IN VIVO BEHAVIOR OF ^{99m}Tc-FIBRINOGEN AND ITS POTENTIAL AS A THROMBUS-IMAGING AGENT

Sylvia S. L. Harwig, John F. Harwig, R. Edward Coleman, and Michael J. Welch

Edward Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Missouri

We have investigated the in vivo behavior of ^{99m}Tc-fibrinogen, prepared by a mild and efficient electrolytic method employing tin electrodes. The clearance mechanisms of this agent were studied, and its efficacy for imaging deep-vein thrombi in dogs with an Anger camera was determined. The ^{99m}Tc-fibrinogen preparations, which are stable in vitro, undergo partial rapid exchange of the technetium with other plasma proteins and with anions of the blood buffer system in vivo, resulting in an early drop in the percent of radioactivity associated with clottable protein. However, very little or no oxidation to pertechnetate occurs. The nonclottable material is much more rapidly cleared from the blood than the remaining ^{99m}Tc-fibrinogen, and the proportion of clottable protein activity increases with time. The fraction of ^{99m}Tc-fibrinogen that remains intact in vivo is biologically active and will incorporate into thrombi. Higher thrombus-to-blood activity ratios are obtained with ^{99m}Tc-fibrinogen than with radioiodinated fibrinogen when both agents are injected into dogs 4 hr after induction of femoral vein thrombosis. Clearly delineated images of the thrombi are obtained, beginning about 2.5 hr after injection. Thus, ^{99m}Tc-fibrinogen may be of clinical use as a thrombus-imaging agent in patients undergoing active thrombosis, especially in regions of high blood pool.

The fibrinogen uptake test employing ^{125}I-fibrinogen and external probe monitoring has proved to be an accurate method of detecting deep-vein thrombosis in the calf and neighboring thigh (*1–4*). More recently, ^{131}I-fibrinogen has been used to detect deep-vein thrombi by scintiscanning (*5*). Both of these radiopharmaceuticals, however, deliver a high radiation dose and neither is ideal for imaging with a scintillation camera. Since ^{99m}Tc is superior in terms of radiation dose and compatibility with the Anger camera, fibrinogen labeled with this emitter may be useful for imaging thrombi. We have developed a mild and efficient method of preparing ^{99m}Tc-fibrinogen with good in vitro stability and physicochemical properties (*6*). To be useful clinically, these preparations must also possess satisfactory in vivo characteristics. The ^{99m}Tc should remain firmly bound to the fibrinogen after administration, and the ^{99m}Tc-fibrinogen should remain in circulation long enough to be incorporated into a thrombus, but clear fast enough to minimize blood background. Although in vivo studies have been made on such ^{99m}Tc-labeled radiopharmaceuticals as pyrophosphate (*7*), polyphosphate and diphosphonate (*8,9*), and albumin (*10,11*), no in vivo studies of ^{99m}Tc-fibrinogen have been reported (*12*). Two ^{99m}Tc-labeled enzymes, urokinase and streptokinase, have been studied as agents for thrombus visualization (*13–17*), but the results have not been reproducible (*14*) and little is known about the in vivo fate of these preparations. This paper reports our investigation of the in vivo behavior of ^{99m}Tc-fibrinogen, including stability, clearance mechanisms, incorporation into thrombi, and potential as a thrombus-imaging agent.

METHODS AND MATERIALS

Preparation of ^{99m}Tc-fibrinogen. The canine fibrinogen used in this study was isolated from pooled dog plasma as the Blombäck I-2 fraction through glycine precipitation and alcohol fractionation (*18,19*). The rabbit fibrinogen was isolated from fresh rabbit plasma by the ammonium sulfate precipitation method (*20*). The spectroscopic clottability (*21*) of

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For reprints contact: Michael J. Welch, Div. of Radiation Sciences, The Edward Mallinckrodt Institute of Radiology, 510 South Kingshighway, St. Louis, Mo. 63110.

the canine and rabbit fibrinogen was 96% and 89%, respectively.

