A rapidly produced ^{125}I labelled autologous fibrinogen: *in vitro* properties and preliminary metabolic studies in man

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SYNOPSIS The properties of fibrinogen extracted by a precipitation method using glycine at ambient temperatures near neutral pH are described. The simple and reproducible method gives a 73% yield of high purity plasminogen-free fibrinogen in 45 minutes from small volumes of plasma. The protein extract was labelled with ^{125}I using chloramine-T under conditions optimal for fibrinogen stability. The extraction procedure, radio-iodination, desalting, and sterilization take only 70 minutes for completion from the time donor blood is received in the laboratory. The methods, using a specially developed extraction vessel and desalting/sterilizing column, can be used in a small hospital laboratory. Autologous fibrinogen can thus be extracted from patients' blood, eliminating the risk of transmitting hepatitis when it is re-administered. The autologous material, which is 97% clottable and contains less than 0.05% free iodide, is being routinely used as a diagnostic tool in the detection of deep vein thrombosis. The high purity of the preparation facilitates metabolic studies and *in vitro* experimental work. *In vivo* results show a mean half-life in three normal volunteers of 3.95 days and a catabolic rate of 25.23% per day with the extravascular space estimated as 24.86%. In 30 surgical patients an expected reduced half-life in plasma was determined with a mean of 3.1 days.

The highly sensitive and specific method for the detection of deep vein thrombosis, that of the ^{125}I fibrinogen technique, has been criticized because of the risk of transmitting serum hepatitis when pooled human plasma is used for the extraction of fibrinogen (Kakkar, 1972; Hume, 1973). Hicks and Hazell (1973) have reported the use and follow-up of this technique in large numbers of patients and there seems little evidence to support this criticism although their techniques have been questioned (Goudie *et al*, 1973). This hepatitis risk has prevented its widespread use as a diagnostic tool in both Britain and, by legislation, the United States. The use of autologous plasma would eliminate the risk of introducing hepatitis. However, previous methods suitable for extracting purified autologous fibrinogen from plasma have been lengthy or too complex for routine diagnostic use (Bettigole *et al*, 1969; Ingraham *et al*, 1969; Peabody *et al*, 1974). The few rapid methods that have been reported contain protein and free radio-iodide contaminants, making them unsuitable for metabolic studies or

laboratory assays (Roberts *et al*, 1972; Hagan *et al*, 1974). Materials used for previous metabolic studies suffer from the same faults (McFarlane, 1963; Takeda, 1966; Collen *et al*, 1972; Bradley and Hickman, 1975).

Kazal *et al* (1963) showed that glycine in an aqueous system has a salting-in action on all plasma proteins with the exception of fibrinogen and a cold insoluble beta globulin; by using controlled conditions of temperature and glycine concentration fibrinogen alone could be precipitated. This simple precipitation procedure produced a high yield of clottable protein with little degradation or contamination of the product (Walker and Catlin, 1971). The removal of plasminogen during fibrinogen extraction from plasma has proved particularly difficult, although Blombäck and Blombäck (1957) showed glycine to have a greater solubilizing effect on plasminogen than on fibrinogen and produced a fibrinogen low in the pro-enzyme. The addition of zinc ions (Zn^{2+}) as well as ϵ -amino-n-caproic acid (EACA) to samples during preparation has been shown by Brown (1972) to assist the removal of plasminogen. The use of EACA to inactivate

fibrinolysis has ensured the absence of fibrinogen degradation product contaminants and has stabilized the preparation.

The present study details the methods for extraction, radio-iodination, desalting, and sterilization of an autologous fibrinogen suitable for metabolic studies. The *in vitro* properties and *in vivo* behaviour are outlined.

