ultra-high-purity AHF concentrates are reported.

Addendum

Since this manuscript was submitted, we have found PEG-4000 preferable to PEG-6000. The optimal PEG-4000 concentration in the first PEG precipitations ranges from 4.0—5.5%, depending upon the desired degree of purification and yield (higher concentrations result in a more highly purified AHF but with a lower yield).

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