"KIT" PREPARATION OF RADIOIODINATED AUTOLOGOUS FIBRINOGEN USING ^{131}I-MONOCHLORIDE

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A rapid method for the production of radioiodinated autologous fibrinogen (RAF) is given. The method is complete within 1 hr, can be conveniently handled in kit form, produces fibrinogen that is 85–90% clottable, and eliminates the risk of serum hepatitis transmission. Iodination yields of 48–60% were obtained over a range of iodine-to-fibrinogen ratios of 0.5–3.0 using the Helmkamp modification of the iodine monochloride labeling procedure. The physicochemical damage to the fibrinogen molecule owing to the radiolabeling procedure was determined by chromatographing RAF on Bio Gel A 1.5 m and 15 m. The biologic authenticity of RAF was assayed employing both plasma clearance and tissue-distribution studies in dogs. Plasma half-lives of 52–58 hr were found corresponding to the known rate of fibrinogen catabolism in dogs. Three and one-half, 6, and 7.6% of the injected RAF was found in the thyroid, stomach, and liver, respectively, at 24 hr. The results indicate that this rapid method produces biologically active radiolabeled fibrinogen suitable for routine clinical use.

Fibrinogen labeled with radioactive iodine has been used for the detection of blood clots in their early stages of formation. Several investigations using ^{125}I-fibrinogen have demonstrated the efficacy of this radiopharmaceutical in detection of deep-vein thrombosis in the lower extremities (*1,2*). Recently, application of ^{131}I-fibrinogen for imaging emboli in the ileofemoral region and lungs has been proposed (*3,4*). Its use has been limited in the United States because there is a significant risk of serum hepatitis associated with the use of iodinated fibrinogen prepared commercially from pooled human blood sources. Fibrinogen may be obtained from screened donor blood, certified hepatitis-free, but is available in only a few major centers. In addition, the time required for preparation of labeled autologous fibrinogen by previously published methods varies from 2–12 hr, and the methods are not readily amenable to a kit system (*5–7*). Various procedures for preparing radioiodinated fibrinogen have appeared in the literature since McFarlane (*8*) originally reported the iodine monochloride (ICl) method. Although the chloramine-T (*9*), electrolytic (10), and enzymatic (11) labeling methods have been used to radioiodinate fibrinogen, recent evidence of Krohn, et al (*12,13*) and Metzger, et al (*14*) indicates that concomitant protein alteration is minimal when the ICl technique is used.

The following report describes a method for preparing radioiodinated autologous fibrinogen (RAF) utilizing a convenient "kit" providing reagents and materials pretested for purity, sterility, and apyrogenicity. The procedure combines the fibrinogen separation technique of Roberts, et al (*5*) with the iodine monochloride labeling procedure as modified by Helmkamp, et al (*15*). Advantages of the present method are: (A) the entire procedure can be completed in 1 hr, (B) there is no risk of serum hepatitis transmission to the patient, and (C) the final components of the "kit" intermediate used to label autologous human fibrinogen meet the USP requirements for i.v. injectables.

MATERIALS AND METHODS

Components of the kit. The following quantities are sufficient to prepare ten individual doses of RAF: 5 cc of 3×10^{-4} *M* iodine monochloride solution containing 3–5 mCi ^{131}I/cc; 50 cc of 3 *M* ammonium sulfate solution; 80 cc of 0.1 *M* phosphate buffer, pH 7.4, containing 0.05 *M* E-aminocaproic

Received Jan. 18, 1974; revision accepted June 4, 1974.

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acid (EACA) and 0.38% trisodium citrate; 10 Hopkins double-closure resin vials containing 2 gm of Dowex 1X-4 (50–100 mesh) anion-exchange resin (chloride form) and a 3 × 10-mm Teflon-coated magnetic stirring bar; 50 cc of 1% human serum albumin with 0.1 mg/cc $Na_2S_2O_5$; and 10 10-cc sterile evacuated siliconized serum tubes.

