Improved Protein Labeling By Stannous Tartrate Reduction of Pertechnetate

William A. Pettit, Frank H. DeLand, Sidney J. Bennett, and David M. Goldenberg

Veteran's Administation Medical Center and University of Kentucky Medical Center, Lexington, Kentucky

A procedure has been developed whereby small amounts of protein—specifically human serum albumin and immunoglobulin G—can be labeled with Tc-99m. Artifactual problems associated with electrolytic and stannous chloride labeling procedures are virtually eliminated. The procedure is satisfactory for labeling human serum albumin, normal goat immunoglobulin G, and goat anti-carcinoembryonic antigen immunoglobulin G.

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Technetium-99m-labeled proteins of radiochemical quality potentially have a variety of in vivo uses. Those methods of protein labeling with Tc-99m considered include the stannous chloride reduction and electrolytic reduction procedures. Problems associated with these methods, however, are often masked by current assay techniques. In previous work the necessity to use an appropriate assay procedure for product analysis has been demonstrated (*1*). The application of gel filtration, specifically with Sephacryl S-200, has proven to be a reliable but lengthy procedure for the identification of two principal problems that can arise in protein-labeling procedures (*2,3*). Tc-99m-labeled colloid formation has been found to occur in procedures using stannous chloride (*1*) or electrolytic reduction with electrodes (*3,4:* W. A. Pettit, unpublished results). By many of the currently used assay methods these colloids are indistinguishable from the labeled protein. Additionally, with currently used procedures, if another ("foreign") protein with a molecular weight different from that of the original labeled protein is added to the reaction mixture, it too may be labeled. This observation implies that in vivo labeling of blood components may also occur. Although these problems may contribute only to somewhat inferior scintillation images and inaccuracies in blood-pool studies, they would be unacceptable in cases where a specific biological function is to be studied. The procedure described in this communication circumvents these problems satisfactorily and provides labeled protein that may be useful for functional or metabolic studies in vivo.

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For reprints contact: William A. Pettit, Nuclear Medicine Service, Veterans Administration Medical Center, Lexington, KY 40507.

MATERIALS AND METHODS

Materials. Human serum albumin* (HSA) was diluted in saline to a concentration of 5 mg/ml, and lyophilized normal goat immunoglobulin G† (IgG) was reconstituted in saline (pH 5.6), 10 mg/ml. Affinity-purified goat anti-carcinoembryonic antigen (CEA) IgG (5) was dialyzed against saline (pH 5.6) before use. The small Sephacryl S-200 columns (1.5 × 6.0 cm) used in the final step of the labeling procedure were specially prepared.‡ Labeled protein samples were assayed by elution from a 2.5- × 87.8-cm Sephacryl S-200‡ column with 0.04 *M* phosphate buffer, pH 7.4.

Methods. *Labeling procedure.* A 1-ml mini-vial,§ fitted with screw cap and septum, is used as the reaction vessel. The labeling procedure is as follows:

A. add 0.2 ml stannous tartrate (2×10^{-5} *M*, pH 3.1–3.2)

b. add protein (100 μg) to be labeled

c. let stand at room temperature for 20 min

d. add 0.2 ml $^{99m}TcO_4^-$ (<5 mCi) in saline

e. incubate vial and contents for 10 min at 40°C

f. add 10 μl saturated $NaHCO_3$ solution

g. incubate vial and contents for 20 min at 40°C

h. place contents of vial over 1.5 × 6.0 cm Sephacryl S-200 column and collect protein fraction

The stannous tartrate solution was prepared by dissolving the reducing agent in nitrogen-purged distilled water adjusted to pH 3.1–3.2 with hydrochloric acid. The final solution had a pH of 3.8. The small Sephacryl column was prepared for use by washing with 10 ml of phosphate buffer, followed by 0.2 ml of normal goat serum and 20 ml of buffer. (Goat serum should be replaced by HSA before consideration for human use.) This method of column preparation provided approximately 90% recovery of small amounts of protein. The first 3.0 ml of eluate from the column were discarded after sample application and the next 3.5 ml (containing the protein) were collected. Typically, 100 μg of protein in saline was used in this procedure.

