RAPID PREPARATION OF AUTOLOGOUS RADIOIODINATED FIBRINOGEN

Ronald C. Roberts, Carolyn O. Sonnentag, and James H. Frisbie

Marshfield Clinic Foundation for Medical Research and Education, Marshfield, Wisconsin, and University of Connecticut Medical School, Farmington, Connecticut

The use of radioiodinated fibrinogen (RIF) for detection of postoperative venous thrombosis has been successfully developed in the past three years (*1–4*). Extended use of RIF is hampered, however, by the risk of hepatitis in commercial fibrinogen from pooled plasma and by the inconvenience of autologous preparation. Bettigole, et al (*5*) reported a preparation of autologous RIF requiring 3–5 hr to yield 20% of radioactivity bound to the final purified fibrinogen. The method of Ingraham, et al (*6*) requires 7–12 hr of preparation and yields 70% of radioactivity bound to protein. We have found that through gentle handling it is possible to shorten the method to 2 hr and that the preparation is clinically suitable for detection of deep vein thrombosis by the usual criteria.

METHODS

Reagents and materials. All solutions were prepared with commercial sterile injectable water*. Phosphate-buffered saline was 0.1 *M* sodium phosphate buffer, pH 7.4, in 0.9% NaCl. The 1 *M* $(NH_4)_2SO_4$ was prepared by diluting 4 *M* $(NH_4)_2SO_4$ with phosphate-buffered saline. Carrier (stable) iodide contained 9 mg NaI/liter. All salts were analytical reagent grade†. The $Na^{125}I$ was obtained carrier-free and preservative-free in minimum volume‡ and diluted to 200 μCi/100 μl with 1 *N* NaOH. Chloramine T|| was dissolved in phosphate-buffered saline immediately before iodination. Sterilization was accomplished immediately before injection by filtration through a 0.22-micron Millipore Swinney filter.

Assays. The Ratnoff-Menzie method (*7*) was used for assaying the fibrinogen content of plasma, the prepared fibrinogen solution, and the iodinated fibrinogen solution. Clottability was determined by comparing clottable protein with total protein. Total protein was determined by omitting the clotting step from the Ratnoff-Menzie assay.

The protein concentration of the fibrinogen preparation was estimated by absorption at 280 mμ. The absorbance at 280 mμ was determined on 0.1 ml diluted to 3.0 ml with saline with a Zeiss PMQ-II spectrophotometer. The absorptivity was considered to be $A^{1\text{ cm, }0.1\%}_{280\text{ m}\mu} = 1.55$ (*8*).

Distribution of radioactivity in the reaction mixture (RIF assay) was assessed in a manner similar to that of Regoeczi (*9*). Ten-microliter samples of the radioiodinated fibrinogen solutions were added to 1 ml of fresh, pooled, decalcified plasma in 12 × 75-mm clear plastic tubes* which supplied a source of carrier fibrinogen. Tubes were counted in a deep-well autogamma counter to determine total plasma radioactivity. The fibrinogen was then clotted by addition of 10 μl (5 units) of topical thrombin† and removed by spinning out on an etched glass stirring rod, carefully squeezing out the clot liquor. The tube was recounted to determine serum radioactivity. The amount of unbound iodide absorbed to the clot was statistically insignificant (1.7% ± 3.5 s.d., n = 15) as determined by adding $Na^{125}I$ to fibrinogen preparations before clotting. Finally the nonclottable protein-bound radioactivity was assessed by adding 0.5 ml of 30% (w/v) trichloroacetic acid (TCA). The sample was centrifuged at 500 G for 5 min, the supernatant decanted as thoroughly as possible, and the precipitate counted. Cor-

* Cutter Laboratories, Berkeley, Calif.
† Mallinckrodt Chemical Works, St. Louis, Mo.
‡ Mallinckrodt Nuclear, St. Louis, Mo.
|| Eastman Organic Chemicals, Rochester, N.Y.

Received Mar. 31, 1972; revision accepted June 26, 1972.
For reprints contact: Ronald C. Roberts, Marshfield Clinic Foundation for Medical Research and Education, 510 N. St. Joseph Ave., Marshfield, Wis. 54449.