The radioactive iodine monochloride reagent was prepared by adding 20–25 mCi of protein iodination grade $Na^{131}I$ of high specific concentration to 5 cc of 3×10^{-4} *M* ICl stock solution contained in a 10 cc multidose vial. The stock solution was made by diluting an 0.2 *M* ICl solution (*15*) 1:66 with 2 *M* NaCl, giving a final composition of about 3×10^{-4} *M* ICl_2^-, 4.0×10^{-4} *M* KIO_3, 0.015 *M* HCl, and 2 *M* NaCl. This solution provided sufficient radioactivity for ten individual doses of 300 μCi each. The stability of the solution was judged by determining its fibrinogen labeling efficiency and its percent $^{131}IO_3^-$ as a function of time. Helmkamp's method was used to determine the latter.

Three molar ammonium sulfate, 0.1 *M* phosphate buffer, pH 7.4, and 1% human serum albumin solutions were prepared with water for injection USP and sterilized by passage through a 0.22-μm membrane filter into 30 cc vials.

The Hopkins double-closure resin vials with magnetic stirring bar were prepared following the procedure of Cooper, et al (*16*).

Preparation of radioiodinated autologous fibrinogen. Twelve milliliters of the patient's heparinized blood was centrifuged at 1,900 G for 10 min and 4 cc of plasma transferred to another tube. Fibrinogen was precipitated from plasma by gradual addition of 2 ml of 3 *M* $(NH_4)_2SO_4$ accompanied by gentle mixing and centrifugation at 200 G for 5 min. Following removal of the supernatant, the precipitate was dissolved in 4 ml of 0.1 *M* phosphate buffer, pH 7.4. Fibrinogen was once again precipitated from this solution by addition of 2 ml of $(NH_4)_2SO_4$, centrifuged, and the supernatant removed. The purified fibrinogen was redissolved in 2 ml of phosphate buffer in preparation for iodination.

Iodination was accomplished by addition of 0.35 cc of radioactive iodine monochloride solution to the buffered fibrinogen and the solution mixed for 3 min to promote uniform labeling. Nonprotein-bound radioiodine was removed using an anion-exchange resin contained in a Hopkins double-closure vial. Prior to addition of fibrinogen, 4 cc of 1% human serum albumin containing $Na_2S_2O_5$ were added to the vial. After stirring gently at a slow speed for 5 min, the iodinated fibrinogen was withdrawn from the end of the vial having the glass frit and prepared for injection into the donor patient by filtering through a 0.22-μm Millipore filter. The yield of RAF was determined by assaying the activity in the resin vial before and after removal of the protein mixture.

The final, formulated ^{131}I-RAF contains not more than 0.56–1.9 mg RAF, 0.2 mg EACA, 6.3 mg human serum albumin, 1.2 mg of sodium citrate, and 0.06 mg sodium metabisulfite per milliliter of phosphate buffer, pH 7.4.

Iodine-to-fibrinogen ratio. The amount of fibrinogen precipitated from 4 ml of plasma was determined in 17 normal volunteers and the mean value and standard deviation determined. This was achieved by measuring the ultraviolet absorbance of the purified fibrinogen solution at 280 nm with absorptivity taken to be 1:55 (*17*). The range of iodine-to-fibrinogen ratios produced with this method of labeling was then calculated and the iodination yield and clottability of RAF determined over that range. For comparison, RAF with known iodine-to-fibrinogen ratios was prepared by determining the amount of autologous fibrinogen present immediately prior to iodination followed by labeling with an appropriate amount of ICI solution.

Physical methods. The percent protein-bound radioiodine in the final product was determined by precipitation with 20% trichloracetic acid. Clottability was measured using the procedure of Regoeczi (*18*). Topical thrombin and EACA were dissolved in neutral phosphate buffer prior to addition of the fibrinogen. The measurement employed 45 units of thrombin and approximately 3 hr clotting time at room temperature. The clot was removed with an etched glass rod.