The electrolytic labeling procedure employed in this study is similar to the method we have reported elsewhere (*6*). The electrolysis cell consisted of two high-purity tin wire electrodes penetrating through a rubber diaphragm into a 5-ml vial. The reaction mixture contained 3 mg of fibrinogen, 0.1–1.5 ml of $Na^{99m}TcO_4$ of desired activity direct from a generator, 1 ml of a buffer (0.03 *M* NaH_2PO_4 and 0.15 *M* NaCl, pH = 6.0), and sufficient normal saline to bring the total volume to 3 ml. A charge of 0.05 coulomb (100 μA for 500 sec) was passed through the cell, following which the reaction mixture was stirred for an additional 5 min. The labeled fibrinogen was then separated by ammonium sulfate precipitation at 30% saturation and redissolved in a solution of 0.02 *M* $NaHCO_3$ and 0.12 *M* NaCl (pH 7.4).

Biologic clearance studies. These were performed with intravenous administrations of 5–6 mCi of canine ^{99m}Tc-fibrinogen in anesthetized dogs (30 mg sodium pentobarbital/kg) and with 1–2 mCi of rabbit ^{99m}Tc-fibrinogen in unanesthetized rabbits. Three animals in each group were studied. The syringe was weighed before and after injection, and a weighed aliquot was kept as a standard. Blood samples were drawn into sodium citrate tubes at 10, 60, 120, and 240 min after injection, with two additional blood samples taken on the second day. The blood samples were processed as follows:

1. An aliquot of whole blood was counted in an automatic well scintillation counter (Searle Radiographics) at the same time as the weighed standard. The whole-blood clearance curve, expressed as percent of the injected dose in circulation with respect to time, was constructed.
2. The red cells of a known volume of blood were counted after thorough washing of the cells with saline. The percent of circulating activity associated with the red cells at various times after injection was calculated.
3. An aliquot of plasma was clotted by the addition of buffered ϵ-aminocaproic acid and thrombin solution at a concentration of 1 NIH unit per milligram of clottable protein. The clot was removed after 1 hr incubation and both the clot and supernatant were counted. The clottable-protein activity of each plasma sample was then expressed as a percentage of the 10-min sample, from which the curve for clottable-protein clearance was constructed by plotting the sample percentage with respect to time.
4. The protein fraction of the plasma was precipitated with ammonium sulfate at 70% saturation. Both the precipitate and supernate were counted and the percent of protein-bound activity in the plasma was calculated.
5. Electrophoresis on Corning ACI agarose special-purpose film was performed on the plasma from the first two blood samples (Arthur H. Thomas Co. electrophoresis power supply and chamber). Approximately 1 μl of plasma was applied to the film and electrophoresis was performed at 150 volts for 40 min in 0.05 *M* sodium barbital buffer (pH 8.6). Duplicate samples underwent electrophoresis under the same conditions. One of the samples was stained with amido black and destained in 5% acetic acid to visualize the relative electrophoretic mobility of each band. The bands in the second sample, which were not stained, were located by alignment with the stained sample. Each band in the second sample was separated and counted. The relative radioactivity associated with each protein fraction and the total recovery of applied radioactivity were then calculated.
6. Biogel P-100 column chromatography was performed on the plasma from the first blood sample. The column (0.9 $\times$ 42 cm) was calibrated individually with fibrinogen, $^{99m}TcO_4^-$, and ^{99m}Tc-Sn-phosphate. A 0.01 *M* NaH_2PO_4 and 0.15 *M* NaCl buffer (pH 6.0) was used for elution. One milliliter fractions were collected and the radioactivity in each fraction was measured.

Thrombus uptake studies. A thrombus was induced in one femoral vein in three anesthetized dogs by the technique of alteration of the intima with an electric current (*22,23*). Four hours after thrombus induction, 6–9 mCi of ^{99m}Tc-fibrinogen and 100 μCi of ^{125}I-fibrinogen prepared by the iodine monochloride method (*24*) were injected intravenously. Scintillation camera imaging of the thrombus was performed at 2.5 and 18 hr after injection.

