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HUMAN ANTIHÆMOPHILIC FACTOR

THE PREPARATION IN A HOSPITAL OF A CONCENTRATE FOR CLINICAL USE

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NOT long ago bleeding in a hæmophiliac was regarded as a danger to life; dental extractions were avoided, and major operations were almost unthinkable. Even with fresh blood available the situation was not greatly improved. When animal antihæmophilic-globulin concentrate was produced (MacFarlane et al. 1954) the situation was changed; it became possible to arrest hæmorrhage in a hæmophiliac with certainty. There were only two disadvantages to its use; these were (1) its antigenicity, and (2) its loss of efficacy when given for a second course. Since in a hæmophiliac only one incident could be treated successfully, there was always the worry as to whether the incident justified its use or whether it should be saved for a possible greater emergency in the future. The production of human concentrate (Kekwick and Wolf 1957, Blombäck and Nilsson 1958) removed this worry and permitted not only lifesaving operations but also operations for the correction of deformities, dental clearances, and other measures to improve the health of the hæmophiliac (Blombäck and Nilsson 1958, Wolf 1959a). Its use also shortened the lengthy periods of disability from joint and tissue hæmorrhages, and reduced the likelihood of permanent disability (Wolf 1959b). With increasing experience it became clear to us that much could be done with fresh plasma, stored in the frozen state, providing concentrate was available if plasma

failed to control the hæmorrhage. Nevertheless the infusion of a small quantity of concentrate in a short time had many advantages over the administration of a large quantity of plasma during several hours.

Being convinced that it had become possible to keep most of our hæmophiliacs reasonably well and at school or at work instead of at home or in hospital, we realised that we wanted more concentrate than we could expect to receive. We could not accept the view that the production of adequate supplies for general treatment was impossible. This paper describes a method of producing human antihæmophilic-factor (A.H.F.) concentrate at local treatment centres or blood banks in sufficient quantities to meet their own demands, without incurring a large initial financial outlay or imposing too great a strain on the donor panel. In 18 months, fifty-two batches of this concentrate, each of 300 ml. and prepared from 3 litres of plasma, have been made at Lewisham Hospital for clinical use.

Method of Preparation

The essential method of preparation is that of Kekwick and Wolf (1957), using cold ethyl ether as a protein precipitant. The preparative procedures and the apparatus are basically those described by Kekwick and Mackay (1954) for the preparation of plasma fractions under sterile conditions. Modifications have been introduced to adapt the original process to the limits of the material and manpower that would be expected in a large hospital laboratory without incurring an initial or running expenditure out of proportion to general routine laboratory requirements.

The main modifications to the original process are:

1. The precipitation is made from undiluted acid-citrate-dextrose (A.C.D.) plasma which can be processed immediately or frozen within 2 hours of bleeding and thawed just before fractionation.

2. The equilibration period with ether at 0°C has been reduced from 18 hours to 20 minutes. Such a short time is, of course, possible only when processing small quantities.

3. The salt content of the solutions used to wash the precipitate has been increased.

4. The final precipitate is brought into solution in a volume of concentrated salt solution equal to 1/100 of the original plasma-volume.

Details of the Process

Blood is collected from donors in the ordinary way, using

Fenwal double-plastic bags and A.C.D. as the anticoagulant. In this experimental period we have collected some plasma from donations intended for patients requiring fresh packed red cells, some from malarial donors, and the remainder from A, B, or AB donors. In the last group the red cells have gone into the blood-bank for use. The blood is centrifuged within 2 hours of collection, at +4°C and 2000 r.p.m. for 15 minutes in a refrigerated M.S.E. major centrifuge. At +4°C the cells pack well, and the plasma is separated into the second bag, using the Fenwal separator. The red cells are stored at +4°C until required for transfusion. The bag of plasma is placed in a paper bag to prevent tearing when frozen, and it is stored at −25°C.