The amount of radiolabeled gamma-globulin along with the percent aggregation was estimated by Gel permeation chromatography on Bio Gel A 15 m and A 1.5 m columns. These columns were eluted with a saline citrate buffer consisting of 0.15 *M* NaCl, 0.05 *M* sodium citrate, and 0.05 *M* EACA, pH 7.0. The amount of aggregated fibrinogen resulting from the ICl labeling procedure was determined using a Bio Gel A 15 m column whereas the amount of iodinated gamma-globulin and radioactive I^- in the RAF preparation was determined on a Bio Gel A 1.5 m column. These Gel permeation chromatograms were obtained over a range of iodine-to-fibrinogen ratios from 1.0 to 3.0.

Biologic methods. Experiments were performed in 12 dogs to determine the plasma clearance curve, tissue distribution, and arterial and venous clot-to-blood ratio following intravenous injection of 300 μCi of RAF.

Plasma clearance. Three animals were anesthetized

with i.v. sodium pentobarbital and 12 ml of blood were withdrawn for preparation and labeling of RAF. Three hundred μCi of RAF were reinjected into each animal and venous blood samples withdrawn at intervals until the sixth day after injection. Blood samples were prepared by centrifugation at 1,900 G for 10 min and the plasma separated. Two-milliliter plasma samples were counted in a well counter for 2 min or until a minimum of 10,000 counts were obtained. All samples were counted simultaneously with a standard prepared by diluting RAF 1–3,000. Plasma clearance curves were constructed by calculating plasma volume from serial hematocrit measurements and blood volume, estimated as 7% by weight.

Tissue distribution. Tissue distribution was measured in six dogs at 2 hr and 24 hr following injection of radioiodinated autologous fibrinogen. (One of four animals in the 2 hr-study was subsequently excluded when pneumonia was found at autopsy.) Individual organs in each animal were dissected, exsanguinated, and washed, and the activity counted with a scintillation detector. The blood content was determined by deriving the blood volume as equivalent to 7% of the animal's body weight. Tissue distribution was expressed as a percent of the injected dose per organ and the mean values calculated.

Clot-to-blood ratio. The clot-to-blood ratio of RAF was determined for arterial and venous clots as a function of the age of the clot prior to injection of the RAF. Thrombi were prepared in the femoral veins and arteries of a dog by suturing thrombin-soaked threads (50 I.U./ml in physiologic saline) through the walls and lumen of the vessels according to the method of Rhodes, et al (*19*). The thrombi were allowed to form on the threads for intervals from 2 min to 3 hr. Three hundred μCi of RAF were injected intravenously and all thrombi exposed to circulating labeled fibrinogen for 2 hr. The thrombi on the threads were removed by dissection, washed in saline, dried, weighed, and their activity determined by counting in a well counter. The radiolabeled fibrinogen level in the venous blood was measured at the time each clot was removed and the clot/blood ratio calculated.

RESULTS

From 4 cc of plasma 8.12 ± 2.0 mg of fibrinogen were recovered. This represents 73% of the available fibrinogen (*20*). Ninety-five percent of the RAF had an iodine-to-fibrinogen ratio varying between 1.3 and 3.8 with a mean ratio of 1.9. When RAF was compounded without maintaining a constant ratio, the labeling yields varied from 48 to 60% with the radiopharmaceutical being 85–90% clottable and 98% TCA precipitable. The labeling yield as determined by the resin vial agreed within ±2% of that obtained by TCA precipitation.

The ^{131}ICl iodinating reagent was found to have a constant iodinating efficiency over a week's period with the extent of unreactive $^{131}IO_3^-$ being less than 1%. The parent, nonradioactive 3×10^{-4} *M* ICl solution has shown no decrease in labeling efficiency during the past 8 months. Helmkamp (*15*) quotes the stability of this solution at greater than 1 year. The shelf-life of the remaining components is at least 8 months.

