BASIC SCIENCES

RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

Gallium-68 Labeling of Albumin and Albumin Microspheres

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Because of the high stability constant of gallium transferrin, the formation of a protein that will be stable in vivo and labeled with gallium-68 (a positron emitter) requires preliminary coupling of a strong chelating group to the protein. In the present study, we have used a reaction developed by Krejcarek and Tucker, in which DTPA is coupled to proteins by the formation of an amide bond. Using human serum albumin (HSA) as a model, we have studied the efficiency of the reaction of HSA with the mixed acid anhydride of the quarternary triethyl ammonium salt of DTPA and butyl formate, as a function of the ratio of albumin to DTPA. After purification of the DTPA-labeled HSA, it is possible to prepare Ga-68-labeled albumin in high yield by chelation of the Ga-68 with the DTPA-labeled protein. In vitro and in vivo stability studies showed that the labeled protein was stable over a period of several hours. The same type of bifunctional chelate has been used to attach Ga-68 to HSA microspheres.

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A variety of approaches have been used to attach bifunctional metal-chelating groups to macromolecules. Sundberg and coworkers (*1*) first conjugated diazophenyl EDTA to human serum albumin (HSA). They then labeled the bifunctional molecule with indium-111 for use as a tracer in biologic processes. More recently several investigators have extended this method of labeling with chelating agents to smaller drug molecules and biochemicals. Both EDTA and DTPA have been attached to palmitic and other fatty acids (*2*), as well as to several sulfonyl ureas (*3*). Nitrogen-substituted iminodiacetic acid (IDA) derivatives have been evaluated as chelates for incorporation into molecules of biologic interest (*4*). Krejcarek and Tucker (*5*) developed an alternative method to attach DTPA to proteins, and it appears to be simpler than the Sundberg procedure using reagents common in peptide synthesis.

In this communication, a modification of the Krejcarek-Tucker method has been used to attach the positron-emitter Ga-68 to albumin.

MATERIALS AND METHODS

Attachment of DTPA to proteins. DTPA was attached to HSA according to the method of Krejcarek and Tucker (*5*). DTPA (100 mg) and triethylamine (172 μl) were mixed in a ratio of 1:5 in a minimum volume of distilled water. The solution was then lyophylized and the resulting glassy residue was dissolved in acetonitrile. The reaction mix-

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ture was cooled in an ice bath, and isobutyl chloroformate (28 μl) was added. The reaction mixture was stirred for 30 min, during which time the excess triethylamine hydrochloride precipitated. The mixed acid anhydride was then added to a cooled solution of HSA (250 mg), refrigerated, and stirred for 12 hr. The DTPA-protein purification procedure described by Krejcarek and Tucker was modified, since attempts to radiolabel the protein using the original procedure gave results that were variable and suggested the presence of unbound DTPA in the reaction mixture. Since DTPA appears to adsorb to the protein, a complex separation procedure was required. In the modified procedure, ultrafiltration through Diaflo PM 10 ultrafiltration membranes*, which retain MW > 10,000, was used to concentrate the reaction volume. The protein was then further purified by biofiber dialysis against 0.9% sodium chloride for 5 hr, followed by gel partition chromatography on Sephadex G-50/150 using a 48- by 2.5-cm column eluted with 0.9% sodium chloride. The fractions corresponding to DTPA-protein were collected and the volume reduced by ultrafiltration. This sample was dialyzed for 15 hr in a Biofiber 50 minibeaker† against 0.1 *M* acetate buffer (pH 5.0) and was finally subjected to membrane dialysis in dialyzer tubing that retains MW > 12,000‡, for 24 hr, also against 0.1 *M* acetate buffer.

In order to determine the yield of the reaction to bind DTPA to albumin, C-14-labeled acid was used. Carbon-14 DTPA is not available commercially, so C-14 EDTA was used to study the binding efficiency of the ligand to human serum albumin. Both EDTA and DTPA contain iminodiacetic acid groups and bind to the protein with amide group; they should therefore have similar protein-binding efficiencies. By counting aliquots of the reaction mixture after each purification step and by measuring the protein concentration by uv absorbance, the efficiency of binding could be determined.

