RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

Stoichiometric Tc-99m RBC Labeling using Stable Kit Solutions of Stannous Chloride and EDTA: Concise Communication

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An in vitro Tc-99m labeling method is described, which utilizes stable stock solutions of stannous chloride and disodium edetate (EDTA). The kit procedure requires as little as 1 ml of patient blood, can be performed in only 15 min, and gives labeling yields in excess of 98%. By using EDTA, the binding capacity of RBCs for technetium is sufficient to produce Tc-99m RBC doses with specific concentrations greater than 50 mCi/ml for first-pass cardiac studies. Scintigrams reveal a slight amount of bladder activity and splenic uptake, but at no time has the thyroid, stomach, or normal bowel been visualized. The predominant 20-hr blood-clearance half-time results in excellent image quality for over 24 hr—an essential property for following intermittent GI bleeding or for performing repeat cardiac function studies over a several-hour time interval.

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Technetium-99m-labeled red blood cells (RBCs) have become the radiopharmaceutical of choice for blood-pool scintimaging in large part because of the convenience of the in vivo labeling procedure of Pavel and Zimmer (*1*). But as clinical use expanded to include studies of intermittent GI bleeding, variable amounts of gastric, urinary, and colonic activities were seen with RBCs labeled in vivo (*2*). In a detailed study of the in vivo labeling kinetics, Callahan et al. (*3*) determined that these pertechnetate distribution artifacts could be reduced significantly by incubating the desired pertechnetate activity with a volume of in vivo-tinned patient blood in a closed in vitro system before reinjection. A similar modification by Vyth et al. (*4*) was shown to improve image quality in cardiac studies. In these methods, less of the Tc-99m activity is injected as pertechnetate ion. However, an often overlooked side effect of the in vivo tinning procedure is the long-term in vivo survival of stannous RBCs, effectively contraindicating the use of sodium pertechnetate as a radiopharmaceutical for several weeks (*5*). The in vitro labeling procedures do not tin the entire red cell volume of the patient and, as a result, do not affect subsequent nuclear medical studies.

Since its introduction in 1976 by Smith and Richards (*6*), the "Brookhaven RBC kit" has generally been regarded as the optimal method for the in vitro labeling of red blood cells with Tc-99m. The entire handling and labeling procedure is easily performed in 20 min and produces an average labeling yield of nearly 97% (*7*). Unfortunately, the kit still has not become commercially available, and its preparation is beyond the capability of the majority of potential users. A further restriction of the kit is its limited binding capacity for pertechnetate. Following the recommended procedure (*6*), 2 ml of the tinned and washed RBCs will effectively bind only 1.48×10^{14} atoms of Tc, or the quantity of chemical Tc produced from the decay of 11.65 mCi of Mo-99. In more familiar terms, the specific activity of [^{99m}Tc]pertechnetate from a generator having a 24-hr buildup is such that 1.48×10^{14} atoms of Tc is equal to 35.4 mCi of Tc-99m at the time of elution, or only 17.7 mCi 6 hr later. Regardless of the Tc-99m concentration, the

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maximum specific concentration of labeled RBCs would be less than 20 mCi/ml, unless additional generator elutions were routinely performed. For many cardiovascular nuclear medicine procedures it is desirable to have a bolus of less than 0.5 ml (*8*).

The primary purpose of this paper is to describe an in vitro labeling kit that uses materials and techniques common to most nuclear medicine laboratories, and avoids the limitations of the Brookhaven kit. The critical reagents of this protocol are (a) an acidic stock solution of stannous chloride, which is easily prepared and remains stable for several months, and (b) a dilution of commercial disodium edetate (EDTA). The critical labeling parameters are described in sufficient detail to enable the user to modify the procedure for specific clinical needs.

MATERIALS AND METHODS

Preparation of stock tin solution. As previously reported (*9*), stock stannous chloride solution is easily prepared by dissolving 2.0 g $SnCl_2 \cdot 2H_2O$ in 8.3 ml concentrated hydrochloric acid and diluting to 100 ml with sterile water for injection. The solution is clarified by 0.45-μ membrane filtration into clean unstoppered serum vials. The filtration unit should not contain metal needles, as the hydrochloric acid may dissolve some metallic components and contaminate the tin solution. The vials are then stoppered and autoclaved at 121 °C for 20 min. An alternative packaging method is filtering with 0.2-μ membrane filters directly into sterile vials using a sterile Teflon catheter instead of a metal needle. The stock tin solution typically contains 9.5 to 10.5 mg Sn^{2+} per ml of 1 *N* HCl, as determined by iodometric titration.