Gel permeation chromatography. A radioactive tracing from a typical gel permeation chromatogram obtained with Bio Gel A 1.5 m and A 15 m are shown in Figs. 1 and 2. The dashed line in Fig. 1 indicates the molecular weight profile of the clottable protein. The shoulder on the iodinated fibrinogen peak and the radioactive peak at an elution volume of 25 ml correspond to labeled nonclottable protein and I^-, respectively. RAF consists of 3–8% nonclottable protein and less than 2% I^-. The nonclottable protein may represent fibrinogen degradation products.

Gel permeation chromatography reveals no evidence of labeling-induced aggregation with 100% of the activity being eluted from a Bio Gel A 15 m column (Fig. 2) and with no high molecular weight tailing. The molecular weight profile assessed by

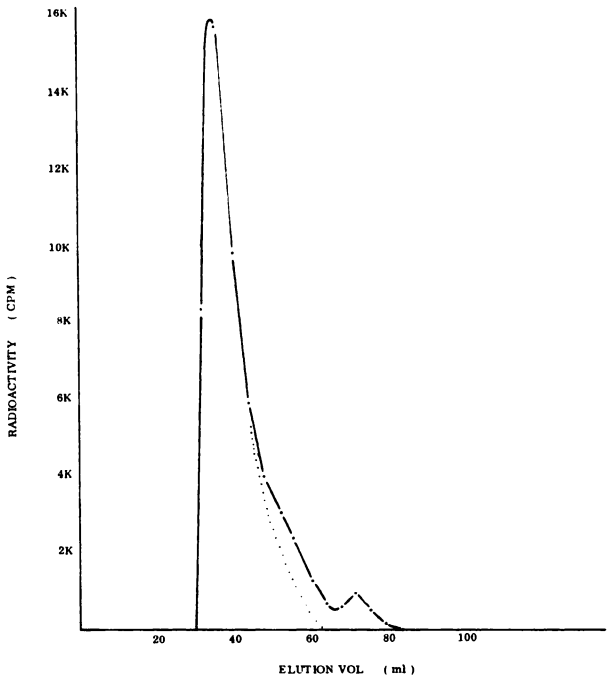

FIG. 1. Gel permeation chromatogram of radioiodinated autologous fibrinogen obtained on Bio Gel A 1.5 m. Dashed line was obtained by measuring clottability on individual eluant fraction between 40 and 70 ml. Peaks are 38 ml, fibrinogen; 55 ml, nonclottable protein; 73 ml I^-.

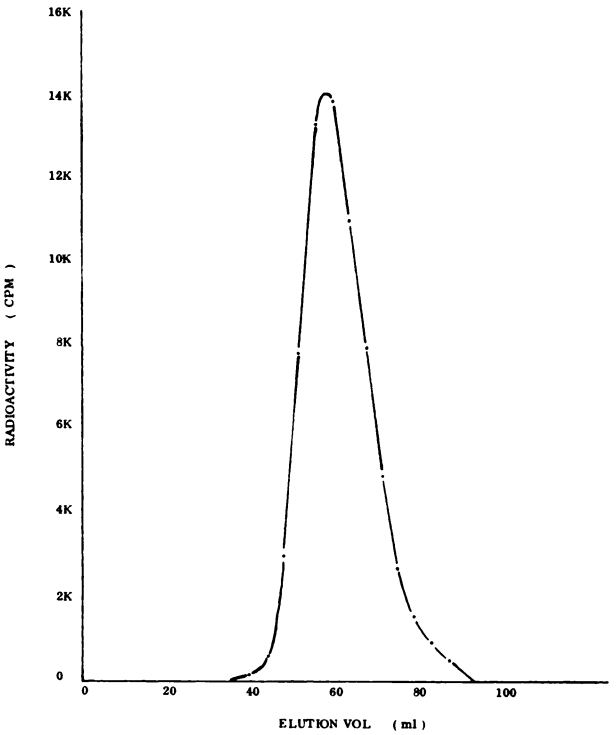

FIG. 2. Gel permeation chromatogram of radioiodinated autologous fibrinogen obtained on Bio Gel A 15 m. Molecular weight profile corresponds to that of fibrinogen with no significant high molecular weight tailing. Void volume is 23 ml.

gel permeation chromatography is invariant of the iodine-to-fibrinogen ratio over the range of 1.0–3.0.