An attempt was also made to optimize the amount of DTPA attached to HSA. The reaction conditions were studied by varying the starting amount of DTPA from 17 to 70 times the molar amount of HSA. Twenty μCi of C-14 EDTA were added simultaneously with each of the varied amounts of DTPA during the condensation with triethylamine. Total C-14 activity was measured after each protein purification step using a liquid scintillation spectrometer. The protein concentrations were measured by ultraviolet spectroscopy at 280 nm.

Radiolabeling. This was accomplished using $^{111}InCl_3$ or Ga-68 oxine (*6*) obtained from a solvent-extraction generator. The pH of the DTPA-HSA solution was lowered to 3.1 with 0.1 *M* HCl. The Ga-68 oxine residue was dissolved in 50 μl of 95% ethanol and 150 μl of 0.9% sodium chloride, then added to the protein solution. Typically, 1 mg of protein in 125 μl of buffer was used. After stirring the reaction mixture for 30 min, the final pH was adjusted to 5.5 with 0.1 *M* NaOH. The percentage of radioactivity bound to protein was determined by gel partition chromatography using a Sephadex G-50/150, 30- by 1.5-cm column eluted with 0.9% sodium chloride. Forty-five 1-ml fractions were collected and counted using a NaI well scintillation detector. The peak corresponding to labeled DTPA-HSA eluted in fractions 8–16, and the free labeled DTPA eluted in fractions 23–29.

In vitro exchange of gallium and indium to transferrin. DTPA-HSA was labeled using In-111 chloride or Ga-67 citrate as described above. The final pH was adjusted to 5.5 with 0.1 *M* NaOH. The labeled protein solution was mixed with physiologic concentrations of transferrin (250 mg/100 ml) and stored at 37°C. Over a period of several days, samples were withdrawn and spotted on agarose gel electrophresis film. The electrophoresis was carried out in 0.1 *M* barbital buffer (pH 8.4) at 200 volts, 40 mA per gel, for 40 min. The gels containing the radiolabeled proteins were allowed to dry, then cut into 1-cm segments. Each segment was counted using an auto gamma counter. The migration positions of the labeled proteins were compared with those of albumin and transferrin standards. The standard gels were dyed using 1% amido black in methanol:acetic acid:H_2O in ratios of 8:12:1.

In vivo clearance of Ga-68 DTPA-HSA. Simultaneous injections of Ga-68 DTPA-HSA and I-125 HSA were administered to rabbits. Blood samples were withdrawn at 30 min, and at 1, 1½, 2, 3, 4, and 5 hr postinjection. The protein was separated using the Sephadex G-15 and counted using a NaI well scintillation counter.

In vivo stability of Ga-68 DTPA-HSA. In vivo stability was measured by injecting rhesus monkeys with the labeled protein and withdrawing blood samples at specified times postinjection to determine the amount of Ga-68 attached to transferrin as a function of time. The labeled transferrin was separated from the serum by affinity chromatography using anti-human transferrin. Electrophoresis had been used in the in vitro study to separate the labeled DTPA-HSA from the exchange-labeled transferrin. This technique was not adaptable to the in vivo study, since the 3-μl serum samples necessary for the electrophoresis gels did not contain sufficient Ga-68 activity to allow completion of the electrophoresis and counting of the gel segments.

The in vivo blood clearance was carried out using rabbits, which were the original animal model for

the in vivo stability. Since the analytical technique for separating the DTPA-HSA and transferrin used affinity chromatography with a rabbit anti-human antibody, it was necessary to find an animal model in which the animal transferrin cross-reacted with the human antibody. This is not the case with rabbits or dogs, but the rhesus monkey was found to be an acceptable model.