Material and Methods

MATERIAL FOR EXTRACTION OF FIBRINOGEN

The extraction procedure was carried out at room temperature (not less than 20°C) and has been centred around the separation vessel (fig 1) which

Fig 1 Separation vessel.

was specially developed for rapid salting-out techniques. The vessel was made from a 20 ml disposable syringe having a sintered filter set into a central position in the barrel and has been described in detail elsewhere (Hawker, 1975). Precipitation was carried out in the upper chamber, with the vessel in the stirring assemblage (piston directly beneath the sinter, preventing loss of solutions). The supernatant could be drawn to waste by withdrawing the piston, leaving the precipitate on the porous disc where it could be repeatedly washed.

All chemicals were obtained from British Drug Houses Ltd unless otherwise stated. The solutions required were as follows:

0.05 M Alkaline-glycine buffered saline citrate 1 litre of aqueous solution containing 3.75 g glycine, 6.5 g sodium chloride, and 7.5 g trisodium citrate, adjusted to pH 8.6 with 2N NaOH.

Anticoagulant solution 400 mg ϵ -amino-n-caproic

acid, 352 mg zinc sulphate $7H_2O$, and heparin as sodium heparinate (Sigma), 200 units/ml final solution, made to 100 ml with buffer pH 8.6 and adjusted to a final pH of 8.1 with 2N NaOH.

Dilute anticoagulant solution Prepared as a solution containing 20% anticoagulant solution diluted with buffer pH 8.6 and adjusted to pH 8.6 with 2N NaOH.

Washing solution Prepared as a solution containing 20% anticoagulant solution diluted with buffer pH 8.6 before the addition of 32.86 g glycine/200 ml diluted anticoagulant solution. The pH was adjusted to 7.5 with 2N NaOH.

Saline citrate 1 litre of aqueous solution containing 5.75 g trisodium citrate $2H_2O$ and 27.25 g sodium chloride adjusted to pH 7.0 with 1N HCl.

METHOD OF EXTRACTION

Fifteen millilitres of blood were taken by clean venepuncture and diluted with 4.5 ml of 3.8% aqueous trisodium citrate. After centrifugation at $3000 \times g$ for 5 min, 8 ml of the plasma was magnetically mixed with 1 g powdered glass and 2.0 ml anticoagulant solution in the separation vessel. 1.47 g glycine was dissolved in the mixture and after 8 min of constant stirring the supernatant was drawn to waste through the sintered base of the separation vessel. To prevent the sintered disc becoming clogged by the gelatinous precipitate stirring was stopped and 15 s delay was allowed for the powdered glass to settle before the supernatant was drawn off. The glycine concentration at precipitation is equivalent to 1.79 M.

The retained precipitate was washed repeatedly with the washing solution (1.98 M glycine) before the vessel was returned to the stirring assemblage. Ten millilitres of diluted anticoagulant solution was added, followed, after 8 min of stirring, by 1.47 g glycine to reprecipitate the fibrinogen. After precipitate collection and washing as before, this purified precipitate was redissolved in 5 ml of saline citrate pH 7.0 and was collected after being pulled through the sintered disc.

The clear-to-opalescent fibrinogen solution had a final pH of 7.4. The timing used and illustrated in fig 2a gave a purified product in 45 min from the time whole citrated blood was received in the laboratory. Protein concentration was calculated from the optical density at 280 nm of a 1/10 saline-citrate dilution using an extinction coefficient $E_{1.0\%}^{1.0\text{cm}}$ of 15.5.

MATERIAL FOR LABELLING FIBRINOGEN WITH ^{125}I

Radioactive iodine ^{125}I as iodide, prepared at the Radiochemical Centre, Amersham, for protein iodination (code IMS 30)

Fig 2 Timing of (a) fibrinogen preparation (0-45 minutes); (b) radiolabelling, desalting, and sterilization (45-70 minutes).