The animals were reanesthetized 24 hr after injection of the labeled fibrinogen preparations, and the thrombus was removed and washed with saline. A blood sample was taken at the same time. Both the thrombus and blood sample were weighed and counted, and the thrombus-to-blood ratio [(cpm/gm thrombus)/(cpm/gm blood)] was calculated.

The weighed standard of the injected solution was

counted at the same time as the thrombus. The percent of the injected dose of labeled fibrinogen per gram of thrombus was calculated from the formula:

$$\frac{(\text{cpm/gm thrombus})}{(\text{cpm/gm standard}) \times (\text{gm injected})} \times 100.$$

Organ distribution studies. In one of the thrombus uptake studies, the ^{99m}Tc-fibrinogen was injected with the dog positioned beneath a scintillation camera (Radicamera, Nuclear Data, Inc.). Serial images were taken during the first 30 min to determine the organ distribution of ^{99m}Tc-fibrinogen with respect to time. A similar study was performed with a normal rabbit without thrombosis.

The following experiments were designed to evaluate further the fate of ^{99m}Tc-fibrinogen in vivo.

1. Two rabbits were injected with 2 mCi of $Na^{99m}TcO_4$ direct from the generator. Blood samples were taken at 10 min, 1 hr, and 3 hr after injection. The percent of protein-bound activity in the plasma was determined by ammonium sulfate precipitation. Electrophoresis was also performed on the 10-min and 1-hr samples.
2. Technetium-99m-Sn-phosphate complex was produced in the absence of fibrinogen by the same electrolytic reaction described for preparation of ^{99m}Tc-fibrinogen. An aliquot of the reaction mixture was analyzed by Biogel P-100 column chromatography. Approximately 1 mCi of ^{99m}Tc-Sn-phosphate was injected into a rabbit positioned under a scintillation camera. Serial images were taken to determine organ distribution with respect to time.
3. An injection of 150 μg of stannous ion in the form of stannous DTPA (Diagnostic Isotopes, Inc.) was given to each of two rabbits prior to injection of ^{99m}Tc-fibrinogen. Blood samples were taken at 10 min and at 1, 2, and 20 hr after injection. An aliquot of plasma from each sample was clotted and the percent of clottable radioactivity was determined.

RESULTS

The labeling efficiency of canine or rabbit ^{99m}Tc-fibrinogen, determined by ammonium sulfate precipitation, ranged from 70 to 80%. The radioactivity bound to clottable protein, determined by the method of Regoeczi (*25*), was 55% for canine and 65% for rabbit ^{99m}Tc-fibrinogen.

The clearance behaviors of the labeled canine and rabbit fibrinogens were identical. The whole-blood clearance curve is shown in Fig. 1, while the curve

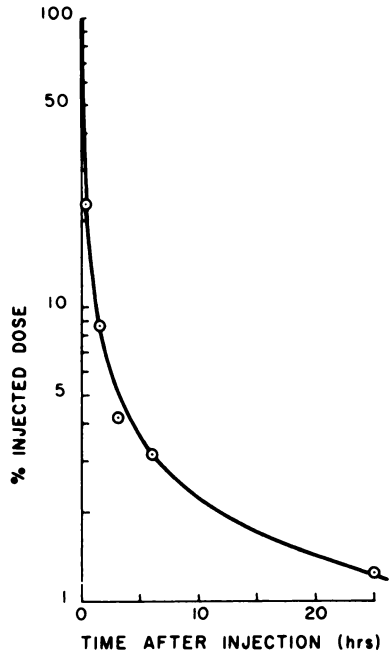

FIG. 1. Whole-blood clearance curve of typical ^{99m}Tc-fibrinogen preparation. Activity clears very rapidly from blood, with only 25% of injected dose remaining in circulation at 10 min after injection.