Product analysis. A 2.5- × 87.8-cm Sephacryl S-200 column was used to determine the percentage labeling of the protein as well as the detection of colloid formation and possible labeled "foreign" protein. After the labeling procedure "foreign" protein was added in large excess to the labeled protein recovered from the small column. The protein mixture was incubated at room temperature for 1 hr before the gel filtration assay was performed. The effluent from the column was continuously monitored by uv absorbance, and the radioactivity in the collected fractions counted in a scintillation well counter to determine if the "foreign" protein were labeled. The assay procedure and apparatus have been described in detail elsewhere (*1*).

Binding and stability studies. Four-tenths milliliter of HSA in saline (100 mg/ml) was dialyzed against 2×10^{-3} *M* $HgCl_2$ in saline for 1 hr and then dialyzed against saline. A control experiment was performed by dialyzing HSA against 2×10^{-3} *M* $MgCl_2$ in saline followed by dialysis against saline. After dialysis, aliquots (100 μg) of both protein samples were subjected to the labeling procedure.

One milliliter of normal goat IgG (5 mg/ml) was allowed to stand at room temperature with 2.0 mg of dithioleryritol (DTE) for 1 hr, and the sample was then dialyzed against saline. A portion of this sample (100 μg) was subjected to the labeling procedure. Goat anti-CEA IgG (0.2 ml, 4 mg/ml) was treated similarly.

Goat anti-CEA IgG was labeled and collected in the usual manner and tested for immune reactivity with CEA and anti-goat IgG. Tc-99m anti-CEA IgG (0.07 μg) was incubated with excess of CEA (0.13 μg) for 45 min at 40°C in 0.01 *M* ammonium acetate (pH 6.3). The reaction mixture was assayed by gel filtration. Tc-99m normal goat IgG was treated in a similar manner. Tc-99m anti-CEA IgG was incubated for 15 min at room temperature with excess donkey anti-goat IgG bound to a solid support (Kynar)‖. After centrifugation the resulting pellet was assayed for Tc-99m activity.

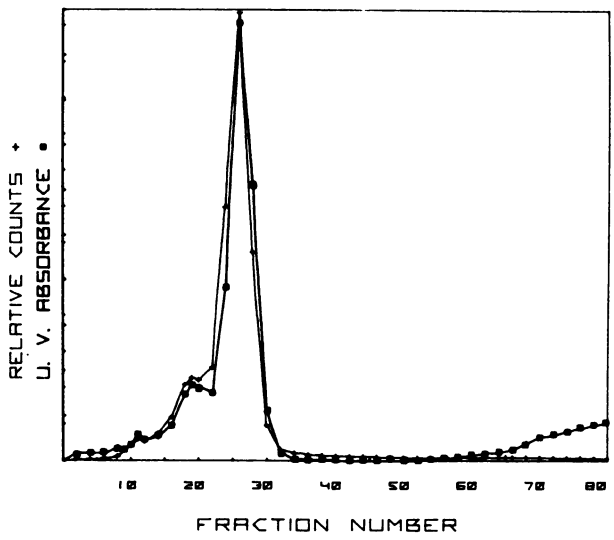

FIG. 1. Sephacryl S-200 column chromatographic results following labeling of HSA with Tc-99m. Counts of each fraction (+) show coincidence with uv absorbance (■).

To determine the stability of Tc-99m HSA labeled by this procedure, aliquots of the labeled protein were analyzed by Sephacryl S-200 chromatography, immediately following preparation and again at 4 hr and 20 hr later. The labeled protein remained at room temperature between chromatographic assays. Normal goat IgG was labeled and assayed in a similar manner.