* Falcon Plastics, Los Angeles, Calif.
† Parke-Davis, Detroit, Mich.

rection was made for absorption of unbound iodide to the TCA precipitate which was 17% (±4.0 s.d., n = 15). This correction factor was determined by addition of approximately 1 μCi of $Na^{125}I$ to unlabeled fibrinogen. The data thus obtained allowed the determination of the clottable radioactivity, non-protein-bound radioactivity, and the percent clottability of the protein-bound radioactivity.

Preparation of radioiodinated fibrinogen. Twenty milliliters of fasting whole blood were collected by venipuncture into heparinized tubes, transferred to sterile glass centrifuge tubes, and centrifuged at 6,000 G for 5 min. The plasma was transferred to new tubes and recentrifuged at 6,000 G to remove any remaining platelets and cells. The fibrinogen was precipitated from 4 ml of this plasma by addition of 1.3 ml of 4 *M* $(NH_4)_2SO_4$ in a sterile, disposable, 17 × 100 mm, polystyrene tube*. The solution was mixed by gentle inversion of the tube and was centrifuged at 500 G for 5 min. Then the precipitate was dissolved in 4 ml of phosphate-buffered saline, reprecipitated with 1.3 ml of 4 *M* $(NH_4)_2SO_4$, and redissolved in 1 ml of phosphate-buffered saline. The concentration of fibrinogen was determined by measuring absorbance at 280 mμ and was adjusted to 3 mg/ml by dilution (the concentration has always been in the range of 4–12 mg/ml). Samples of this solution have been examined for homogeneity by various methods reported in the results.

One milliliter of this fibrinogen solution was iodinated. The iodination mixture finally adopted as routine contained 1 ml of the fibrinogen solution (3 mg fibrinogen/ml or 8.6×10^{-9} moles); 10 μl NaI carrier solution (6×10^{-10} moles); 10 μl $Na^{125}I$, 200 μCi (trace); and 10 μl chloramine T (0.034 gm/10 ml or 12×10^{-7} moles). These were added in the order listed to a 17 × 100-mm clear polystyrene tube, mixed gently, and allowed to react for 6 min. The reaction was essentially stopped by addition of 2 ml of the original plasma or (in some experiments in which the homogeneity of the RIF was evaluated) phosphate-buffered saline.

The non-protein-bound ^{125}I could most conveniently be removed by reprecipitating the fibrinogen with 1 ml of 4 *M* $(NH_4)_2SO_4$, washing the precipitate once with 1 *M* $(NH_4)_2SO_4$, and redissolving the precipitate in 1 ml of phosphate-buffered saline. The clottability of the protein-bound ^{125}I and the non-protein-bound radioactivity were then determined on duplicate 10 μl samples of this solution. Preparations in which the clottability of protein-bound ^{125}I was greater than 90% and non-protein-bound ^{125}I less than 1% were considered acceptable for injection and sterilized.

Physical and chemical methods. Disk electrophoresis on polyacrylamide gels was performed using the method of Davis (*10*) and a 5% gel. Radioautographs of the disk electrophoresis gels were obtained by exposing Kodak RP x-ray film for 48 hr. Immunoelectrophoresis was done by the method of Schiedegger (*11*) using rabbit antiplasma antisera prepared in our laboratory and commercial anti-fibrinogen antisera prepared in rabbits*. Sedimentation velocity analysis was carried out in a Beckman Model E analytical ultracentrifuge. The sedimentation coefficient was determined from measurements on the maximum ordinate using a Nikon shadowgraph (*12*).

Half-life studies. Half-life studies were carried out on serum and plasma samples drawn 15 min after injection and then daily for 8–10 days. The difference between the plasma and serum counts was considered to be fibrinogen radioactivity. The half-life was determined from semilogarithmic plots of percent of the radioactivity remaining versus time by extrapolating the terminal linear portion to zero time. The slope and the y intercept were determined by least-squares analysis.

RESULTS

Isolation. The yield of fibrinogen from 4 ml of plasma was 4.5–7.5 mg from individuals with normal

* Falcon Plastics, Los Angeles, Calif.

