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Site-Specific ^{99m}Tc-Labeling of Antibody Using Dihydrazinophthalazine (DHZ) Conjugation to Fc Region of Heavy Chain

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The development of an antibody labeling method with ^{99m}Tc is important for cancer imaging. Most bifunctional chelate methods for ^{99m}Tc labeling of antibody incorporate a ^{99m}Tc chelator through a linkage to lysine residue. In the present study, a novel site-specific ^{99m}Tc labeling method at carbohydrate side chain in the Fc region of 2 antibodies (T101 and rabbit anti-human serum albumin antibody (RPAb)) using dihydrazinophthalazine (DHZ) which has 2 hydrazino groups was developed. The antibodies were oxidized with sodium periodate to produce aldehyde on the Fc region. Then, one hydrazine group of DHZ was conjugated with an aldehyde group of antibody through the formation of a hydrazone. The other hydrazine group was used for labeling with ^{99m}Tc. The number of conjugated DHZ was 1.7 per antibody. ^{99m}Tc labeling efficiency was 46~85% for T101 and 67~87% for RPAb. Indirect labeling with DHZ conjugated antibodies showed higher stability than direct labeling with reduced antibodies. High immunoreactivities were conserved for both indirectly and directly labeled antibodies. A biodistribution study found high blood activity related to directly labeled T101 at early time point as well as low liver activity due to indirectly labeled T101 at later time point. However, these findings do not affect practical use. No significantly different biodistribution was observed in the other organs. The research concluded that DHZ can be used as a site-specific bifunctional chelating agent for labeling antibody with ^{99m}Tc. Moreover, ^{99m}Tc labeled antibody via DHZ was found to have excellent chemical and biological properties for nuclear medicine imaging.

Key words: ^{99m}Tc, Site-specific labeling, Radiopharmaceutical, Dihydralazine, Dihydrazinophthalazine, Antibody, Bifunctional chelating agent

INTRODUCTION

Tumor-specific antibodies have been developed for targeting cancers. Radiolabeling of these antibodies has been studied for imaging or therapy (Hinkle *et al.*, 1990; Iznaga-Escorbar, 2001). ^{99m}Tc is the most widely used radionuclide for radiopharmaceuticals due to its excellent physical and economical advantages. The labeling of tumor specific antibody with ^{99m}Tc has been studied extensively. ^{99m}Tc labeling of antibody can be classified into two main methods: the direct labeling method and the indirect labeling method (Hnatowich *et al.*, 1993).

Direct labeling is a method that doesn't use any bifunctional chelating agent. ^{99m}Tc is directly linked to a high-affinity binding site of the antibody. This concept was established by Paik *et al.* (1985). The intramolecular sulfhydryl groups generated by reducing protein with β-mercaptoethanol or stannous chloride play a major role for binding ^{99m}Tc (Jeong *et al.*, 2004; Mather and Ellison, 1990; Pettit *et al.*, 1980). Although direct labeling method shows good biodistribution, it is limited in improving the biodistribution.

In indirect labeling, bifunctional chelating agents are used for labeling ^{99m}Tc. Many sulfhydryl containing bichelating agents have been designed and tested for the high stability of their technetium chelates (Fritzberg *et al.*, 1988). However, sulfhydryl-containing bifunctional

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chelating agents have been difficult to handle due to a high reactivity and instability. Furthermore, the antibody itself has disulfide groups that can be reduced to sulfhydryl compounds during the labeling procedure. To prevent the problems, a method that conjugates antibody with a prelabeled bifunctional chelating agent was developed (Kasina *et al.*, 1998). Kasina and coworkers labeled a chelating agent containing 3 nitrogen atoms and 1 sulfur atom (called N_3S ligand) with ^{99m}Tc, and then conjugated it to amino group of antibody using an *N*-hydroxysuccinimide ester (Fig. 1a). Although the labeling of antibodies by ^{99m}Tc showed good biodistribution and stability, this method is of limited practical use because its labeling yield is low. Furthermore, it is time consuming.

Another bifunctional chelating agent that employs a hydrazine-containing linker instead of a sulfhydryl-containing linker was reported (Schwartz *et al.*, 1991) (Fig. 1b). The chemical structure of the complex ^{99m}Tc with antibody conjugated hydrazino compound can be deduced from Nicholson and Zubieta (1988) article about the rhenium complex and hydrazine. The hydrazinonicotinic acid (HYNIC) was conjugated to a non-specific human polyclonal IgG. HYNIC-IgG was labeled with ^{99m}Tc and successfully used for imaging focal sites of infections (Abrams *et al.*, 1990). Extensive studies about