The rabbit anti-human transferrin|| was bound to cyanogen-bromide-activated Sepharose 4B following a previously described procedure (*7*). The buffer systems used for the elution of the affinity column were based on the method of van Eigk and van Noort (*8*). Blood samples were withdrawn from the monkey at 1, 2.5, 3.5, and 4.5 hr postinjection. Serum samples of 400 μl each were mixed with 400 μl of the application buffer and applied to the affinity column. Thirty-five 2-ml fractions were collected. The activity in each fraction was counted using a NaI well scintillation detector, and the absorbance of each fraction was measured with a spectrophotometer at 280 nm. At each postinjection time, an additional blood sample was drawn and the activity per gram of blood was determined.

Radiolabeling of DTPA-HSA microspheres. Five milligrams of DTPA-HSA microspheres were prepared by the Krejcarek-Tucker procedure§ by attaching the DTPA to the surface of human serum albumin microspheres. The DTPA microspheres were labeled using Ga-68 oxine. The oxine residue was dissolved in 50 μl of 95% ethanol and 150 μl of saline, then added to the microspheres. The vial was sonicated for 30 min and the microspheres washed with saline. The microspheres were then administered intravenously to two dogs and images were obtained using the PETT IV positron tomograph (*9*). Serial blood samples were withdrawn at 5-min intervals. Each sample was weighed and the blood activity measured with a single-well, NaI scintillation detector.

Labeling efficiency was determined using an aliquot of the microsphere suspension that was passed through a 0.22-μ Millipore filter. Size range of the DTPA microspheres is 15–30 μ, so any activity passing the Millipore will be activity not associated with the protein.

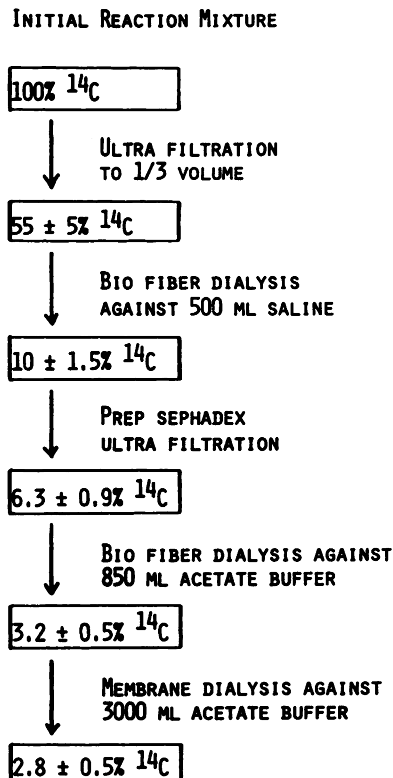

FIG. 1. Purification of DTPA-HSA using a C-14-labeled ligand.

RESULTS AND DISCUSSION

Attachment of DTPA to protein. Figure 1 indicates the percentage of the initial C-14 activity lost after each of the purification steps, starting with 100 mg of DTPA and 250 mg of HSA in the original reaction mixture. These data indicate that 2.1 moles of DTPA are attached per mole of albumin (100% yield would be 70 moles of DTPA/mole of protein). By reducing the amount of starting DTPA it was possible to increase the amount of DTPA attached to human serum albumin. Addition of 50 mg of DTPA gave a 56% attachment, indicating that 17 moles of DTPA are attached per mole HSA. With an initial 25 mg of DTPA, the final attachment was >98% of the original activity, indicating that 17 moles of DTPA are attached per mole HSA. These results show that by increasing the amount of DTPA, one decreases the attachment to the protein. This could be due to polymerization of the DTPA at high concentrations of the mixed acid anhydride used in the initial reaction conditions. Although the EDTA may not be an ideal tracer for DTPA, these results are in good agreement with preliminary data obtained by an isotope-dilution technique (*10*).