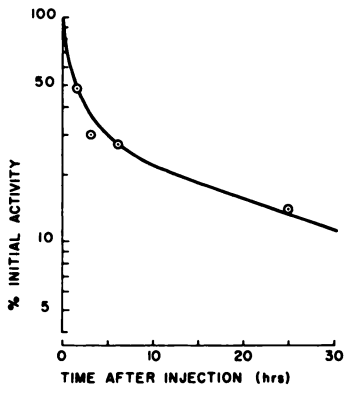

FIG. 2. Clottable-protein clearance curve of typical ^{99m}Tc-fibrinogen preparation. Radioactivity bound to clottable protein of each plasma sample is expressed as percentage of 10-min sample. Radioactivity bound to clottable protein decreases rapidly initially; biologic half-life of latter component is 25 hr.

for clottable-protein turnover is shown in Fig. 2. Figure 3 shows the percent of clottable radioactivity in the plasma as a function of time. The whole-blood clearance was very rapid, with only about 25% of the injected dose remaining in circulation 10 min after injection. The percent of radioactivity associated with the clottable protein in this first blood sample was only 18%, compared to the in vitro value of 55–65% prior to injection. However, the blood clearance of nonclottable material was also rapid, resulting in a gradual increase in the relative amount of ^{99m}Tc-fibrinogen in circulation. Accordingly, the percent of radioactivity associated with

clottable protein in plasma gradually increased, reaching a value of about 55% on the second day after injection. The serial scintillation camera images taken immediately after injection of ^{99m}Tc-fibrinogen showed that this nonclottable ^{99m}Tc activity cleared rapidly through the kidneys and bladder, with no liver accumulation. No difference in the clottable-protein turnover (Fig. 2) or in the percent of clottable activity in the plasma (Fig. 3) was observed when stannous ion was administered prior to injection of the ^{99m}Tc-fibrinogen.

The percent of protein-bound radioactivity in plasma, determined by ammonium sulfate precipitation, was 85 ± 5% for all blood samples. The gel chromatogram of the first blood sample (Fig. 4) suggests that the other 10–20% of plasma radioactivity was in the form of ^{99m}Tc-Sn-phosphate chelate and not in the form of $^{99m}TcO_4^-$. Less than 2% of the whole-blood activity was found to be associated with the red cells at any time. The radioactivity in the combined saline washings was found to be negligible when compared to the red cell counts.

The electrophoretic pattern of the 10-min blood samples is shown in Fig. 5, and an identical pattern was obtained from the 1-hr plasma sample. The percent of recovered radioactivity bound to each protein fraction is listed under each protein band, with the α_2-globulin band containing the largest fraction of activity. The relative amount of recovered radioactivity in each band may not represent the actual relative radioactivity bound to each protein in the plasma since the technique of electrophoresis can cause some loss of radioactivity, depending on the relative strengths of the ^{99m}Tc–protein bonds. Only 60–80% of the applied radioactivity was recovered in the various protein fractions. The most important observation is the increase with time of the proportion of radioactivity bound to fibrinogen, which agrees with the increase with time in the percent of radioactivity associated with clottable protein (Fig. 3).

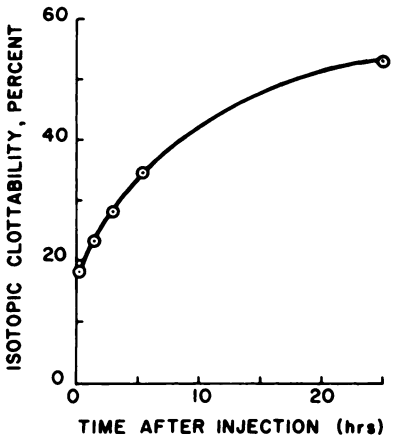

FIG. 3. Characteristic curve shows percent of radioactivity in plasma associated with clottable protein (isotopic clottability) as function of time.