Stability of iodinated material. The stability of the RAF radiopharmaceutical at 22°C was estimated utilizing gel permeation chromatography on both Bio Gel A 15 m and A 1.5 m as a function of time. The results are shown in Table 1 where the activity has been divided into four groups: iodinated fibrinogen, aggregated fibrinogen, nonclottable protein, and I^-. The results indicate that RAF need not be injected immediately subsequent to preparation.

Both stability and clottability were improved by the addition of EACA, citrate, and albumin. EACA inhibited plasma-induced degradation of fibrinogen whereas albumin inhibited the hydrolysis of RAF into protein and free I^- maintaining the $^{131}I^-$ concentration at 4% for up to 22 hr. The latter is in agreement with the observations of Krohn, et al (*12*) who found that the initial daily rate of hydrolysis in vitro for iodinated fibrinogen was reduced from 25 to 2% upon the addition of either plasma or albumin.

Plasma clearance, tissue distribution, and clot uptake. Plasma clearance curves of the percent TCA precipitable radioactivity in the plasma as a function of time are shown in Figs. 3 and 4 for iodine-to-

TABLE 1. IN VITRO STABILITY OF RAF

Time after preparation (hr)	Clottability (%)	Distribution of RAF (%)*		
		Iodinated fibrinogen	Non-clottable protein	I^-
0.5	87.2	93	5.0	2.1
3.5	85.0	91	4.7	3.6
9.0	79.6	85	10	3.9
22.0	76.8	83	12	3.7

* Aggregated fibrinogen <0.5.

fibrinogen ratios of 1:1 and 3:1, respectively. Table 2 contains a list of the in vivo and in vitro parameters used to evaluate RAF for three representative iodine-to-fibrinogen ratios. Between 40 and 50% of the injected RAF clears the plasma compartment with a $t_{1/2}$ of 52–56 hr corresponding to the known rate of fibrinogen catabolism in dogs (*14,21,22*). No significant trends in either the percent cleared or the $t_{1/2}$ of RAF are found over a range of iodine-to-fibrinogen ratios of 1:1 or 3:1. The results from the 2- and 24-hr whole-body distribution studies are summarized in Table 3 and show that a maximum of 3.5, 6, and 7% of the injected dose is found in the thyroid, stomach, and liver, respectively, over the first 24 hr.

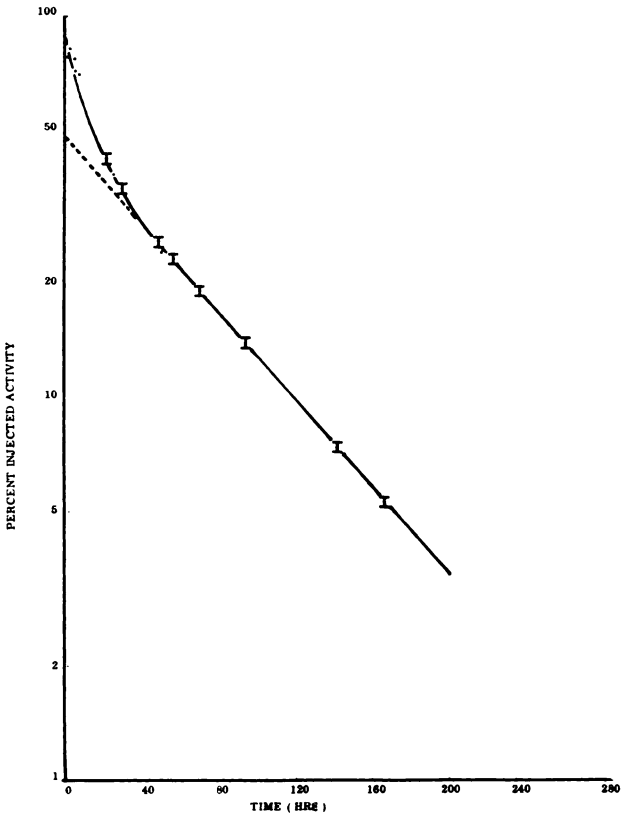

FIG. 3. Plasma clearance curves of TCA precipitable activity for RAF preparation at ratio of 1 atom I per molecule fibrinogen.