* Behring Diagnostics, Inc., Woodbury, N.Y.

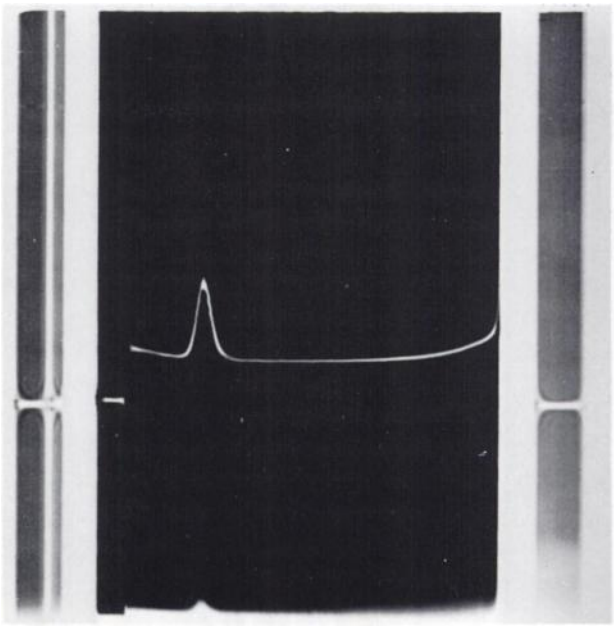

FIG. 1. Analytical ultracentrifuge pattern of sedimentation velocity run on fibrinogen preparation (before iodination). Centrifugal force left to right; photograph taken after 32 min at 56,000 rpm.

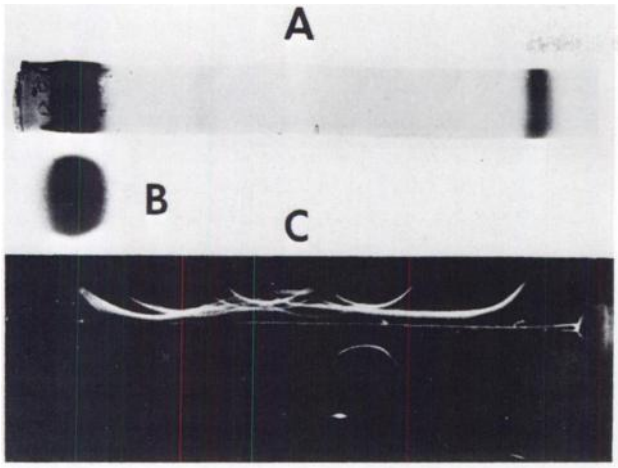

FIG. 2. A, disk electrophoresis of a radioiodinated fibrinogen preparation, pH 8.9, 5% polyacrylamide gel. Anode is at right, band on far right represents dye front. B, radioautograph of polyacrylamide gel electrophoretogram shown in A. C, immunoelectrophoresis of fibrinogen preparation, photograph of obtained precipitin lines. Anode is on left. Top sample is normal human plasma, bottom sample was iodinated fibrinogen preparation. Precipitin pattern was developed using rabbit antihuman plasma antisera.

levels of fibrinogen. This represents about 50% of the available fibrinogen. The clottability of the preparation ranged from 90 to 97% as determined by the Ratnoff-Menzie assay. The analytical ultracentrifuge pattern of a representative preparation of 95% clottability showed a single symmetrical peak (Fig. 1). The sedimentation coefficient for this sample was $S^{0.5\%}_{20,w} = 6.63$. Disk electrophoresis of several preparations showed a major component and a minor component (Fig. 2A).

Iodination. We studied the conditions for iodinating the fibrinogen. The yields of clottable radioactivity with time at three different concentrations of chloramine T are shown in Fig. 3. The iodination mixtures contained 17, 34, and 68 μg of chloramine T. The concentration of carrier NaI was 6×10^{-10} moles in these experiments. Varying the fibrinogen concentration from 1 to 3 mg/ml did not affect the degree of iodination observed. Damage to fibrinogen by chloramine T was determined by loss of clottability. Six reaction mixtures with ratios of protein/chloramine T varying from 82/1 to 13/1 by weight were assayed for clottability. There was no detectable loss of clottable protein at 6 min reaction time at a protein/chloramine T ratio as low as 41 to 1.