RESULTS

Stannous chloride reduction of $^{99m}TcO_4^-$ in the

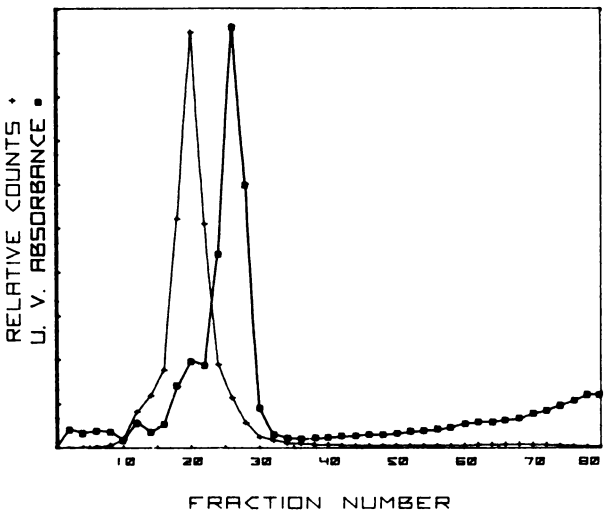

FIG. 2. Sephacryl S-200 column chromatography of labeled Tc-99m normal goat IgG to which large excess of HSA has been added. Activity (+) is associated primarily with IgG peak whereas uv absorbance (■) indicates excess HSA present, no evidence of "foreign" protein labeling.

presence of HSA can result in tin-technetium colloid formation, particularly at low protein concentrations (1). Similarly, electrolytic reduction also can lead to colloid formation. Figure 1 shows the results of Sephacryl S-200 analysis of Tc-99m HSA prepared by the procedure reported here. Recoveries were typically 20–60% of the activity applied to the small column and were inversely related to the amount of pertechnetate used initially. Assay of the protein fraction from the small column gave recoveries of 80–100% on the large column. Assay results of labeled normal goat IgG to which "foreign" protein (HSA) had been added are shown in Fig. 2.

Following dialysis of HSA against mercury (II) ion, attempts to label the protein demonstrated that less than 0.5% of the activity used in the procedure was protein-associated. Normally, 20–60% of the activity was protein-bound following the procedure. Dialysis against magnesium ion did not significantly affect the labeling yield. Typical labeling results for HSA, normal goat IgG, and goat anti-CEA IgG are given in Table 1. Pretreatment of HSA, normal goat IgG, and goat anti-CEA IgG with DTE increased specific activities of the Tc-99m protein complex to 11–18 μCi/μg.

Two immunological testing systems were used to determine the effects of the labeling procedure and the Tc-IgG complex on the immunological characteristics of anti-CEA IgG. The first system utilized the reaction of Tc-99m anti-CEA IgG with CEA. Incubation of Tc-99m anti-CEA IgG and CEA produced a complex that, upon gel filtration, appears in the void volume of S-200. The amount of Tc-99m activity associated with components in the void volume was used to determine the degree of complex formation and the retention of immunological activity following the labeling procedure. These determinations showed that slightly more than 60% of the recovered activity appeared in the void volume. The remaining activity was primarily associated with the IgG fraction. Control experiments using Tc-99m normal goat IgG instead of Tc-99m anti-CEA IgG provided no labeled component in the void volume. Similar antibody preparations of iodinated anti-CEA IgG (chloramine-T method) provided labeled antibody with 60–70% retention of immunological activity (*6*).

The other system employed donkey anti-goat IgG bound to a solid support. Incubation of Tc-99m-labeled anti-CEA IgG followed by centrifugation demonstrated that 78% of the Tc-99m activity was associated with the pellet. The value indicated the amount of labeled antibody immunologically recognizable as goat IgG.

