RADIOACTIVE UROKINASE FOR BLOOD CLOT SCANNING

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A radiopharmaceutical which specifically localizes blood clots would be useful in the diagnosis and management of patients with thromboembolic disease. Several substances are being evaluated for this purpose. These include iodinated fibrinogen, antifibrin and plasmin, technetium-labeled platelets or white blood cells, and iodinated or technetium-labeled streptokinase (*1*). Radioactive particles have been proposed for blood clot scanning (*2*) but have been found to be nonspecific (*3*). Another potential substance is labeled urokinase. This thrombolytic enzyme which is found in human urine has been isolated and purified and is currently used in the treatment of pulmonary embolism (*4*).

Urokinase was chosen for labeling and evaluation as a radiopharmaceutical because it has potential advantages over the other proposed agents. It is less likely to be antigenetic than streptokinase or antifibrin since it is a native human protein. Because of its rapid clearance from the blood stream, blood background should be less of a problem than it is with antifibrin (*5*) or plasmin (*6*). Its safe use in humans has been documented as a result of its use in pulmonary embolism therapy. Urokinase has a potential advantage over fibrinogen because fibrinogen must be injected before clots form as it labels clots primarily during their formation. Also fibrinogen is a difficult protein to process because it can easily be converted to an irreversibly insoluble form and because it is difficult to free from hepatitis virus. Urokinase also has a potential advantage over tagged platelets or white blood cells because they must be isolated and labeled without destruction of their viability.

The purpose of this study was to determine if urokinase could be labeled with ^{131}I and ^{99m}Tc and to determine if the labeled protein retained its enzymatic activity and immunoreactivity and if it localized in blood clots in vivo.

METHODS

Urokinase (Winkinase, Sterling-Winthrope, hereafter denoted by SW) of 20,000–100,000 CTU's in 0.2 cc of saline, was labeled with ^{99m}Tc using the iron-ascorbate method (*7*) or with ^{131}I using the chloramine-T method (*8*). For each reaction 1–5 mCi of radioisotope was used. Some reactions were carried out with Abbott urokinase (hereafter denoted by A). With both procedures the labeled protein was separated from the unbound radioisotope by precipitating the proteins with 3–5 ml of saturated ammonium sulfate. After centrifugation at 2,000 rpm for 10 min, the supernate was decanted and the precipitate was dissolved in 3 ml of 0.9% saline and passed through a 0.45-micron Millipore filter. Seven milliliters of 0.9% saline were used to rinse the centrifuge tube and filter. These washings were added to the original filtrate to give a final volume of approximately 10 ml.

Comparing initial to final radioactivities it was found that 70% or more of either ^{99m}Tc or ^{131}I was incorporated into the protein. Paper chromatography of the final product in 85% methanol showed approximately 95% of the radioactivity at the origin and usually less than 2% contamination with either radioiodide or pertechnetate.

Recovery of enzymatic activity was determined from the time required for the labeled protein to lyse fibrin clots. At the same time standards, i.e., dilutions of the original urokinase in the range of 100–10,000 CTU's, were tested. The lysis times of the standards gave straight line calibration curves when plotted against enzyme activity on semilogarithmic paper. This simple urokinase assay system was validated by Celander et al (*9*) and has been used in other laboratories (*10*). The fibrin clots used in this assay were prepared by mixing 0.1 ml of 0.9% saline containing 1 unit of thrombin of bovine origin (Parke-Davis) with 0.1 ml of 1% bovine fibrinogen

Received Jan. 17, 1972; revision accepted Apr. 3, 1972.

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(Pentex, Miles Laboratories) also in 0.9% saline. The clots were aged 1 hr at 4°C then warmed to 37°C by 3–4-min incubation in a waterbath. The urokinase to be tested along with the dilutions of the standards was added in 0.1-ml volumes to the clots. Barbital buffer (0.4 ml) of pH 7.6 was also added. The incubation was carried out at room temperature; the tubes were periodically tilted to facilitate observation of the progress of clot lysis. The times when lysis was complete were recorded.

With all the preparations of urokinase 70–100% of the enzymatic activity was recovered in the final product when either isotope was used for the label. In other tests using the enzyme assay system, it was found that incubating the labeled protein at room temperatures for ½ hr in solutions of pH ranging from 2.5 to 10.0 did not diminish the enzymatic activity nor did passing the material through a 0.22-micron Millipore filter.

To determine whether the labeling process altered the urokinase, double-diffusion experiments were performed in 1.1% agarose in pH 8.6 veronal buffer. All experiments were carried out at room temperature. Concentration of 1,000 CTA units/0.1 ml were used. The antiurokinase antisera was prepared in New Zealand white rabbits against urokinase (A). The antisera employed was the IgG fraction of the whole rabbit serum.