The maximum incorporation of radioactivity into clottable protein was 60%, and the optimal reaction time was about 6 min. The lowest chloramine T concentration producing maximum iodination was 34 μg/ml/3 mg of fibrinogen, a protein/chloramine T ratio of 88 to 1. This is more than twice the estimated minimum ratio for preserving high clottability.

Immunoelectrophoresis of the radioiodinated product diluted with phosphate-buffered saline instead of plasma generally showed a single component, fibrinogen (Fig. 2C), but occasionally a trace amount of another protein of gamma mobility was observed. Radioautography of disk electrophoretic gels showed only one band of radioactivity corresponding to the major protein band (Fig. 2B).

Clottability of protein-bound radioactivity and half-life studies. The clottability of the protein-bound radioactivity in the final RIF preparation and the percentage of non-protein-bound radioactivity for six normals is presented in Table 1. RIF was prepared as described in the Methods section, using 200 μCi of carrier NaI in the iodination reaction mixture. Also the results of half-life studies on these normals are presented in Table 1 in terms of the slope and y intercept of the terminal linear portion of a plot of log p versus time (*13*) in which p is

TABLE 1. RADIOIODINATED FIBRINOGEN HALF-LIFE STUDIES

Subject	Plasma fibrinogen (mg%)	Clottability of RIF (%)	Non-protein bound ^{125}I (%)	a_1*	C_1*	Estimated s.e.†	$T_{1/2}$ for a_1 (days)
FS	240	95	0.6	0.182	0.780	0.0247	3.85
RCR	269	95	1.1	0.2184	0.643	0.040	3.17
GR	306	94	0.6	0.1728	0.739	0.0423	4.01
CH	299	93	1.1	0.1800	0.540	0.0454	3.85
JP	248	94	0.6	0.1416	0.569	0.0536	4.89
HR	276	94	0.6	0.1584	0.616	0.04669	4.38
Avg.	273.00	94.17	0.7666	0.1755	0.6478		4.03
s.d.	24.17	0.6962	0.2360	0.0223	0.0866		0.5270
s.e.	10.81	0.3113	0.1055	0.0099	0.0387		0.2356

* Parameters from usual two-compartment equation (Ref. 13) $p = C_1e^{-a_1t} + C_2e^{-a_2t}$, where p = fraction of original dose in plasma; t = time; and C_1, C_2, and a_1, a_2 are constants defining the relative volumes of distribution and the decrease in fibrinogen radioactivity, respectively. a_1 and C_1 refer to the y intercept and slope of the terminal linear portion of the disappearance curve.
† Standard error of estimate of points from 3 days from the least-squares line.

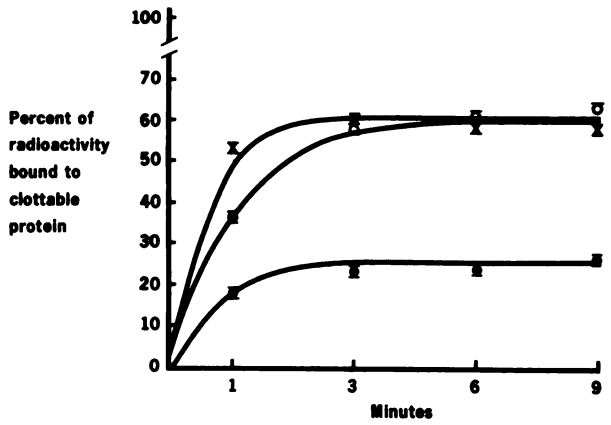

FIG. 3. Effect of time and chloramine T concentration on recovery of clottable radioactivity. Reaction mixtures consisted of 3 mg of 90% clottable protein; carrier iodide; tracer amounts of ^{125}I and 17 (●), 34 (0), and 68 (x) μg chloramine T in 1 ml phosphate saline pH 7.4. Each point represents average of two assays.

the fraction of the original concentration of radioactivity remaining.