Preparation of dilute tin solution for RBC labeling. The high tin and acid concentrations of the stock solution prevent its direct addition to small volumes of whole blood or washed RBCs, so, a fresh dilution is prepared as needed. Adding 0.05 ml of the stock solution to a 10-ml single-dose vial of sterile preservative-free saline for injection USP (which actually contains 11 ml) yields a dilute solution containing a nominal 45 μg Sn^{2+} ion per ml of 4.5 m*N* HCl in saline. If used within one hour of preparation, 0.05 ml of the dilute solution contains a nominal 2 μg of Sn^{2+} ion.

Preparation of EDTA solution. Stock EDTA solution is easily prepared by diluting a 20-ml ampoule of edetate disodium injection, USP (150 mg/ml) with 40 ml sterile water for injection and repackaging in sterile 10-ml serum vials. The resulting solution contains 5mg Na_2EDTA in 0.1 ml.

Standard in vitro RBC labeling protocol. Having been refined over the last 6 yr, the following RBC handling and labeling procedure is presented as a reliable and routine in vitro labeling method. The stoichiometric studies discussed later are also based upon this standard protocol.

Using aseptic techniques:

1. Add 2 ml heparinized blood to a sterile 7-ml Vacutainer tube (BD-6531 or 6542).
2. Using a tuberculin syringe, add 0.05 ml of the *dilute* tin solution (~2 μg Sn^{2+} ion), mix gently, and allow to stand for 1 min.
3. Add 0.1 ml of the 5% EDTA solution (5 mg) and 5 ml sterile saline. Use a vent needle to remove any residual vacuum or positive pressure. Remove vent needle and mix tube gently.
4. Place in balanced centrifuge and spin inverted (stopper down) at 1,000 *g* for 2 min.
5. Remove tube carefully to maintain the softly packed RBCs. Using a 18- or 20-gauge needle, barely penetrate the stopper and slowly remove 0.5 ml of the tinned RBCs.
6. Transfer the RBCs to a second syringe containing the desired Tc-99m activity, mix gently, and allow 5 min for incubation. If the ratio of pertechnetate volume to RBC volume exceeds 2:1, allow additional incubation time.
7. Perform quality control before patient injection either by (a) assaying a one-drop aliquot in a dose calibrator both before and after a 7-ml saline wash, or (b) by assaying both sections of a microhematrocrit tube broken at the cell-plasma interface.

Studies on the critical labeling parameters. The following studies are presented to provide some insight on the stoichiometry of the Tc-99m RBC reaction.

The effect of EDTA on RBC labeling yield. Several workers have shown that RBCs have a limited capacity to take up stannous tin (*6,10–13*). Unless removed by washing, chelation, or oxidation, the extracellular Sn^{2+} ion in the trapped plasma of an RBC pellet can reduce the pertechnetate before it can penetrate the RBC membrane and lead to impurities such as Sn:Tc colloid and/or labeled plasma proteins. Either 0, 1, or 5 mg EDTA (a strong chelating agent for divalent metal cations) was added to triplicate 2-ml blood samples at the time of the 5-ml saline wash (Step 3). Equal tracer levels of Tc-99m in 0.5 ml saline were reacted for 10 min with 0.5 ml RBCs. Labeling yields were determined by dividing the RBC activity remaining after two 7-ml saline washes by the decay-corrected activity assayed during the incubation.

The effect of EDTA on in vivo clearance of Tc-99m RBCs. The labeling method presented above differs from the Brookhaven kit procedure in that EDTA is used to bind the extracellular tin before the labeling step. This study compares the blood clearance of Tc-99m RBCs prepared with and without the use of EDTA, using only 0.5 μg Sn^{2+} ion to pretin 2-ml blood samples. For one volunteer, 5 mg EDTA was used to stop the tinning re-

action, and 0.5 ml RBCs were labeled with 10 mCi Tc-99m with a 99.6% yield. For the second volunteer, EDTA was not used and, instead, the final 10 mCi Tc-99m RBC suspension was washed with saline before injection. After washing, the radiochemical purity was also 99.6%. An injection standard was prepared and serial blood samples taken for 24 hr. Triplicate blood samples were counted against the injection standard for each time interval, and the values obtained for fractional dose per ml were multiplied by the estimated blood volume to get the percent circulating activity. Blood-clearance data were analyzed using a slight modification of Cornell's method (*14*). Images were made of the cardiac pool and abdomen at 1, 3, 6, and 24 hr. Urine was also collected for 20 hr.