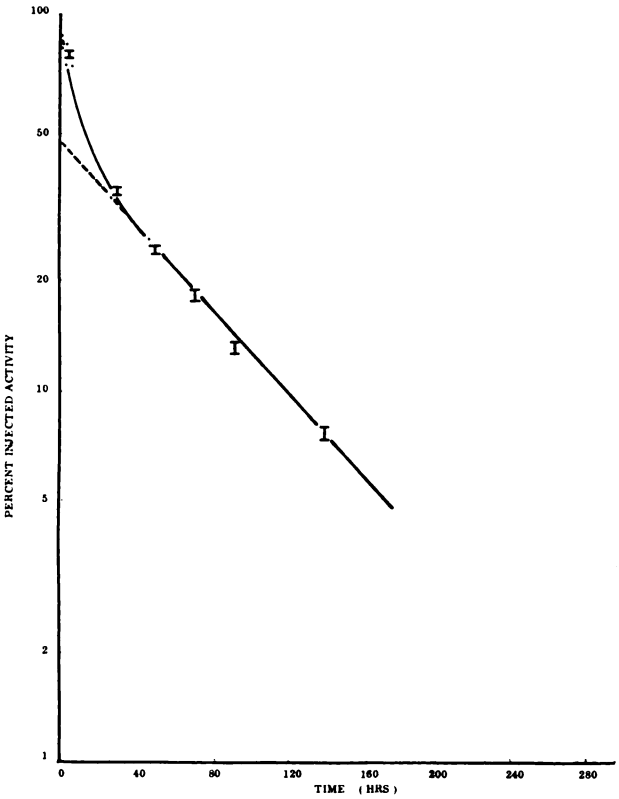

FIG. 4. Plasma clearance curves of TCA precipitable activity for RAF preparation at ratio of three atoms I per molecule fibrinogen.

Figure 5 contains data showing the clot-to-blood ratio in both arterial and venous clots as a function of age of the clot prior to injection of RAF. The clot weights (mean 75 mg, range 14–240 mg) were sufficient to calculate clot-to-blood ratio within a 10% error limit.

DISCUSSION

The clinical usefulness of radioiodinated fibrinogen for the detection of thrombi has been established by several investigators using nonimaging techniques. Its value as a means of imaging thrombi and thromboemboli using ^{123}I and ^{131}I is being currently investigated. In this country use of fibrinogen prepared from pooled blood sources carries a significant risk of transmission of serum hepatitis. Type B hepatitis has been estimated to occur in 1 of 85 transfusions where blood is utilized from pooled sources and may be higher in metropolitan areas (*23*). The risk becomes insignificant when carefully selected and screened donor blood is used. In Britain there have been no reports of hepatitis transmission from fibrinogen prepared at a center where pooled and screened blood is utilized (*24*). Nevertheless, a method which allows rapid labeling of autologous fibrinogen overcomes the practical difficulties which have heretofore limited the widespread clinical application of the radiopharmaceutical in this country.