Aqueous sodium iodide solution at $2.95 \mu\text{g}/\text{ml}$ as diluent to give required specific activity in $50 \mu\text{l}$

Chloramine-T, $200 \text{ mg}/100 \text{ ml}$, and sodium metabisulphite, $400 \text{ mg}/100 \text{ ml}$, freshly prepared in saline citrate pH 7.0 or freshly prepared, aliquoted, and kept at -20°C until required

Sephadex G25 medium grade (Pharmacia) pre-swollen for a minimum of 3 h in saline citrate pH 7.0.

PREPARATION OF A DESALTING COLUMN

A polypropylene 10 ml syringe barrel containing a sintered polythene disc as a gel retainer was filled with swollen Sephadex slurry, sealed with parafilm, and spun at $1000 \times g$ for 10 min to remove the void volume. The packed column (8-10 ml) was sealed and stored at 4°C until required.

^1MSE supermagnum centrifuge swingout head, $60 \times g = 550 \text{ rev}/\text{min}$, $1000 \times g = 2200 \text{ rev}/\text{min}$.

METHOD OF IODINATION

To 0.75 ml of prepared fibrinogen in a flat-bottomed disposable tube (Luckhams PT/1054) $50 \mu\text{l}$ of suitably diluted ^{125}I (normally $200 \mu\text{Ci}$ of activity in $50 \mu\text{l}$) was added. The mixture was stirred in an ice bath using a magnetic flea. $50 \mu\text{l}$ of freshly prepared chloramine-T solution was added rapidly with stirring from an Eppendorf pipette followed after 60 s by $50 \mu\text{l}$ of $\text{Na}_2\text{S}_2\text{O}_5$. After a further 3 min of stirring $100 \mu\text{l}$ of $1.7 \text{ g}\%$ aqueous potassium iodide was added.

To a prepared desalting column with attached sterilized filter unit and bottle (containing a $0.2 \mu\text{g}$ Gelman GA8 filter) 1.0 ml of a one plus one dilution of donor plasma in saline citrate was carefully added to the Sephadex surface followed by the contents of the fibrinogen-labelling tube (also 1.0 ml). The plasma had a twofold function: to prevent non specific adsorption of the labelled fibrinogen to both the Sephadex column and filter; to prevent biochemical changes and radiation damage to the fibrinogen in solution.

The filter unit consisted of a Swinnex 13 type Millipore filter holder modified for the retention of Sephadex gels and the sterilization of filtrates during centrifugation (Hawker, 1974).

The column was sealed with parafilm and spun at $60 \times g^1$ for 5 min followed by a further 10 min at $1000 \times g$. The bottle containing sterile filtrate was detached from the column, sealed, and identified ready for *in vivo* or *in vitro* use. The timing can be seen in fig 2b.

IN VIVO METHODS

In all recipients of autologous ^{125}I fibrinogen, the thyroid uptake of ^{125}I was blocked by oral administration of 120 mg potassium iodide daily for 7 days pre- (if possible) and 21 days post-injection.

In vivo half-life studies were carried out on whole blood samples drawn 1 h after injection (used as a base line) and then daily for up to 10 days. The difference in radioactive content between a 15 min sample, as used in previous studies (Takeda, 1966), and the 60 min sample used here was determined.

The half-life in each case was calculated by regression analysis from the radioactivity remaining ($\log_{10}$ percentage of baseline value) versus time. By extrapolating the graph to zero time the capacity of the extravascular compartment could be derived.

Surgical and medical recipients of fibrinogen ^{125}I were scanned daily with a one-inch NaI crystal detector for accumulating radioactivity, ie, fibrin deposition, indicative of thrombus formation (Kakkar, 1972).

Results

IN VITRO

The extracted material was shown to contain only one protein species, fibrinogen, by immunoelectrophoresis, double diffusion (Wolf and Walton, 1965), and SDS polyacrylamide gel electrophoresis (Weber and Osborn, 1969). The material had a single ultracentrifugation peak S_{20w} of 6.8 (MSE Centriscan 75). The absence of plasminogen was confirmed by the use of fibrin plates (Wolf, 1968) and by clot lysis tests which showed the inability of a clotted specimen to lyse during five days at 37°C even after the addition of plasminogen activators.