Required incubation times. Triplicate 2-ml blood samples were incubated with either 1 or 10 μg Sn^{2+} ion for 1 to 10 min and the incubations stopped by adding 5 mg EDTA and proceeding with the standard protocol. Equal tracer levels of Tc-99m (5–10 pmole) in 0.5 ml saline were added to each 0.5 ml RBC sample.

The rate of pertechnetate labeling was determined by applying the competing ligand method of Callahan et al. (*3*). A commercial stannous DTPA kit was reconstituted with 10 ml saline, and 0.5-ml aliquots added to each Tc-99m RBC suspension from 1 to 10 min after the pertechnetate addition. The Sn^{2+} concentration was constant (0.5 μg/ml blood) as was the amount of chemical pertechnetate added in the timed labeling step (5–10 pmole).

The effect of carrier Tc on RBC labeling. In preliminary studies designed to determine the optimal Sn^{2+} concentration for in vitro RBC labeling, we observed a wide range of percent labeling yields for a given pretinning concentration. However, having overlooked the potential Tc-99 carrier effect, we repeated the labeling studies in triplicate using six different chemical concentrations of pertechnetate—from 45 to 870 pmole of total technetium per ml RBCs. The chemical pertechnetate concentrations were calculated using the method of Lamson et al. (*16*).

Labeling was also studied as a function of the Sn^{2+} concentrations used to pretin whole blood, with five different pretinning concentrations used for each of the six Tc concentrations. To minimize variables in blood chemistry, stannous tin concentrations, and pertechnetate concentrations, the following controls were placed on the study:

1. All studies were conducted over a 3-day period using blood from a single donor.
2. For each of the six study groups, fresh dilute or double-dilute tin solutions were prepared from the same vial of stock tin solution.
3. The same stock solution of expired pertechnetate was used to adjust the specific activity of the Tc-99m test solutions.
4. All incubation times were extended to 15 min to maximize the tin and pertechnetate uptake.

Percent labeling efficiencies were determined as before by radioassay, then multiplied by the chemical quantity of total technetium in the labeling suspension, thus yielding pmoles Tc bound per ml RBCs. The percent Sn^{2+} retention by RBCs was not measured. Instead, Tc binding was plotted against the tin concentration added to the heparinized whole-blood samples (Fig. 4).

RESULTS AND DISCUSSION

Stability of stock tin solution. Having used this stock stannous chloride formulation for over 5 yr, we have observed several factors that affect its long-term stability. If stored at 4 °C, an unused vial of the autoclaved solution routinely assays >9 mg Sn^{2+} per ml after 6 mo. The stock vial used initially in this labeling study assayed 9.6 mg Sn^{2+} per ml at 4 mo after formulation. When small volumes are repeatedly withdrawn, the integrity of the rubber stopper is more easily maintained when 25-gauge or smaller needles are used. Because no preservative is present other than the high Sn^{2+} and HCl concentrations, we recommend that a given vial be discarded after 2 mo of repeated use. We routinely discard batches of stock tin solution after 6 mo.

EDTA effect on labeling yield. When labeling RBCs with trace levels of pertechnetate, the labeling yield falls off rapidly as the pretinning Sn^{2+} concentration increases (Fig. 1). Using EDTA to bind the excess extracellular tin effectively enables more of the pertechnetate to enter the RBCs and become bound. The practical value is that one can pretin the whole blood with known excesses of stannous chloride and still obtain clinically acceptable labeling yields by using EDTA. The chemical

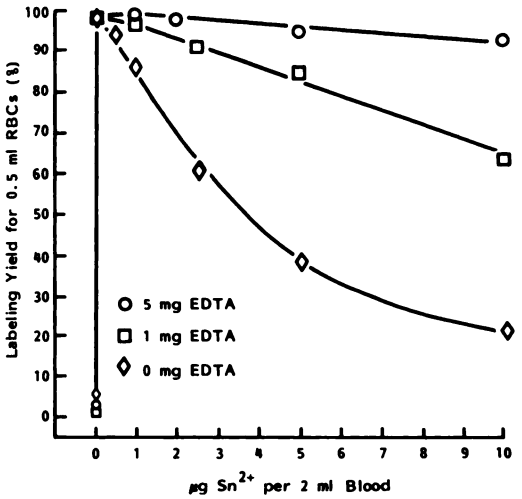

FIG. 1. Effect of EDTA on Tc-99m RBC labeling yield. Note small quantity of Sn^{2+} ion required to obtain nearly 100% labeling yield. EDTA effectively eliminates negative effect of excessive Sn^{2+} concentration.