Although an average of 2–17 moles of DTPA per mole of protein appears high, it should be noted that at these levels it takes only 1.1–9.6 μg of iron to saturate the DTPA binding sites on 1 mg of protein. Great care must therefore be taken to use solutions and glassware containing as small an amount of trace metals as possible. In order to obtain protein with 5–10 moles of DTPA per mole of protein, the amount of DTPA should be reduced to even less than 25 mg of DTPA per 250 mg of albumin.

Radiolabeling. The time for reaction of Ga-68 with the DTPA-albumin was varied up to 60 min, and it was found that 30–60 min gave a 98% labeling efficiency, whereas at times <30 min, lower and variable yields were obtained.

After the 30-min labeling reaction, 98% of the radioactivity eluted from the Sephadex G50–150 column with an elution volume corresponding to human serum albumin, as shown in the elution profile (Fig. 2).

In vitro exchange of gallium and indium to transferrin. In order to assess the in vivo exchange of the metal from the DTPA-protein to transferrin, in vitro studies were done with In-111 and Ga-67. The studies (Fig. 3) showed an exchange of approximately 9% per day from the DTPA-HSA to transferrin. This rate of exchange would be insignificant in studies with proteins labeled with Ga-68. Note that both gallium and indium exchange at approximately the same rate. This suggests that the relative stability constants of DTPA and transferrin are very similar for gallium and indium. This exchange, although greater than that observed for the exchange of In-111 from In-111 azoalbumin to albumin in serum (*11*), is such that in five half-lives of Ga-68 only a few percent have exchanged.

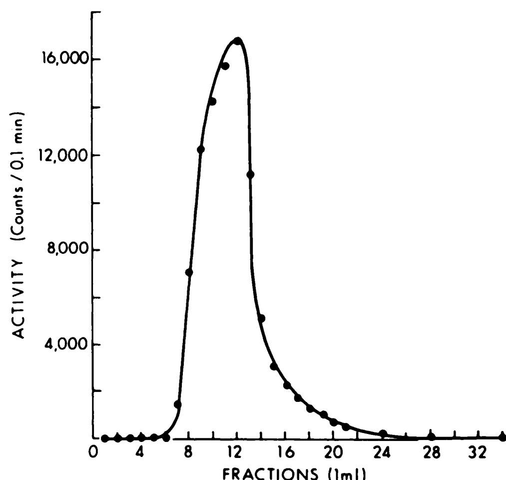

FIG. 2. Chromatogram of Ga-68 DTPA-HSA eluted from Sephadex G-50/150 using 0.9% NaCl.

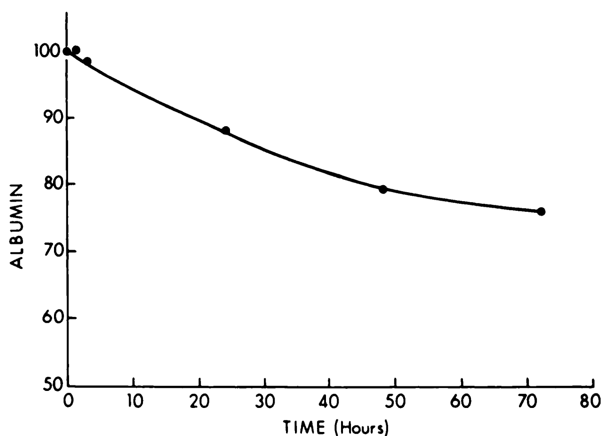

FIG. 3. In vitro exchange of Ga-67 and In-111 from DTPA-HSA to transferrin.