In the double-diffusion experiments, single precipitation lines of identity were seen when comparing the labeled and unlabeled enzyme. Also, single precipitation lines of identity were seen when the labeled urokinase (SW) preparations were examined over a pH of 3.0–9.0. At pH less than 3.0 or greater than 9.0 the labeled urokinase (SW) did not react with the IgG antisera. The same results occurred with immune electrophoresis in 1.1% agarose. Urokinase (A) was studied in an identical manner, and it was found that the preparation contained large quantities of albumin. In this case (urokinase A) approximately 85% of the radioactivity appeared in the albumin fraction and 15% in the enzyme fraction. Because this urokinase (A) preparation is diluted in human serum albumin (*10*), it is not suitable for preparing the radiopharmaceutical (Fig. 1).

The in vivo behavior of ^{99m}Tc-urokinase was studied by determining the distribution of the radioactivity in mice, the urinary excretion in dogs, and the uptake into clots implanted and retrieved from the femoral vessels of dogs.

RESULTS AND DISCUSSION

In initial tests, ^{99m}Tc-urokinase was injected into mice and 3 min later blood was withdrawn from their hearts and allowed to coagulate. The clots were separated from the serum by centrifugation. A mean of 84.5 ± 4.41% (1 s.d., n = 4) of the radioactivity remained with the clot. For comparison, when $^{99m}TcO_4^-$ is tested in this way, the radioactivity is equally distributed between clot and serum. In later tests tissue distribution in six mice was determined at various time intervals. Thirty minutes after an intravenous dose, 40% of the radioactivity was found in the liver, 12% in the kidneys, 9% in the blood, and lesser amounts in other tissues. In testing four dogs, the animals were anesthetized with sodium pentobarbital and given saline intravenously, at a rate of about 500 ml/hr. Urine was collected for 3 hr by an indwelling catheter to the bladder. Seventy to 80% of the injected doses of ^{99m}Tc was collected in the urine during this time.

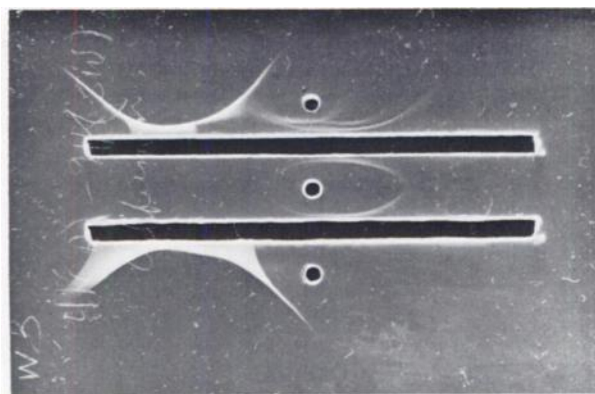

FIG. 1. Immunoelectrophoretic analysis of ^{99m}Tc-urokinase. Top well—Abbott urokinase (A); center well—Sterling Winthrope urokinase (SW); bottom well—human serum albumin. After electrophoresis, bands were developed with antiserum against urokinase (A). Intense bands on upper and lower left are human serum albumin which is used as stabilizer in preparation (A). Urokinase (A) shows multiple protein components. Urokinase (SW) shows two components.

The in vivo uptake into blood clots was determined in four anesthetized dogs. Femoral arteries and veins were surgically exposed. Silk threads soaked in thrombin solution (1–10 units/ml of 0.9% saline) were laced through the lumens of the vessels and the vessels partially constricted by loosely tying with heavy string. One-half to one hour later, ^{99m}Tc-urokinase (1 mCi, 10,000–50,000 CTA units) was administered intravenously. One and one-half to three hours later, the vessels were occluded and the strings with attached clots removed from the vessels by dissection. The clots were isolated, dried in air, and weighed. Blood samples obtained at the same times were prepared in the same way. The concentration of radioactivity in the clots was then compared to that of the blood samples. The results are listed in Table 1.

CONCLUSIONS

In summary, urokinase was labeled with both radioiodine and technetium. The labeled protein re-

TABLE 1. UPTAKE OF ^{99m}Tc-UROKINASE IN BLOOD CLOTS IN DOGS

Experiment	cpm/mg clot / cpm/mg blood
1. 2 hr arterial	15.8
2 hr venous	1.6
2. 2 hr venous	22.0
3. 2 hr arterial	12.2
2 hr venous	8.6
2.5 hr arterial	10.0
3 hr venous	6.0
4. 2 hr arterial	1.5
2 hr venous	1.3
2.5 hr arterial	3.2
	$\bar{x} = 8.2$

tained both its enzymatic activity and immunoreactivity over a wide pH range. When injected intravenously it concentrated in the liver and kidneys and was rapidly excreted into the urine. Preliminary tests in dogs indicated that the labeled enzyme does localize in blood clots. Further tests are underway to determine if this localization is sufficient for scanning and if this can be used as a specific test for blood clot detection and localization.

ACKNOWLEDGMENTS

This research was supported by U.S.P.H.S. Grant GM 10548. We wish to thank L. J. Lewis, Department of Molecular Biology, Abbott Laboratories, North Chicago, Illinois, who prepared and supplied the urokinase antisera. We wish to thank Malcolm Cooper who provided helpful advice and continuous encouragement.

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