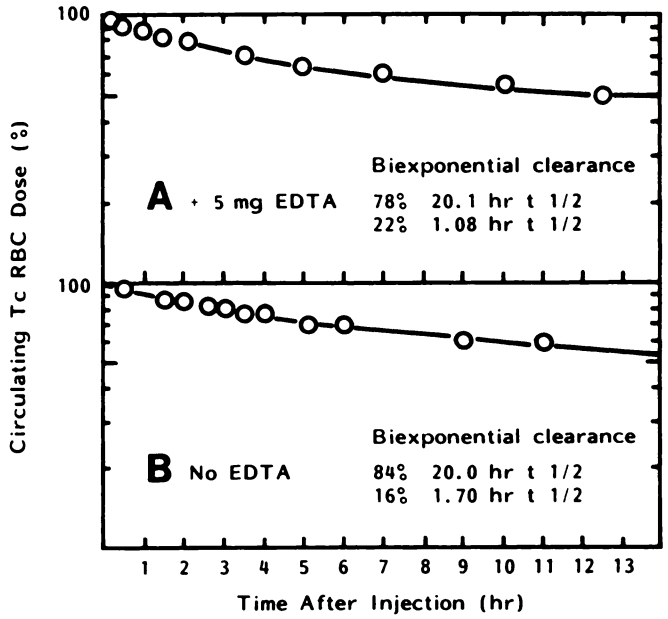

FIG. 2. Blood clearance of Tc-99m RBCs labeled in vitro with (A) and without (B) use of 5 mg EDTA.

form(s) of the unbound Tc-99m activity has not been fully characterized; however, <10% of the free activity migrates as pertechnetate when spotted on ITLC-SG chromatography strips and developed with acetone or 85% methanol.

Tc-99m RBC behavior in vivo. EDTA has been implicated by Ryo et al. (*16*) as causing an unusually rapid blood clearance of labeled RBCs. However, our 5-yr clinical experience with the standard protocol does not show such an adverse effect. For the present study, only slight differences were found in the short-lived component of blood clearance for Tc-99m RBCs labeled with or without the use of EDTA (Fig. 2). No difference was found in the long-lived component. The predominant 20-hr half-time compares favorably with the 29-hr half-time reported by Larson et al. (*7*) for the Brookhaven kit method. The 20-hr urinary excretion of radioactivity totaled 26% when EDTA was used and 37% when not. The scintigrams revealed some slight early bladder accumulation and minimal spleen uptake, but at no time did we see thyroid, stomach, or bowel activity. The images also support the theory that the short-lived component of blood clearance represents early urinary excretion and splenic uptake.

Minimum incubation times. No differences in the labeling were seen over the range of 1 to 10 μg Sn^{2+} over

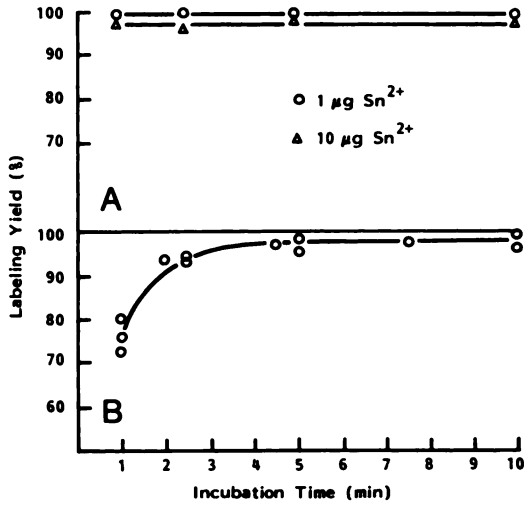

FIG. 3. Effect of Sn^{2+} incubation time (A) and TcO_4^- incubation time (B) on Tc-99m RBC labeling yield. Sufficient Sn^{2+} is absorbed by RBCs in whole blood within 1 min to bind nearly 100% of added TcO_4^-, whereas pertechnetate uptake requires about 5 min to be quantitative.