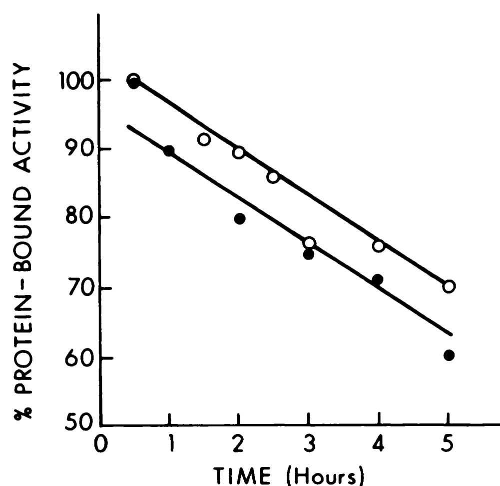

FIG. 4. Comparative behavior of Ga-68 DTPA-HSA (open circles) and I-125 HSA (solid circles) in a rabbit.

In vivo behavior of Ga-68 DTPA-HSA. Figure 4 compares the in vivo clearance of I-125 HSA and Ga-68 DTPA-HSA in rabbits. Over the time interval feasible with Ga-68, the behavior of the two tracers is very similar. The separation of transferrin from other plasma proteins by affinity chromatography is shown in Fig. 5. All the proteins except transferrin have retention volumes of ≈9 ml, whereas the transferrin is eluted when the pH of the eluate is decreased to pH 2.8. By application of this technique to plasma samples from the rhesus monkey, the amount of Ga-68 present as gallium-transferrin was found to be 2%, 2.7%, 6%, and 6% at 1, 2.5, 3.5, and 4.5 hr, respectively. This showed that the Ga-68 DTPA protein is stable over the time span of studies possible with Ga-68.

Radiolabeling of DTPA-HSA microspheres. The labeling efficiency of Ga-68 to the DTPA-HSA microspheres was 95% as determined by separation of the microspheres with Millipore filtration. After injection of the labeled protein spheres, the percentage of radioactivity per gram of blood stayed constant for 45 min during the imaging time. This is consistent with the presence in the preparation of a small amount of free Ga-68, which then becomes associated with the transferrin in the blood and maintains a constant blood activity level.

Images obtained from PETT IV are shown in

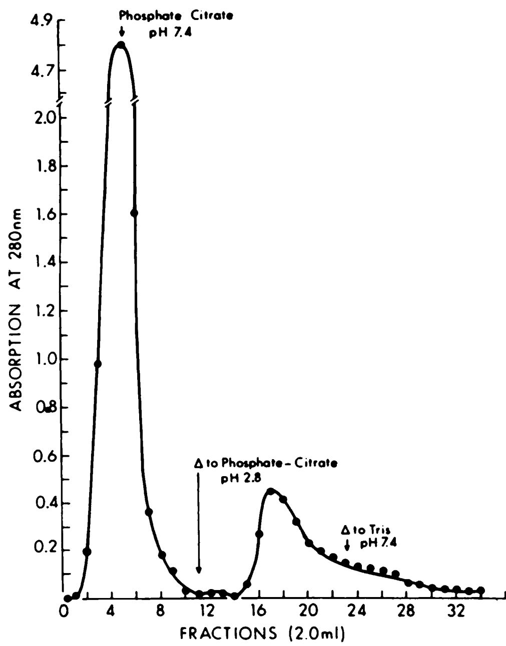

FIG. 5. Separation of albumin (and other plasma proteins) from transferrin using anti-human transferrin attached to Sepharose by cyanogen bromide.

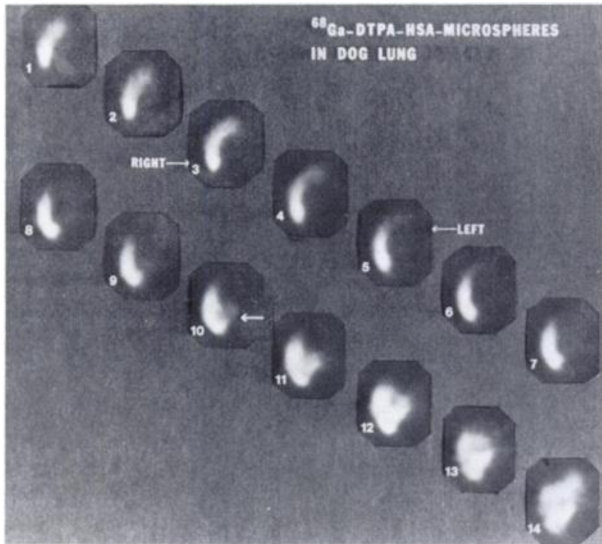

FIG. 6. PETT IV transaxial cross sections of dog's lung, obtained with Ga-68 DTPA-HSA microspheres in dog having one lung surgically ligated. Arrow indicates diaphragmatic lobe.