Quick's one-stage prothrombin time was used to determine fibrinogen stability after extraction; the clotting time remained constant after 6 hours at 37°C. In all samples, factor XIII was present as determined by the inability of 5 M urea to lyse a clot, unless Mersalyl (Evans) was added before clot formation.

The extinction coefficient was calculated from gravimetric analysis to be $E_{280nm}^{1.0\%}$ of 15.5, which is in agreement with the literature (Dellenback and Chien, 1970).

The percentage yield was $73 \pm 8\%$ as calculated by comparison of clot weight of fibrinogen with that of donor plasma.

Gel permeation studies were carried out on iodinated samples using continuous flow monitoring over a 2-inch thallium-activated NaI crystal detector. In all cases the eluent was saline citrate, pH 7.0, containing 0.05% Tween 20 to prevent non-specific adsorption of proteins to the dextran gels.

The percentage labelling with ^{125}I was determined as 30% to 60% by G25 chromatography. The free iodide content was shown by chromatography on G25 Sephadex to be less than 0.05% in all samples after passage through the desalting and sterilizing unit. Gel chromatography on Sephadex G200 showed only one peak of radioactivity, that of fibrinogen ^{125}I . Autoradiography of ^{125}I labelled material on immunoprecipitin plates confirmed the presence of a single labelled protein, fibrinogen. The absence of aggregated material (less than 0.1%) was demonstrated by passage through Sepharose 4B; this confirmed the ultracentrifugation analysis.

Owing to the rapidity of preparation, sterility testing could be performed only retrospectively, but on no occasion have recipients of fibrinogen shown any pyrogenic side effects. After sterilizing filtration, laboratory samples showed no bacterial growth. The apparatus used in the procedure was sterilized before use with Cidex (Ethicon Ltd), eliminating the risk of transmitting hepatitis, although the apparatus could be considered disposable.

Fig 3 Autologous ^{125}I fibrinogen: plasma half-life data.

IN VIVO

The half-life of the autologous fibrinogen (fig 3) in three normal human volunteers was shown to be 94.8 ± 2.4 hours (3.95 ± 0.1 days) with an extravascular compartment of 24.86% and a catabolic rate of 25.23% per day. The material has to date been used for the detection of deep vein thrombosis in approximately 70 patients. In 30 surgical patients, for whom there are complete data, a fibrinogen half-life of 74.8 ± 11.37 hours (3.12 ± 0.47 days) and a decreased value for the extravascular space (14.35%) was calculated. Figure 4 shows the typical plasma clearances of two recipients of autologous fibrinogen. Quoted figures show the very high degree of correlation (r), the calculated plasma half-life ($T_{1/2}$), and the estimate of extravascular space (a). The difference in radioactive content between a one-hour sample and that taken 15 minutes after injection was only 1.29% in a medical patient with bilateral deep vein thrombosis.

External monitoring using a counter-ratemeter (J & P Engineering Ltd, Reading) and hand-held crystal detector was used to localize radioactivity in patients (Kakkar, 1972). The injected ^{125}I fibrinogen was shown to be incorporated into thrombi developing in the deep veins of the legs, and a raised radioactive count over surgical wounds was seen, consistent with fibrin deposition at this site.

Autoradiographs of histological sections of thrombi removed from patients clearly demonstrated the incorporation of radioactivity in intercellular strands corresponding to the fibrin network. An association of radioactivity with leucocytes

Fig 4 Autologous ^{125}I fibrinogen: typical plasma survival (semi-log scale) in (a) a normal human and (b) a surgical patient.

(Edwards and Haynes, 1974) was only found in specimens removed after a period of several biological half-lives of fibrinogen, suggesting that leucocytes were responsible for the removal, by phagocytosis, of degradation products (Hawker *et al*, 1975).