TABLE 1 RESULTS OF PROTEIN LABELING

Protein	Amount of protein (μg)	Activity used (mCi)	Specific activity (mCi/μg)
HSA	100	2.3	3.5
HSA	100	3.0	13.0
HSA	100	1.5	2.4
HSA	100	1.0	2.0
HSA	500	5.5	6.1
HSA	1,000	10.0	6.7
N.G. IgG	400	2.0	1.5
N.G. IgG	100	3.0	7.8
N.G. IgG	400	3.0	2.7
N.G. IgG	100	2.3	6.0
N.G. IgG	100	2.2	4.0
anti-CEA IgG	115	0.88	2.8
anti-CEA IgG	115	2.4	6.0
anti-CEA IgG	115	2.0	3.9
anti-CEA IgG	115	7.0	5.0

DISCUSSION

It has been demonstrated previously that an appropriate assay procedure is imperative for adequate analysis of labeled proteins (*1*). The two principal problems encountered in labeling proteins with Tc-99m are the production of colloid and the labeling of "foreign" protein. The procedure presented here satisfactorily overcomes these problems and provides a relatively nonlabile Tc-99m protein derivative of good radiochemical purity. Tc-99m HSA obtained by this procedure (Fig. 1) is characterized by lack of tin-Tc colloid contamination. The activity curve and uv absorbance curve coincide closely even to the amounts of dimer- and polymer-HSA (fractions 20 and 8) present in the original sample. Results indicate that the Tc-99m IgG (fraction 20) is uncontaminated with colloid; and when a large excess of HSA (fraction 26) is added following the procedure, it is not labeled (Fig. 2).

The pH of the tartrate solution had a most pronounced influence on the labeling yield. Either above or below the pH range specified (i.e., pH 3.1–3.2), the incorporation of radionuclide into protein falls sharply. Although other conditions in the labeling procedure were varied, their effect on the radiochemical yield was not as dramatic as was the variation of the pH. The addition of bicarbonate ion sufficiently raised the pH of the reaction mixture to limit the possibility that other materials (e.g., "foreign" protein) might be labeled.

In our previous evaluation of several commercially available Tc-99m HSA kits (*1*), the use of stannous tartrate as the reductant (Union Carbide) effectively reduced the amount of tin-technetium colloid produced during labeling. Stannous tartrate greatly retards oxidation and hydrolysis of the stannous ion and subsequent colloid formation (*7*).

Mercury (II) salts are known to bind to sulfhydryl (SH) groups on proteins (*8*). Steigman (*9*) first demonstrated that pretreatment of HSA with $HgCl_2$ or N-ethylmaleimide effectively inhibited Tc-99m labeling by the iron-ascorbic acid procedure. Conversely, pre-

treatment of protein samples with DTE increased labeling efficiency two- to fourfold. Since DTE is known to cleave disulfide linkages of proteins to sulfhydryl groups (*10*), these results strongly indicate that the free SH groups are the sites of attachment of reduced Tc-99m to the protein. Interestingly, the maximum incorporation of activity into HSA is almost twice that incorporated into IgG. This observation closely reflects the relative number of potential sulfhydryl groups (i.e., cystine and cysteine residues) in the two protein species (*11*).

The stability of Tc-99m HSA labeled by this procedure appears to be satisfactory for most applications. At 20 hr following preparation, only 10–15% of the activity has dissociated from the Tc-99m HSA complex. Long-term studies with Tc-99m IgG may be limited by the lower specific activity obtainable and greater lability of the Tc-99m-protein complex. DTE may provide an approach to increasing the specific activity and stability if its use does not significantly alter the biological function under study. Preliminary results indicate that more activity can be used in the procedure provided the amounts of protein, stannous tartrate, etc. are increased.

FOOTNOTES

* Cutter Laboratories, Berkeley, CA.
† Miles Laboratories, Elkhart, IN.
‡ Pharmacia Fine Chemicals, Uppsula, Sweden.
§ Reliance Glass Works, Bensenville, IL.
‖ Provided by Dr. Hans Hansen, Hoffman-LaRoche, Nutley, NJ.

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