The radiopharmaceutical is compounded utilizing a rapid kit method in which the reagents are stable and autoclavable, the final product is membrane filterable, and its in vitro stability is sufficient to allow for clottability and pyrogen testing prior to intravenous administration. The citrate ions and the EACA present in the phosphate buffer inhibit the formation of thrombin and plasmin, respectively. The presence of both inhibitors is essential during the purification and labeling procedure in order to avoid degradation of the fibrinogen molecule. EACA is present in a concentration one five-thousandth of that used therapeutically and no adverse reaction

TABLE 2. VARIATION IN RAF AS A FUNCTION OF THE IODINE-TO-FIBRINOGEN RATIO

Dog	Iodine-to-fibrinogen ratio	Labeling yield (%)	TCA precipitability (%)	Clottability	Plasma clearance: last component	
					$t_{1/2}$ (hr)	% cleared
A	1:1	60	98	85.1	52	48
B	2:1	58	98	90.2	56	41
C	3:1	64	97	88.1	55	46

TABLE 3. DISTRIBUTION OF RAF IN DOGS

Time after injection (hr)	% of injected dose									
	Blood	Lungs	Spleen	Kidneys	Stomach	Bladder and urine	Heart	Liver	Thyroid	Carcass
2.0	63.3	1.66	0.66	1.39	2.13	0.32	0.63	9.87	0.22	81.2
24.0	39.8	2.38	0.30	1.16	6.06	0.88	0.83	7.58	3.58	62.6

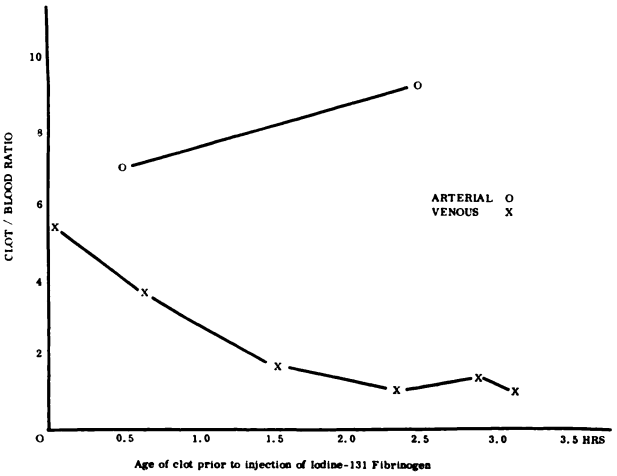

FIG. 5. Clot-to-blood ratio of RAF as function of age of arterial or venous clot. Fibrinogen exposure time was 2 hr. O, arterial; X, venous.

need be anticipated from its use or that of the other components of the kit.

The results show that the size of the intravascular component of RAF in dogs does not change over a range of iodine-to-fibrinogen ratios varying from 1.0 to 3.0. This is in agreement with the work of Zetterqvist (*21*) in which the size of the intravascular component in humans was independent of the iodine-to-fibrinogen ratio over a range of 0.8–2.7. However, McFarlane (*24*), working with rabbits, observed that the size of the intravascular component decreased when the iodine-to-fibrinogen ratio exceeded 0.5.

The intravascular component of RAF was found to be 45% of the injected material, which compares with 66% found by Zetterqvist using pooled fibrinogen and the ICl labeling procedure. Metzer, et al (*14*) found that the component size varies from 34 to 50% using the ICl procedure, pooled fibrinogen, and an iodine-to-fibrinogen ratio of less than 0.5.

The plasma clearance and tissue-distribution studies show RAF appears to be neither quickly hydrolyzed in vivo nor rapidly sequestered by the liver, and both the plasma half-time and percent cleared are in agreement with recent data of Metzer, et al (*14*). The high clot/blood ratios obtained in freshly forming clots indicate that despite the presence of labeled nonclottable, low molecular weight fragments (3–8%), their presence is in no way deleterious for clinical studies. The molecular weight of these fragments suggests they are labeled gamma-globulins. RAF was shown to be stable in vitro for 9 hr. If longer storage times are desired, then additional citrate ions should be added to replace those absorbed by the anion-exchange resin.

This rapid kit which eliminates the risk of serum hepatitis transmission while producing an authentic fibrinogen molecule should allow routine clinical use of iodinated autologous fibrinogen in humans.

ACKNOWLEDGMENT

The authors would like to thank Ira Miller for his technical assistance.

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