Fractionation procedure.—3 litres of plasma are processed in each batch, since this provides a full load for the M.S.E. centrifuge. The bags of plasma are thawed in a bath at 37°C, and the plasma is then transferred to the 5-litre fractionation vessel under sterile conditions. The undiluted plasma, at pH 7·1, is cooled to 0°C with continuous mechanical stirring, and precipitation is effected by the addition of 330 ml. of diethyl ether over a period of 30 minutes. This ether-plasma mixture is allowed to equilibrate for 20 minutes with mechanical stirring, and it is then transferred, by positive pressure and syphon, to 6 transfusion bottles which have been precooled to −1°C in the centrifuge. The precipitate is separated by centrifugation at 1000 r.p.m. for 30 minutes at −1°C. The supernatant liquid is discarded, and the precipitate in each bottle, quickly resuspended in 150 ml. of ether-citrate saline, is precooled to −1°C. The composition of the ether-citrate saline is: 8·5 g. sodium chloride, 3·7 g. trisodium citrate, water to 1 litre, and 80 ml. of ether. (The salt content of this wash is increased in order to remove more inactive protein from the precipitate.) The suspended precipitate from two bottles is pooled into one, leaving three bottles, which are recentrifuged at −1°C for 30 minutes at 1000 r.p.m. The supernatant fluid is discarded, and the packed precipitate in each bottle is resuspended in 200 ml. of a 0·37 g. per 100 ml. solution of sodium citrate which has been precooled to 0°C. Washing in dilute citrate replaces the water wash, because this prevents traces of prothrombin in the precipitate being converted to thrombin and causing a loss of A.H.F. The resuspended precipitate is then recentrifuged at 0°C for 30 minutes at 1000 r.p.m., the supernatant liquid is discarded, and the precipitate is brought into solution at 37°C by adding 10 ml. of concentrated salt solution to the precipitate in each bottle. The salt solution consists of 17% sodium chloride and 7·4% trisodium citrate. For complete solution the precipitate requires about 15 minutes at 37°C. From each bottle 0·5 ml. of concentrate is withdrawn for sterility tests, and these samples are dried with the main batch.

Freeze drying.—The solution in each bottle is shell frozen at −78°C in a solid carbon-dioxide/acetone mixture, the

transfusion-bottle caps are replaced by drying caps, and the material is then desiccated from the frozen state under high vacuum.

Reconstitution for clinical use.—The dried concentrate in each transfusion bottle, which represents the precipitate from 1 litre of plasma, is reconstituted with 100 ml. of sterile water. The final solution has a tonicity of twice physiological saline, which is required to keep the A.H.F. protein in solution.

Technical Details

Transfusion bottles.—540 ml. boron-silicon transfusion bottles are used. These bottles will not crack in a solid carbon-dioxide/acetone mixture as ordinary transfusion bottles do, but they will not stand a speed of over 1200 r.p.m. in an M.S.E. major centrifuge.

Fractionation vessel.—A 5-litre reagent bottle with a wide neck is used. The accessory fittings required for stirring, temperature control, and maintenance of sterility, which are made of glass, are introduced through a rubber bung. Their arrangement is exactly described by Kekwick and Mackay (1954).

Positive pressure for transfer of the ether-plasma mixture to transfusion bottles is supplied by a compressed-air cylinder. The air filters are made by packing standard transfusion drip chambers with non-absorbent cotton wool, and sterilising before use.

Refrigeration equipment.—This consists of an M.S.E. major centrifuge, which is insulated and fitted with refrigeration coils, and an insulated low-temperature tank, fitted with a circulating pump. Refrigeration is supplied by a 1 h.p. sealed compressor unit, which allows for separate thermostatically controlled cooling of the tank and centrifuge. Temperatures of $-5°C$ can be attained both in the tank filled with glycol and in the centrifuge when spinning at 1200 r.p.m. (room temperature 20°C). This equipment, which was specially constructed for limited refrigeration at an economical price, obviates the necessity for a large capital outlay on ready-made equipment that provides greater refrigeration than necessary.

Freeze-drying equipment.—The assembly consists of a steel vessel, fitted with an O ring seal, in which the transfusion bottles and universal containers holding the frozen concentrate are stood upright, the bottles being fitted with drying caps to preserve sterility. The steel vacuum chamber is supplied with two drying heads for primary and secondary drying. A simplified form of the McCleod vacuum gauge is incorporated in the vacuum circuit to ensure adequate control of the vacuum, and early detection of leaks.