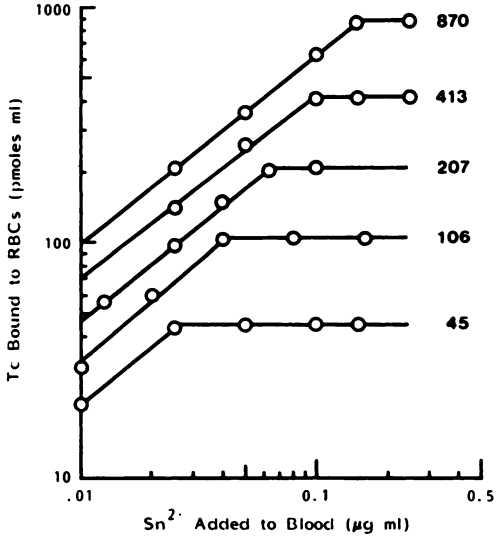

FIG. 4. Technetium binding of EDTA-washed RBCs as function of pretinning Sn^{2+} concentration in whole blood. Each curve illustrates binding obtained with given Tc concentration in washed RBC: pertechnetate incubation mixture (45–870 pmole/ml). Binding curve for one study group has been omitted from figure for visual clarity only.

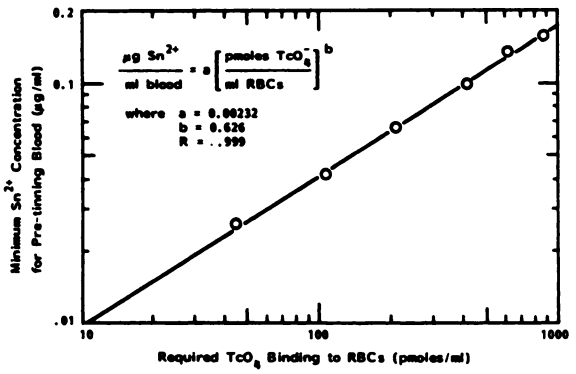

FIG. 5. Minimum pretinning Sn^{2+} concentrations required to obtain nearly 100% binding of technetium by EDTA-washed RBCs. Sn^{2+} concentration in excess of that indicated by figure is not detrimental to labeling yield, due to protective effect of EDTA in RBC wash solution.

10 min of incubation (Fig. 3). The pertechnetate uptake, however, is time dependent at the Tc concentration tested (10–20 pmoles total Tc per ml RBCs), and we recommend a 5-min incubation before quality control and patient administration.

The influence of carrier Tc-99 on RBC labeling. The binding capacity of RBCs for Tc-99m is dependent on both the pretinning Sn^{2+} concentration *and* the chemical concentration of Tc added to the washed RBCs (Fig. 4). For a given Tc concentration, labeling yield increases with increasing Sn^{2+} concentration up to the level where labeling efficiency peaks at nearly 100%. Likewise, for a given pretinning Sn^{2+} concentration, the chemical quantity of Tc bound by the washed RBCs increases as the concentration of Tc in the incubation mixture increases, even though the values for percent labeling may decrease.

The breakpoint in each of the curves in Fig. 4 represents the minimum pretinning Sn^{2+} concentration required in order to achieve nearly 100% binding of the given chemical quantity of technetium added. Replotting these break points in Fig. 5 illustrates the stoichiometry of the in vitro labeling reaction. By calculating the chemical quantity of Tc present in any desired dose of Tc-99m, one can easily determine the minimum pretinning concentration of Sn^{2+} ion required to obtain nearly 100% labeling with a user-selected volume of EDTA-washed RBCs. Using the relationship expressed in Fig. 5, one can also calculate that the Tc binding capacity of the 0.5-ml RBC volume recommended in the standard labeling protocol is 8,080 pmole of pertechnetate. From a typical generator eluant with a 24-hr buildup time, 8,080 pmole is equivalent to 1,163 mCi Tc-99m at time of elution. Even with a 96-hr buildup, a worst-case example, the standard protocol will accommodate over 200 mCi Tc-99m at time of elution. Accordingly, it is doubtful whether the binding capacity of pretinned and EDTA-washed RBCs will ever be exceeded when one prepares Tc-99m RBCs by the method we have presented.

CONCLUSIONS

The data presented herein clearly show the ease and simplicity with which any volume of pretinned RBCs can be quantitatively labeled with any desired Tc-99m activity. The advantages of this in vitro method over the in vivo, the modified in vivo, and even the Brookhaven kit methods are many. The patient's entire red-cell mass is not subjected to the long-term tin retention of the in vivo methods. The use of EDTA preserves high labeling yields in the presence of excess tin concentrations. The presence of carrier Tc-99 in the available [^{99m}Tc]pertechnetate can easily be determined and accommodated, with no loss of labeling efficiency. The long in vivo stability allows for high-quality blood-pool images for at least 24 hr without annoying free-pertechnetate artifacts. Finally, the user is able to prepare a unit dose of Tc-99m RBCs with varying specific concentration requirements unique to the user's clinical needs.

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