TECHNETIUM-99m-HUMAN FIBRINOGEN

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Exogenous fibrinogen has been successfully labeled with ^{99m}Tc using a modified electrolytic method. The exact labeling mechanism has not been determined. Experimental data suggest that the labeling process of ^{99m}Tc-fibrinogen is quite similar to that of ^{99m}Tc-human serum albumin as reported earlier by Benjamin. Technetium-99m-fibrinogen is stable in human plasma or in 1% buffered human serum albumin. A binding efficiency of 76% has been achieved with approximately 25% clottable protein. The entire labeling procedure requires less than 1 hr of preparation time. This short labeling time in a closed system may allow development of a practical method for labeling autologous fibrinogen, thus eliminating the risk of hepatitis transmission.

Fibrinogen is a thermolabile serum protein with a molecular weight of 340,000. Although very sensitive to chemical decomposition as shown by its precipitation by various salts such as ammonium sulfate, sodium acetate, or sodium chloride, fibrinogen has been iodinated with ^{131}I using electrolysis (*1*). Since the reduction of ^{99m}Tc-pertechnetate to a chemically active state requires the presence of reducing agents such as stannous chloride or ironascorbic acid, the salting-out effects of these agents may interfere with the tagging of ^{99m}Tc to the fibrinogen molecule. Because of this and its adaptability to a closed sterile system, low-current and low-voltage electrolysis with zirconium electrodes in a weak acidic medium may be the method of choice (*2,3*). Using controlled electrolysis, less than 1 mg of the powerful zirconium ion is produced (*4*); hence the damage to the fibrinogen molecule should be minimal. We have investigated this possibility and have successfully labeled human fibrinogen with ^{99m}Tc.

MATERIALS AND METHODS

Extraction and purification of human fibrinogen. Pure human fibrinogen was obtained by precipitation from a commercial source, Parenogen® (Cutter Laboratories, Berkeley, Calif.) using either a 4 *M* ammonium sulfate solution or a 2.3 *M* glycine solution. The Parenogen was first reconstituted with 50 ml of water for injection, USP, to a final concentration of approximately 20 mg/ml of fibrinogen. One and three tenths milliliters (1.3 ml) of the 4 *M* ammonium sulfate solution was added to a test tube containing 2 ml of the fibrinogen solution and 2 ml of water for injection. After centrifugation for 10 min at 800–1,000 rpm, the supernatant was removed. The precipitate was washed with 2 ml of distilled water to remove excess ammonium sulfate and redissolved in 4 ml of water. After repeat precipitation, washing, and removal of the supernatant solution, the fibrinogen was finally reconstituted with 2 ml of water for injection.

A similar separation procedure was employed in the extraction of fibrinogen from the reconstituted Parenogen solution with 3 ml of the 2.3 *M* glycine solution. After two separation and washing procedures, the final precipitate was redissolved in 2 ml pH 7.4 phosphate buffer.

Semiquantitative assay of the purified fibrinogen was performed using the Beckman spectrometer with the absorption set at 280 mμ and comparing the reading with a reconstituted and appropriately diluted solution of Parenogen. Data obtained from ultraviolet light spectrometry indicated that the final concentration of the purified fibrinogen solution was approximately 10–15 mg/ml.

Labeling method. The following procedure was used to incorporate ^{99m}Tc into exogenous human fibrinogen. The reconstituted or extracted fibrinogen solution was immediately labeled with ^{99m}Tc using

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electrolysis in a closed system. Prior to electrolysis, 5 ml of 0.05 *N* HCl and 2 ml normal saline containing approximately 30–60 mCi of ^{99m}Tc-pertechnetate were added to a sterile evacuated vial containing two 0.025-in. diam zirconium electrodes. A controlled current of 100 mA with voltage of 5.5–5.7 was passed through the solution for 42–45 sec. Immediately after electrolysis, 0.3–0.4 ml (4–8 mg) of fibrinogen solution was injected into the vial slowly with continuous gentle shaking. Following incubation for 30 min at 37°C, the pH of the labeled fibrinogen solution was readjusted to 7 with 1:2 ratio of labeled solution to filtered human serum or plasma or to 4.5 with 9 ml 1% human serum albumin in pH 7.4 phosphate buffer. The latter buffering agent produced a slightly cloudy solution but with less degree of precipitation of the labeled proteins. Removal of the denatured fibrinogen resulting from incubation and pH adjustment as well as zirconium oxide particles (*3*) was done with a 0.45-mμ Millipore filter.