Primary drying.—The head for this purpose has a large central cylinder, which is filled with a solid carbon-dioxide/acetone mixture. During the primary drying the carbon dioxide requires replenishing at 4-hourly intervals. This process lasts 48 hours, during which the steel chamber is being continuously evacuated with a two-stage diffusion pump. The water vapour is trapped as ice on the inner surface of the steel drying head. When the primary drying head is removed at the end of 48 hours and replaced by the secondary drying head, the ice is automatically removed with it, leaving the main vessel quite dry, so that no defrosting is necessary.

Secondary drying.—The secondary drying head carries three trays which are filled with solid P_2O_5. This process also lasts 48 hours, during which the pump is allowed to run continuously.

Costs.—An estimate is given in the appendix.

The Concentrate

Properties

The reconstituted product shows no significant difference in A.H.F. content or clinical efficacy when compared with the product of the original process. The salt content of the reconstituted product is identical with that of the original material. Electrophoresis on cellulose acetate shows no obvious differences; the main component is fibrinogen (60–70%), and the remaining proteins are γ and β globulins and albumin. The protein concentration is a little less than that of the original material, varying from 1·7 to 2·2%, compared with 2·2 to 2·7%.

The material from the modified process differs from the original in two important respects:

1. The dried material is much more soluble, and it has a smaller insoluble residue.

2. The modified product appears to be free from infusion reactions: to date thirty-five separate infusions have been given, all without reactions. In our previous experience, using materials made by the original process, 9% of all infusions were associated with reactions.

Of the fifty-two batches of concentrate prepared, only two were found unsuitable for clinical use because of bacterial contamination.

Clinical Use

The concentrate has been used to cover five major operations, of which three were orthopædic and two were abdominal. In these it was used for preoperative protection and during the first 2 days postoperatively. After this, fresh frozen plasma was mainly relied on to maintain hæmostasis. The concentrate was used successfully on six occasions to arrest large spontaneous or traumatic tissue hæmorrhages, when treatment with plasma had failed. It was used once, when postoperative treatment with plasma had to be discontinued because of hyperproteinæmia: during the treatment with concentrate the signs and symptoms rapidly subsided.

Discussion

The large-scale process involves the collection of large quantities of blood in sodium citrate, and its rapid transportation to a centre where processing starts some hours later. This limits collection to an area within reasonable distance, and it leads to a loss of the red cells. We hoped to show that A.C.D. plasma was as satisfactory for the production of concentrate; that, if the plasma were

separated promptly and frozen, it could then be processed at leisure; and that the red cells need not be wasted. The first two aims have been achieved. The packed red cells are not suitable for patients who require rapid transfusions or the restoration of volume as well as red cells, though there are many patients who can benefit from them. It would be easier for the blood-bank if one half of the plasma were collected from each pint of blood donated, but this would increase the costs appreciably. The use of plastic bags instead of bottles allows the red cells to be preserved without risk of infection, and it reduces the likelihood of loss of A.H.F.

When fitting a fractionation process into the routine of a laboratory, it is more convenient to freeze the plasma immediately after separation and to thaw it when needed for fractionation. As this entails a certain loss of A.H.F. it is essential that, in adapting the process for small-scale operation, the modifications should aim at minimising the loss of A.H.F. during processing, so that the final product will not be less effective. The two most important causes of loss of A.H.F. during fractionation are undoubtedly protein denaturation and the action of traces of thrombin, which are liberated during processing.

Shortening the speed of processing, including shortening the equilibration-time from 18 hours to 20 minutes, so that the whole process, up to the drying, could be completed in 5 hours, resulted in a clinically more effective product, in the elimination of all infusion reactions, and in a better solubility of the dried product. The effects are probably due to the avoidance of protein denaturation, and they compare with the Swedish A.H.F. concentrate of Nilsson et al. (1962), which also has a short equilibration-time (75 minutes at $-4°C$, with 8% alcohol) and which is completely free from infusion reactions.