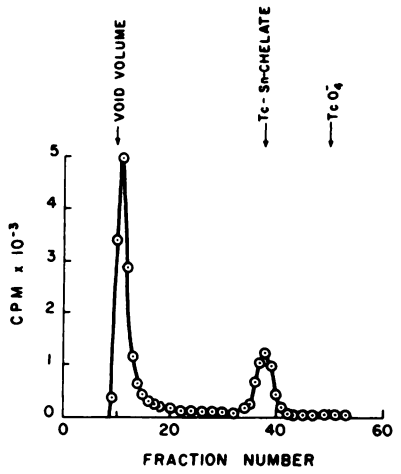

FIG. 4. Biogel P-100 column chromatogram of 10-min plasma sample.

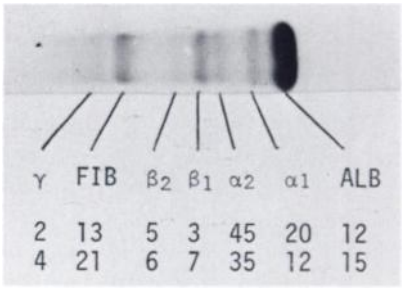

FIG. 5. Agarose film electrophoresis pattern of 10-min plasma sample. Identical pattern was obtained with 1-hr sample. Numbers under each protein band represent percent of recovered radioactivity bound to each protein fraction. Top numbers, 10-min sample; bottom, 1-hr sample.

The turnover studies with $^{99m}TcO_4^-$ also revealed a rapid blood clearance, and ammonium sulfate precipitation showed that more than 80% of the plasma radioactivity was protein-bound. However, when the plasma samples were subjected to the same electrophoretic conditions used for the ^{99m}Tc-fibrinogen studies, less than 1% of the applied radioactivity was recovered in the protein fractions.

The serial scintillation camera studies of organ distribution obtained after administration of ^{99m}Tc-fibrinogen showed blood pool activity and rapid accumulation of activity in the kidneys and bladder. Occasionally some thyroid accumulation was observed. Figure 6 shows a representative image taken in a rabbit at 25 min after injection. The distribution studies of ^{99m}Tc-Sn-phosphate chelate were similar, showing rapid accumulation of activity in the kidneys and bladder and occasional very slight accumulation in the thyroid.

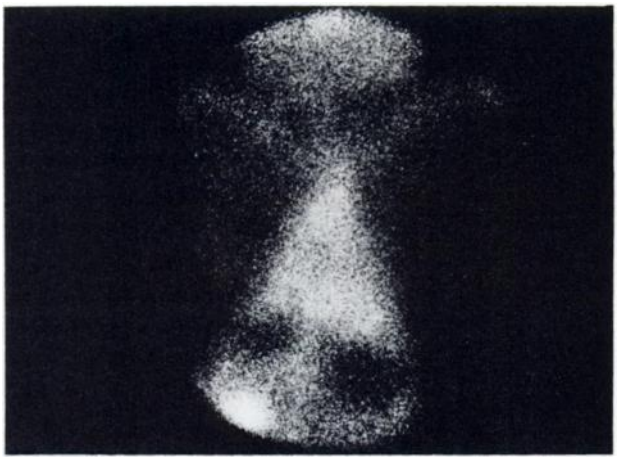

FIG. 6. Scintillation camera image obtained from normal rabbit 25 min after injection of ^{99m}Tc-fibrinogen, showing blood pool and renal accumulation.

Slightly higher thrombus-to-blood activity ratios were obtained for ^{99m}Tc-fibrinogen than for radioiodinated fibrinogen when these preparations were injected 4 hr after thrombus induction, although the actual incorporation, expressed as percent of injected dose per gram of thrombus, is much lower for ^{99m}Tc-fibrinogen. These results are summarized in Table 1. Scintillation camera images obtained from the same animal at 2.5 and 18 hr after injection are shown in Figs. 7 and 8, respectively. The thrombus can be seen clearly in both images.