Assays. Ascending radiochromatography was performed with Whatman No. 1 paper in an 85% methanol medium on which unbound ^{99m}Tc-pertechnetate registered its peak at a R_f value of 0.56. Labeled ^{99m}Tc-fibrinogen as well as any hydrated ^{99m}Tc-zirconium complex remained at the origin with R_f having a value of zero. Percentage of binding of ^{99m}Tc-fibrinogen was determined by counting appropriately cut chromatographic strips in a well scintillation counter. This method was comparable with the results obtained by a modified protein precipitation method utilizing topical thrombin solution (Parke-Davis, 1,000 units thrombin/ml) and 20% trichloroacetic acid (TCAA).

In order to separate ^{99m}Tc-fibrinogen from any hydrated ^{99m}Tc-zirconium complex, which was not possible with paper radiochromatography, column radiochromatography was performed with Bio-Gel P-100,* 50–100 mesh columns. A 0.2-ml radioactive sample was eluted from the column with pH 7.4 phosphate buffer solution. A total of 15 2-ml fractions representing approximately 99% of the activity was collected in the test tubes for later assays. An elution curve as well as percent binding of ^{99m}Tc-fibrinogen was obtained by assaying the radioactive content of each tube in the scintillation counter against a 0.2-ml standard previously diluted to 2 ml with phosphate buffer. An elution pattern for ^{99m}Tc-pertechnetate in normal saline was obtained with a similar technique.

Electrophoresis was performed with a Beckman Microzone® electrophoresis unit utilizing a cellulose acetate supporting medium and Tris-Barbital pH 8.6 buffer with ionic strength of 0.075. Samples of pure fibrinogen and labeled ^{99m}Tc-fibrinogen solution were electrolyzed for 45 min along with a standard containing normal human serum proteins. The separated proteins were stained with Ponceau-S, developed, and analyzed with a Beckman Densitometer equipped with a Microzone® scanning attachment. A curve of color density versus distance along the membrane was obtained with the area under the curve of each component representing the concentration of each protein component.

Semiquantitative assay for zirconium content was determined by adding the labeled solution to a filter paper previously saturated with a 0.1% Alizaren-S solution and allowed to dry in a hood. After washing with a hot solution of 1 *N* HCl and drying, the orange-brown spot of zirconium from appropriate diluted samples was colorimetrically compared with a known standard solution containing 2, 5, and 10 μg/ml of zirconium hydroxide.

In vitro biologic determination of ^{99m}Tc-labeled fibrinogen. The biologic property or behavior of ^{99m}Tc-fibrinogen was assessed by the addition of the labeled protein to fresh as well as clotted blood samples from normal healthy volunteers. Preformed clot was produced by incubating a 2-ml blood sample at room temperature for 3 hr. One-half milliliter (0.5 ml) of ^{99m}Tc-fibrinogen solution was added to test tubes containing 2 ml of fresh blood and also to tubes containing clotted blood. After incubating at room temperature for 3 hr, the serum was completely removed from the test tube by centrifugation and decanting and saved for radioactivity assay. The remaining clot was then washed twice with 1.5 ml distilled water, centrifuged, and separated from the washings. The percent of the ^{99m}Tc-fibrinogen incorporated into the blood clot was then compared with the results using ^{125}I-fibrinogen (Amersham/Searle) under similar experimental conditions. The possibility of coprecipitation or physical trapping of the labeled fibrinogen during in vitro clotting process was evaluated using ^{99m}Tc-pertechnetate and ^{125}I-human serum albumin (RISA) in the same manner.

Identification and clottability determination of ^{99m}Tc-fibrinogen. Percent of antigenically reactive labeled ^{99m}Tc-fibrinogen was determined with 0.05 ml rabbit antihuman fibrinogen antiserum (Hyland Laboratory). Percent clottable labeled ^{99m}Tc-fibrinogen was determined using 0.5 ml (500 units) of topical thrombin solution. Samples containing 0.2 ml of plasma-buffered ^{99m}Tc-fibrinogen and 1.8 ml pH 7.4 phosphate buffer were allowed to react with either the antiserum or thrombin at room temperature for 48 and 24 hr, respectively. After repeated separation and washing procedures, the precipitate,

* Bio-Rad Laboratory. Bio-Gel P-100 column has an operational exclusion limit range of 5,000–100,000 Daltons.