Dilute citrate is substituted for water for the second washing of the precipitate, and a concentrated solution of citrate is used for making the final solution of the product for drying. This prevents thrombin formation which would cause a greater loss of A.H.F. than that due to protein denaturation from the short exposure to a high-salt concentration. Desiccation of a small volume of concentrated salt-protein solution from the frozen state allows for a relatively inexpensive freeze-drying process.

The fact that, in this small-scale process, it is not practicable to try to recover other blood products, such as albumin and globulin, is offset by the fact that the red

cells, which are wasted in the large-scale process, can be preserved for transfusion.

Sweden is at present the only country in the world relying mainly on human A.H.F. concentrate for the treatment of classical hæmophilia (Nilsson et al. 1962). This is possible because, in Sweden with a population of only 7 million, and with an estimated number of about 160 severe classical hæmophiliacs, enough A.H.F. concentrate can be produced at one centre to meet their requirements.

In England, with its greater population, very much more concentrate is required, and the difficulties involved in producing this bulk of concentrate at one centre are considerable. Transportation of blood from collecting centres to the processing centre inevitably results in delays, with consequent loss of A.H.F. activity. The risk of contamination of blood with the virus of infective hepatitis limits a single fractionation batch to a donor pool of 50. Since the concentrate cannot be Seitz filtered, it must be produced under sterile conditions, and any accidental contamination is extremely wasteful if large quantities are involved. It is also more difficult to maintain sterility when processing large batches. For these reasons, in a densely populated country, it seems more advantageous to produce A.H.F. concentrate on a small scale at multiple centres.

Van Creveld and Mochtar (1962) have advocated the small-scale "two donor" fibrinogen preparation of Nitschmann et al. (1957) for the treatment of hæmophilia A, though Nitschmann et al. did not themselves suggest this. This is a simple preparation made by precipitating fresh plasma with 8% ethanol and equilibrating for 12 hours; no attempt is made to wash the precipitate. In our experience, this fraction is very rich in prothrombin, which is liable to be converted to thrombin, causing a very variable A.H.F. content.

Summary

A method is described for preparing, in a hospital laboratory, human antihæmophilic-factor (A.H.F.) concentrate for clinical use. This is a modification of the method of Kekwick and Wolf (1957), using cold ether as the protein precipitant.

The main modifications are: the use of acid-citrate-dextrose plasma; considerable shortening of the time of equilibration with ether; and freeze-drying of the

concentrate from small volumes of solution with a tenfold increase in the concentration of salt and protein.

The resultant concentrate has a better solubility, and higher A.H.F. activity per weight of protein, than the product of the original method. The concentrate was used successfully in thirty-five separate infusions to treat classical hæmophiliacs: none of these was associated with an infusion reaction.

Human A.H.F. concentrate, produced in this way at multiple centres throughout the country, could provide sufficient to treat all the known classical hæmophiliacs in Great Britain.

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Appendix

Equipment

	£	*s.*	*d.*
M.S.E. major centrifuge	298	10	0
6 × 500 ml. head and cups (for transfusion bottles)	50	0	0
4 × 1000 ml. head and cups (for transfusion bags)	67	0	0
Copper tank	65	2	6
Supplying and fitting refrigeration equipment	187	19	0
Leybold D 2-vacuum pump and accessories	80	0	0
Vacuum gauge (Leybold-Elliott)	5	0	0
Drying unit	approximately 50	0	0

The refrigeration equipment was designed and constructed by J. & S. Refrigeration, 33, Victoria Road, Surbiton, Surrey, with the assistance of the hospital engineering department.

The drying vessel and heads, designed and made by Victor Wolf Ltd., Croft Street, Manchester, were donated to Lewisham Hospital.

The 540 ml. boron-silicon transfusion bottles (27*s.* per dozen) and the transfusion-bottle syphons (£1 17*s.* 6*d.* each) were supplied by R. B. Turner & Co. Ltd. of East Finchley.

The separation and processing of plasma from twelve bags requires about 8 hours of a technician's time and yields three 100 ml. units of A.H.F. concentrate. The approximate cost of the consumable items and technical time for this amounts to £21, the major item being the Fenwal JD2 double-pack units at £1 5*s.* 3*d.* each.

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