DISCUSSION

Our preparation method differs from other procedures (*5,14,15*) primarily by omitting refrigeration to remove any cold-insoluble material. Although refrigeration of our preparation overnight before iodination sometimes yielded a small amount of precipitate, no improvement in clottability after iodination was caused by its removal. Also no aggregated material was ever observed upon ultracentrifugation of several of the preparations.

Successful preparation of completely soluble fibrinogen adequately freed of contaminating proteins requires very gentle handling of the ammonium sulfate precipitated protein. The yield was greatly improved by (A) short low-speed centrifugation just sufficient to settle the precipitate without packing and (B) mixing the 1 *M* $(NH_4)_2SO_4$ wash solution by gentle inversion of the tube.

Although immunoelectrophoresis and disk electrophoresis of concentrated (5–7 mg protein/ml) fibrinogen preparations generally showed a trace component in the gamma globulin region, radioautographs of the iodinated preparations showed no radioactivity in this region. The high clottabilities indicate that this contaminant protein is less than 5%.

The half-life data in Table 1 indicate that our preparations behaved in vivo similarly to others from both homologous and autologous sources. In a recent review, Regoeczi (*16*) reports the average half-life and standard deviation for all complete studies so far reported was 4.1 ± 0.5 days. Our value was 4.0 ± 0.5 days for six normals.

We have reinjected our RIF into 35 individuals without any pyrogenic or other untoward reactions.

REFERENCES

1. Flanc C, Kakkar VV, Clarke MB: The detection of venous thrombosis of the legs using ^{125}I-labelled fibrinogen. *Brit J Surg* 55: 742–747, 1968
2. Negus D, Pinto DJ, LeQuesne LP, et al: ^{125}I-labelled fibrinogen in the diagnosis of deep-vein thrombosis and its correlation with phlebography. *Brit J Surg* 55: 835–839, 1968
3. Kakkar VV, Nicolaides AN, Renney JTG, et al: ^{125}I-labelled fibrinogen test adapted for routine screening for deep-vein thrombosis. *Lancet* 1: 540–542, 1970
4. Tsapogas MJ, Miller R, Peabody RA, et al: Detection of postoperative venous thrombosis and effectiveness of prophylactic measures. *Arch Surg (Chicago)* 101: 149–154, 1970
5. Bettigole RE, Himmelstein ES, Clifford GO, et al: Autologous ^{131}I-fibrinogen survival: a useful technique. *J Nucl Med* 10: 322–323, 1969
6. Ingraham SC, Silberstein EB, Kereiakes JG, et al: Preparation and ^{131}I-tagging of autologous fibrinogen at ambient temperatures. *J Nucl Med* 10: 410, 1969
7. Ratnoff OD, Menzie C: A new method for the determination of fibrinogen in small samples of plasma. *J Lab Clin Med* 37: 316–320, 1951
8. Dellenback RJ, Chien S: The extinction coefficient of fibrinogen from man, dog, elephant, sheep, and goat at 280 mμ. *Proc Soc Exp Biol Med* 134: 353–355, 1970
9. Regoeczi E: Measuring the coagulability of fibrinogen in plasma by isotopic means: method and principles for use in in vivo studies. *Thromb Diath Haemorrh* 18: 276–285, 1967
10. Davis BJ: Disc electrophoresis. II. Method and application to human serum proteins. *Ann NY Acad Sci* 121: 404–427, 1964
11. Schiedegger JJ: Une micro-méthode de l'immuno-électrophorèse. *Int Arch Allerg* 7: 103–110, 1955
12. Schachman HK: *Ultracentrifugation in Biochemistry*. New York, Academic Press, 1959, pp 63–103
13. Schultze HE, Heremans, JF: *Molecular Biology of Human Proteins with Special Reference to Plasma Proteins*. Amsterdam, Elsevier, 1966, pp 455–463
14. Takeda Y: Studies of the metabolism and distribution of fibrinogen in healthy men with autologous ^{125}I-labeled fibrinogen. *J Clin Invest* 45: 103–111, 1966
15. MacFarlane AS, Todd D, Cromwell S: Fibrinogen catabolism in humans. *Clin Sci* 26: 415–420, 1964
16. Regoeczi E: Iodine-labelled fibrinogen: a review. *Brit J Haemat* 20: 649–663, 1971