TABLE 1. BINDING EFFICIENCY OF ^{99m}Tc-FIBRINOGEN (AVERAGE OF TEN TRIALS)

Radiopharmaceutical	Column radiochromatography percent bound (mean % ± s.d.)	Paper radiochromatography		Total labeled protein determination* (mean % ± s.d.)
		percent bound (mean % ± s.d.)	Unbound $^{99m}TcO_4$ (mean % ± s.d.)	
^{99m}Tc-fibrinogen incubated at 37°C, 30 min unbuffered	76.43 (10.79)	76.57 (9.95)	23.42 (9.95)	75.08 (6.89)
^{99m}Tc-fibrinogen buffered with human plasma	75.14 (10.70)	90.97 (4.48)	9.06 (4.44)	88.73 (4.01)
Filtered plasma-buffered ^{99m}Tc-fibrinogen	97.65 (3.23)	86.34 (6.17)	13.76 (6.08)	86.03 (2.55)

* With TCAA precipitation.

presumably clottable protein, was collected for assay. Nonclottable labeled protein was precipitated from the supernatant with 0.5 ml 20% TCAA, separated, and washed. The results thus obtained were compared with those using ^{125}I-RISA and ^{125}I-fibrinogen under similar experimental conditions. In order to normalize the plasma content to that of ^{99m}Tc-fibrinogen determination, 0.2 ml fresh plasma was added to the test tube containing 1 μCi of the ^{125}I-labeled protein.

RESULTS

All labeling procedures were carried out with freshly prepared fibrinogen solution. The presence of 0.3–0.4 ml (approximately 4–8 mg) fibrinogen was sufficient to bind most of the radioactivity. The radioactivity could also be bound to zirconium if fibrinogen was not added to the electrolyzed solution. Radiochromatography with Whatman No. 1 paper performed after each step indicated that binding took place after current passage.

Data from column radiochromatography indicate that the sodium salt of ^{99m}Tc-pertechnetate will be eluted from the Bio-Gel P-100 column beginning with the fourth fraction, the main peak occurring at the sixth fraction. The highest activity of ^{99m}Tc-zirconium complex appears at the fifth fraction with a long tailing effect. The exact nature of the ^{99m}Tc-zirconium complex species has not been determined. However, a positive zirconium test can be detected in those fractions containing the highest amount of radioactivity. The percent of technetium binding in a ^{99m}Tc-zirconium complex formed during electrolysis in the absence of fibrinogen is in the range of 14–24% before incubation and 45–47% after 30 min incubation at 37°C. The latter increase may be attributed to additional formation of hydrated ^{99m}Tc-zirconium species.

Each vial of lyophylized powder of commercial fibrinogen (Parenogen) contains as additives 60 mg of glycine, 0.92 gm of sodium citrate, and 2.5 gm of dextrose in addition to 1 gm of pure fibrinogen. Labeling experiments with solution of similar formulation but minus the fibrinogen indicate that these inactive agents were not labeled by ^{99m}Tc after electrolysis. Addition of fibrinogen to the electrolyzed solution of ^{99m}Tc-zirconium will remove most of the radioactivity onto protein. Data from paper radiochromatography indicate the quantity of unbound ^{99m}Tc-pertechnetate is in the range of 0.9–37.4% (mean, 23.42%), comparable to the results obtained with column chromatography (Table 1: column 2, line 1). A majority of the labeled fibrinogen will come down from the column immediately after an initial 2 ml void volume with very little tailing. A negative zirconium test on all eluted fractions containing the labeled protein demonstrates the fact that zirconium is not carried with the fibrinogen during the labeling process. Semiquantitative determination of zirconium content indicates that the final product contains less than 1 mg of zirconium (approximately 30 μg/ml).

Figure 1 illustrates the migration characteristics of normal human serum proteins obtained from electrophoresis. Pure fibrinogen being less electronegative will remain near the negative pole whereas albumin moves rapidly across the cellulose acetate plate. As seen in Fig. 1, the electrophoretic zone of labeled ^{99m}Tc-fibrinogen is identical to that of pure fibrinogen. The exact chemical nature of the labeled protein has not been determined.

Of a total of ten trials, an average binding efficiency of 76.43% (range, 68–93%) assayed by column radiochromatography was achieved prior to pH adjustment (Table 1: column 1, line 1). A wide range of results in binding efficiency could be attributed to partial denaturation of the labeled protein during incubation. In some cases where the binding efficiency fell below 60%, a cloudy colloidal solution was observed with minute particles judged microscopically to be less than 5 microns in size. These insoluble ^{99m}Tc-fibrinogen particles were retained by the column and thus produced a lower percent binding efficiency. Denaturation also occurred during pH adjustment with buffering agents.