Discussion

Fibrinogen extracted and radio-iodinated by the above method has several advantages over the commercially available material. (a) The use of patients' own plasma eliminates the risk of spreading hepatitis; (b) the material can be used for metabolic studies; (c) the *in vivo* half-life is that of native fibrinogen, therefore less radioactivity needs to be administered to achieve good results; (d) the preparative method is extremely reproducible as are the *in vivo* and *in vitro* results; (e) it is cheaper to produce, and the

costly delays and wastage of the radiopharmaceutical available from suppliers are avoided.

The half-life in normal subjects of 3.95 days compares very favourably with that reviewed in the literature for humans (Regoezci, 1971). The plotted data confirm the absence of degraded or denatured material; and the estimate of the extravascular compartment by extrapolation is also comparable with that previously reported.

The shortened intravascular half-life for fibrinogen calculated for surgical patients is to be expected (Hickman, 1971) since the continual formation and lysis of fibrin at the wound site is taking place at a greater rate than in normal states. The partitioning into extravascular space probably does not occur since synthesis of fibrinogen will be extremely rapid and there will be an increased intravascular pool. The underestimate of extravascular space of 14.35% in surgical patients as compared with 24.86% in

normal subjects may not represent extravascular space at all but merely partitioning into a third compartment, ie, the wound or thrombus site.

Fibrinogen is generally labelled by the method of McFarlane (1963) or by one of the many modifications. The methods all require the use of unstable iodine monochloride, accurate pH adjustment, and microlitre volume handling of a very high specific activity radioactive iodide.

The chloramine-T method originated by Hunter and Greenwood (1962) has been used for the labelling of labile proteins, eg, enzymes for radioimmunoassay. The use of less of this powerful oxidizing agent by McConahey and Dixon (1966) reduced protein damage. Krohn *et al* (1972) showed, in a separation technique on Sepharose 4B, that fibrinogen labelled by these methods and by the electrolytic method showed different chromatographic properties, and that the ratio of chloramine-T to tyrosine residues in the labelling mixture was critical if aggregation of fibrinogen was to be avoided.

There are two possible explanations for the differing results of our work and that of these previous authors:

(a) the absence of phosphate ions which, if present, would remove calcium ions necessary for the stability of fibrinogen preparation (Wolf, 1975). Krohn *et al* (1972) investigated the rate of hydrolysis of material labelled by three methods; only one preparation (that of iodine monochloride) was prepared without phosphate buffer, this being the only stable sample. This point is further exemplified by Coleman *et al* (1974), who found that only the iodine monochloride and enzymatic preparations were stable, and these were produced without contact with phosphate buffer. The instability of these chloramine-T iodinated fibrinogens is further seen by the *in vivo* results of Metzger *et al* (1973).

(b) a reduction in the chloramine-T concentrations used in the labelling reaction as judged by the molar ratio of chloramine-T to tyrosine. In our work we have minimized this ratio (max. molar ratio 1.2) and increased the reaction volume of solution from approximately 75 μ l to 850 μ l. Only by using conditions optimal for fibrinogen stability and ignoring labelling yields have we produced an aggregate-free material. The method described in this paper uses chloramine-T with volumes and specific activities of radiolabel that can be handled more comfortably in a small hospital laboratory than that of the iodine monochloride method.

Sterilizing filtration of easily aggregated proteins, eg, fibrinogen, can be a source of large losses and low yields. We have tested methods of filtration of fibrinogen to remove bacterial contaminants (Hawker and Hawker, 1975) and shown that losses

can be avoided by the careful choice of filter type. The traces of heparin (mol wt 17 500) which inevitably pass through the desalting column prevented coagulation in the plasma-diluted 125 I fibrinogen preparation.

The material and equipment required for the extraction are readily available in hospital laboratories and it is possible to produce pure material simply in a very short time. A 'kit' for the production of this radiopharmaceutical, including the 'separation vessel' combined desalting/sterilizing apparatus, and all necessary buffers is at present being tested.

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