DISCUSSION

Although ^{99m}Tc-fibrinogen prepared by the above method is stable in vitro, the in vivo behavior in dogs and rabbits is quite complex. Only a small amount of ^{99m}Tc-fibrinogen, represented by the late component of the clearance curve in Fig. 2, stays in circulation as such, and its half-time of 25 hr is shorter than that of radioiodinated canine fibrinogen (40–72 hr) (*26–28*) and of radioiodinated rabbit fibrinogen (50–60 hr) (*20,29*). The relative amount of ^{99m}Tc-fibrinogen, however, actually increases with time since the remainder of the technetium activity rapidly clears from circulation. Several pathways could lead to the observed in vivo behavior. First, ^{99m}Tc in its reduced protein-bound valence state of +4 or +5 (*30*) can be reoxidized to the more stable +7 valence state in $^{99m}TcO_4^-$. Second, since the binding of technetium to fibrinogen presumably involves formation of a coordination complex with ligands on the protein, other plasma proteins can compete with fibrinogen for binding with the reduced technetium ion, resulting in exchange. Third, anions of the blood buffer system, such as phosphate, are good complexing agents and can also compete for binding with the reduced technetium ion.

Oxidation to pertechnetate is the most likely side reaction when ^{99m}Tc-labeled radiopharmaceuticals exhibit unexpected behavior—especially in our case, in which any excess stannous ion has been removed during the purification step following electrolysis. However, the behavior of ^{99m}Tc-fibrinogen in vivo appears to result almost exclusively from the latter two of the three pathways, based on the following observations:

1. In electrophoresis of the first two plasma samples from the ^{99m}Tc-fibrinogen clearance studies, most (60–80%) of the applied radioactivity is recovered and is associated with proteins other than fibrinogen. Pertechnetate has been shown to bind with plasma proteins (*31*), but the nature of this bind-

TABLE 1. SUMMARY OF THROMBUS UPTAKE EXPERIMENTS

	Thrombus-to-blood ratio			% Injected dose/gm thrombus	
	^{99m}Tc-fibrinogen (A)	^{125}I-fibrinogen (B)	A/B	^{99m}Tc-fibrinogen	^{125}I-fibrinogen
Dog 1	10.5	10.1	1.0	0.007	0.145
Dog 2	12.6	10.3	1.2	0.004	0.121
Dog 3	13.9	8.7	1.6	0.005	0.080

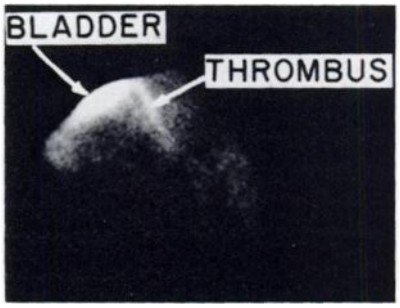

FIG. 7. Scintillation camera image obtained from dog 2.5 hr after injection of ^{99m}Tc-fibrinogen. Thrombus is clearly visualized with little interference from urinary-bladder activity.

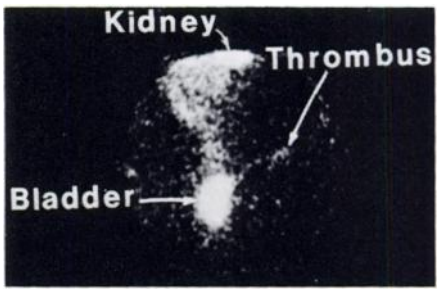

FIG. 8. Scintillation camera image obtained in dog (same as in Fig. 7) 18 hr after injection of ^{99m}Tc-fibrinogen. (Different position than in Fig. 7.)

ing is much weaker than the protein binding of reduced technetium. In electrophoresis of the plasma samples from the pertechnetate-turnover studies, less than 1% of the applied radioactivity is recovered.