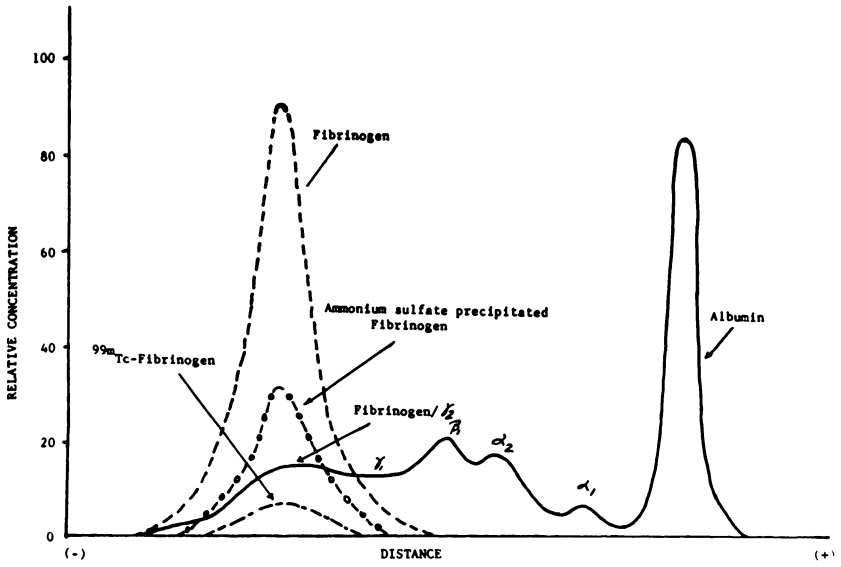

FIG. 1. Migration characteristics of human serum proteins, pure fibrinogen, and ^{99m}Tc-fibrinogen obtained by analyzing electrophoresis with Beckman densitometer.

Stability determination of ^{99m}Tc-fibrinogen was conducted by adding the labeled solution separately to serum and plasma. Radioactive fibrinogen content from each medium was assayed by column radiochromatography. Results from these studies were similar to that of the original solution indicating that ^{99m}Tc did not exchange with plasma or serum proteins during the period it remained in solution. Comparable results were also obtained from analyzing clottability studies using both media. This further suggests that ^{99m}Tc was firmly bound to the fibrinogen molecule. However, data from paper radiochromatography showed a sharp decrease in unbound $^{99m}TcO_4^-$ when labeled fibrinogen solution was added to serum or plasma. An average decrease of 14.3% $^{99m}TcO_4^-$ with a corresponding rise in the total labeled protein content was recorded (Table 1, columns 1 and 2, line 2). This strongly suggests that increased binding efficiency was primarily due to additional tagging of low-molecular-weight serum proteins by an active form of ^{99m}Tc in the fibrinogen solution.

Data from Table 2 demonstrate that both ^{99m}Tc- or ^{125}I-labeled fibrinogen will incorporate into fresh or preformed clots. A higher percentage of ^{125}I-fibrinogen than ^{99m}Tc-fibrinogen is incorporated into clots apparently due to some loss of native clottability of the latter compound. Repeated washing with pH 7.4 phosphate buffer removes less than 1% of the radioactivity from the clots indicating absence of an exchange reaction taking place in vitro. The incorporation of the labeled proteins into the clots appears to be stable.

TABLE 2. IN VITRO DETERMINATION OF CLOT UPTAKE OF ^{99m}Tc-FIBRINOGEN AS COMPARED WITH OTHER RADIOPHARMACEUTICALS

	Percent radioactivity retained in clot	
Radiopharmaceutical	Fresh clots	Preformed clots
^{99m}Tc-fibrinogen	64.20	30.30
^{99m}Tc-pertechnetate	15.22	2.69
^{125}I-fibrinogen	79.23	56.67
^{125}I-RISA	4.31	0.16

Although there is little incorporation of ^{125}I-RISA and ^{99m}Tc-pertechnetate into the preformed clots, coprecipitation of the radiopharmaceuticals will occur with fresh blood samples. However, each successive washing with phosphate buffer will remove approximately 10–12% of the total clot radioactivity. This shows that the radiopharmaceuticals were only temporarily absorbed onto the surface of the clots.

Results from the identity and clottability studies with antiserum and thrombin are provided in Table 3. Experiments with various amounts of antiserum demonstrate that 0.05 ml antiserum will produce the best results under our experimental conditions. Of the tagged protein, 28.34% of the ^{99m}Tc-fibrinogen is antigenically reactive, comparable to the result obtained with ^{125}I-fibrinogen (21.47%).