Figs. 6 and 7. Figure 6 shows a dog having only a right lobe in the lung field. The left lung had been surgically ligated. In slice 10, the diaphragmatic lobe (arrow) begins to appear. Figure 7 shows a dog's lung scan with areas of decreased perfusion apparent. When the animal was killed 3 days later, no reason for the decreased perfusion could be found. These images show detail that is not possible by conventional imaging techniques. Although activity appears to accumulate in the liver, note that each image is normalized to the highest activity distribution in that image. The gallium is bound to DTPA and will not be released over the time of an experiment.

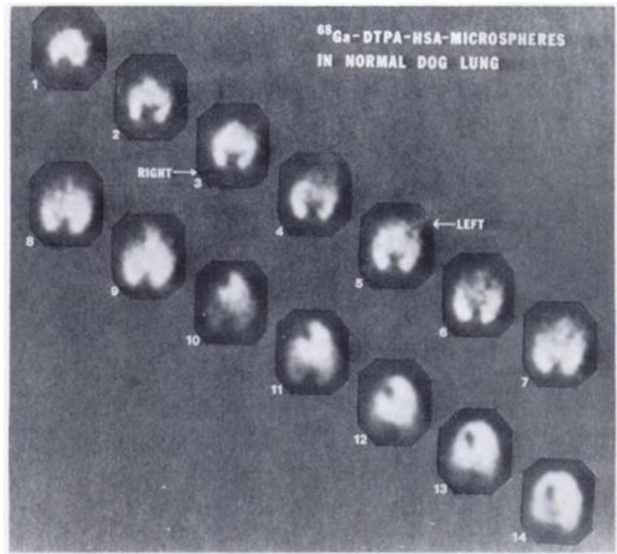

FIG. 7. PETT IV transaxial cross sections of dog's lung, obtained with Ga-68 DTPA-HSA microspheres.

This labeling technique provides stable Ga-68-labeled microspheres. Although other methods for preparing Ga-68-labeled microspheres have been described (*12*), their labeling mechanism is uncertain.

CONCLUSION

In the procedure developed for the labeling of albumin, the addition of the Ga-68 is the last step in the procedure. This is particularly important when radionuclides with short half-lives are used. The labeled albumin could offer an alternative to C-11 albumin as a vascular pool tracer, and the microspheres show potential as a positron lung imaging agent. It offers the possibility of DTPA attachment to other antibodies and proteins. One possibility is fibrinogen for ultimate use in thrombus detection. These agents would extend the number of available positron-emitting radiopharmaceuticals.

FOOTNOTES

* Amicon, Lexington, MA
† BioRad, Richmond, CA
‡ Fisher Scientific, St. Louis, MO
‖ Cappel Labs, Cochranville, PA
§ G. Krejcarek and K. Tucker, 3M Company, St. Paul, MO

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The Scientific Program Committee of the Greater New York Chapter of the Society of Nuclear Medicine solicits abstracts from members and nonmembers of the Society for the 5th Annual Scientific Meeting to be held Oct. 26–28, 1979 at the Sheraton Centre in New York City, NY. In addition to selected scientific papers and commercial exhibits, the meeting will feature survey papers and teaching sessions conducted by invited faculty.

Abstracts should not exceed 300 words. The title, authors, and institutional affiliations should be included at the top of the first page. The name of the author presenting the paper must be underlined. Abstracts should contain a statement of purpose, the methods used, results, and conclusions.

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