2. Gel chromatography of the 10-min plasma samples from the ^{99m}Tc-fibrinogen clearance studies shows that the 10–20% of the non-protein-bound radioactivity is in the form of ^{99m}Tc-Sn-phosphate, and not $^{99m}TcO_4^-$. Furthermore, the scintillation camera studies show accumulation of activity in the kidneys and bladder. Small ^{99m}Tc-chelates of this type are known to act as renal agents. Injection of ^{99m}Tc-Sn-chelate alone produces similar renal images.
3. Little or no accumulation of ^{99m}Tc activity in the thyroid or stomach is observed in the scintillation camera studies at any time.
4. Changes in tissue distribution of $^{99m}TcO_4^-$ have been observed in rats previously given stannous gluconate solution (*32*). These alterations were thought to be due to the chemical reduction and fixation of the technetium to tissue components. Therefore, if ^{99m}Tc-fibrinogen is reoxidized into $^{99m}TcO_4^-$, changes in its in vivo behavior are to be expected when the animals are preinjected with a stannous ion solution. In the experiments with preinjection of stannous DTPA solution, however, no changes in the percent of clottable radioactivity in plasma or in the clearance curves for whole blood and for clottable protein were observed when compared to control animals without preinjection of stannous ion solution.

Similar observations have been made in limited in vivo studies of other ^{99m}Tc-radiopharmaceuticals. A comparison of radiolabeled albumin preparations showed that ^{99m}Tc-albumin clears faster than ^{131}I-albumin (*11*). A study of radiolabeled streptokinase indicated a considerably faster catabolic rate for ^{99m}Tc-streptokinase than for ^{131}I-streptokinase (*14*). Although no attempt was made in either study to explain the faster clearance of the ^{99m}Tc-labeled proteins, a mechanism involving exchange with other blood constituents may be involved. Recently, exchange of label with plasma proteins has been observed in studies of ^{99m}Tc-labeled pyrophosphate, polyphosphate, and diphosphonate (*7–9*). In fact, this type of exchange mechanism may be a general feature of ^{99m}Tc-labeled compounds due to the inherent nature of metal binding in coordination complexes.

The faster clearance and resulting lower blood background with ^{99m}Tc-fibrinogen, compared to radioiodinated fibrinogen, produces slightly higher thrombus-to-blood activity ratios when these agents are injected 4 hr after thrombus induction, even though the actual incorporation (% injected dose/gm thrombus) is lower for ^{99m}Tc-fibrinogen (Table 1). Thus, ^{99m}Tc-fibrinogen may be superior to radioiodinated fibrinogen for detecting active thrombosis in areas of high blood pool, such as the thigh or pelvis. Furthermore, the physical properties of ^{99m}Tc are ideal for scintillation camera imaging, and clearly delineated images of deep-vein thrombi can be obtained beginning about 2.5 hr after injection. The urinary-bladder activity associated with ^{99m}Tc-fibrinogen should not limit its usefulness. The present images obtained in dogs clearly visualize the thrombus in the upper thigh area, despite the presence of bladder activity. Imaging of the pelvic area in patients should be performed after the bladder has been emptied.

A method of ^{99m}Tc-fibrinogen preparation has been reported previously (*12*). The conditions employed in that study, however—especially the low pH of 1–3—could easily denature the fibrinogen (*33*). Favorable biologic behavior of a ^{99m}Tc-fibrinogen species produced in such a manner is not likely. The method of preparing ^{99m}Tc-fibrinogen reported here is very mild and efficient. Despite the rapid exchange mechanism in vivo, a fraction of the injected dose still remains associated with the biologically active clottable protein. Furthermore, this method can also be readily adapted to kit form employing autologous human fibrinogen.

We are currently attempting to alter the nature of the bond between ^{99m}Tc and fibrinogen in order to improve the in vivo stability and thus optimize its incorporation into thrombi. We are also extending our study of ^{99m}Tc labeling to soluble fibrin (*34*), another potential thrombus-imaging agent.

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