Reaction of plasma-buffered ^{99m}Tc-fibrinogen with topical thrombin produced an average of 25.29% clottable protein. However, a much greater clottability (average 84.46%) was recorded for ^{125}I-fibrinogen. This higher percent clottability for radioiodinated fibrinogen was apparently due to the purity of the ^{125}I-fibrinogen. Data from ^{125}I-RISA determinations with thrombin demonstrated that the amount of coprecipitation or trapping was negligible. Less than 2% of the RISA remained with the clot with nonclottable protein accounting for 97% of the radioactivity (Table 3: column 2, line 1).

TABLE 3. PRECIPITATION OF ^{99m}Tc-LABELED FIBRINOGEN, ^{125}I-FIBRINOGEN, AND ^{125}I-RISA BY THROMBIN AND ANTISERUM TO FIBRINOGEN

	Antiserum		Thrombin	
	Antigenically reactive protein	Antigenically non-reactive protein	Clottable protein	Nonclottable protein
Radiopharmaceutical	(mean % ± s.d.)	(mean % ± s.d.)	(mean % ± s.d.)	(mean % ± s.d.)
Plasma-buffered ^{99m}Tc-fibrinogen	28.34 (8.73)	62.85 (9.21)	25.29 (16.77)	60.47 (17.89)
^{125}I-fibrinogen + 0.2 ml human plasma	21.47 (3.30)	74.24 (3.20)	84.46 (1.02)	6.46 (0.32)
^{125}I-RISA + 0.2 ml human plasma	5.78 (0.62)	92.20 (0.37)	1.24 (0.82)	96.97 (0.83)

DISCUSSION

Fibrinogen has an isoelectric point at pH 5.5 and is soluble in a weak solution of 0.05 *N* HCl. To minimize the salting-out effect it should not be buffered above pH 5. The labeled ^{99m}Tc-fibrinogen buffered with plasma, serum, or human serum albumin appears stable. When an acid solution of fibrinogen is adjusted to pH 3–4 with phosphate buffer, it retains its clottability as evidenced by fibrin precipitation with topical thrombin solution. However, we have noted that the rate of fibrin formation is markedly reduced with a corresponding increase in the amount of thrombin needed to induce the reaction. This may be attributed to partial inhibition of the enzymatic action of thrombin in an acidic environment. We have not determined the exact chemical nature of the fibrinogen in the acid solution. Whether the fibrinogen molecule has been broken down to various fragments is not known (*5*). Data from electrophoresis and clottability studies tend to support the hypothesis that a portion of the ^{99m}Tc-labeled fibrinogen retains its molecular integrity after the labeling process. Experimental data from in vitro biologic studies have demonstrated that ^{99m}Tc-labeled fibrinogen behaves similarly to ^{125}I-fibrinogen. In order to determine whether the labeled protein is biologically active in vivo requires further testing with laboratory animals.

Reproducibility of the labeling technique is excellent with reconstituted fibrinogen. We tested the effect of glycine and ammonium sulfate on labeling efficiency since these salts will be used to extract fibrinogen from plasma. Denaturation of the labeled fibrinogen during incubation occurs more frequently when glycine or ammonium sulfate has been used to precipitate and reconstitute the fibrinogen from Parenogen solution than when reconstituted unextracted Parenogen solution alone is used. However, initial attempts with a crude glycine extract from plasma suggest that a similar binding efficiency for fibrinogen can be achieved although albumin remaining in the extract is also labeled. Physical-chemical damage during the extraction and purification process with ammonium sulfate or glycine and the presence as well as the quantity of hydrated ^{99m}Tc-zirconium species formed after electrolysis may contribute to the denaturation. Our experimental data suggest that a combination of both factors is responsible for the decomposition of the labeled protein.

The final product, buffered with plasma and filtered, contains an average of 97.65% labeled protein (Table 1: column 1, line 3) with ^{99m}Tc-fibrinogen accounting for 76.57% of the radioactivity (Table 1: column 2, line 1). Unbound $^{99m}TcO_4^-$ is less than 5% at end of the labeling procedure. Removal of the unbound $^{99m}TcO_4^-$ is unnecessary due to additional tagging by the plasma proteins added as a buffer. Of the total radioactivity used in the labeling procedure, one third to one half of the original activity is lost during filtration process as assayed in the dose calibrator.

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