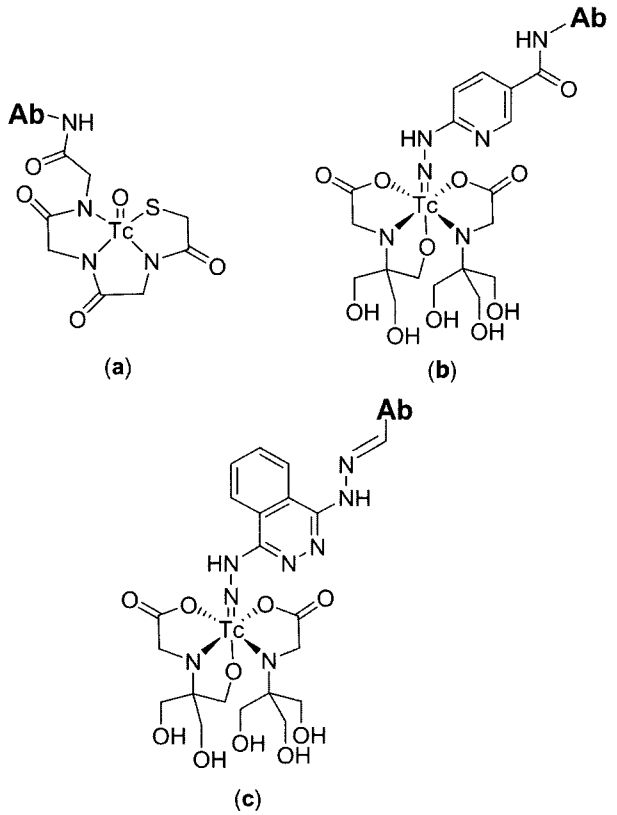

Fig. 1. Chemical structures of ^{99m}Tc-labeled antibodies using bifunctional chelating agents: (a) N_3S, (b) HYNIC, and (c) DHZ.

^{99m}Tc-labeling properties of HYNIC-conjugated monoclonal antibody was reported (Hnatowich *et al.*, 1993). In those studies, the monoclonal antibody B72.3 and C110 were conjugated with HYNIC and labeled with ^{99m}Tc. The immunoreactivity of the indirectly labeled monoclonal antibodies decreased more than that of the directly labeled. In the presence of glutathione or cysteine, the indirectly labeled monoclonal antibodies were more stable than the directly labeled antibodies. Due to its excellent properties and convenience, HYNIC has been used for labeling various kind of peptides for imaging cancer (Riccabona and Decristoforo, 2003; Laverman, *et al.*, 2004).

Site-specific modification of antibody on carbohydrate moieties has been reported by many researchers (O'shannessy *et al.*, 1984; Rodwell *et al.*, 1986). Antibody has carbohydrate moieties in the Fc region of heavy chains that are not important for antigen binding. In the field of antibody labeling with radioisotopes, site-specific modification generally conjugates bifunctional chelating agents with carbohydrate residues.

The antihypertensive drug dihydrazinophthalazine (DHZ) is an approved agent. It has two aromatic hydrazino residues. In the present study, attempts were made to conjugate one of the hydrazino residues with antibody and the other was used for labeling with ^{99m}Tc (Fig. 1c).

MATERIALS AND METHODS

Materials

T101, an $IgG2_a$, murine monoclonal antibody that recognizes CD5 (a human pan-T-cell antigen), was used. The production, purification and characterization of this antibody were previously described (Carrasquillo *et al.*, 1986). The Anti-human serum albumin rabbit antibody (RPAb) was purchased from Boehringer Mannheim Biochemicals. Sodium periodate was purchased from Fisher Scientific Co. The PD-10 ready-made size-exclusion columns and CNBr activated Sepharose CL-4B were purchased from Pharmacia Co. The centricon 30 filter was from Amicon Co. The TSK-GEL 3000 HPLC column was from TosoHaas Co. The cryotubes came from Nunc Co. The ^{99m}Tc-generator was purchased from DuPont Co. The instant thin-layer chromatography (ITLC) plates were from Gelman Sciences. All other reagents, if not specified were purchased from Sigma-Aldrich Co.

The radioactivity on the ITLC plate was scanned using a System-300 Image scanner from Bioscan Co., whereas the radioactivity in the test tubes was counted using a gamma scintillation counter from Packard Co.

Conjugation of DHZ to antibody

The schematic procedure of conjugating DHZ to

antibody is illustrated in Fig. 2. A volume corresponding to 0.1 mL of freshly made 0.3-M sodium periodate solution was added to 1 mL of 6-mg/mL RPAb or 6-mg/mL T101 in PBS (pH 5.5 containing 1 mM EDTA), and incubated for 1 h in the dark at room temperature. To reduce the remaining excess periodate, 200 μL of 0.4-M sodium sulfite was added and incubated 5 min at room temperature in the dark. The resulting mixture was purified with a PD-10 column pre-washed with phosphate buffered saline (PBS, pH 5.5 containing 1 mM EDTA). To the purified oxidized antibody (1.5 mL), 0.1 mL of 80 mM 1,4-dihydrazinophthalazine dihydrochloride monohydrate (DHZ) solution was added while vortexing. The molar ratio of oxydized antibody and DHZ was 1:200. The reaction for conjugating DHZ to the oxydized antibody proceeded for 3 h at room temperature in the dark. The free DHZ in the reaction mixture was removed by a Centricon 30 filter and was purified by a size exclusion HPLC equipped with a TSK-GEL 3000 (7.5 mm×30 cm) column. The fractions containing the monomeric antibody-DHZ conjugate in PBS (pH 6.7) were pooled and concentrated to 1.5 mL. Then 0.2 mL of 0.2 M sodium citrate buffer (pH 5.5) and 0.2 mL of 0.02 M EDTA solution (pH 7.0) were added. The protein concentration of each DHZ-conjugated antibody was determined by the Bradford method, and was adjusted to 2.2 mg/mL (Bradford, 1976). The final product in rubber-capped vial was evacuated and nitrogen gas was filled to replace dissolved oxygen. Each 50 μL

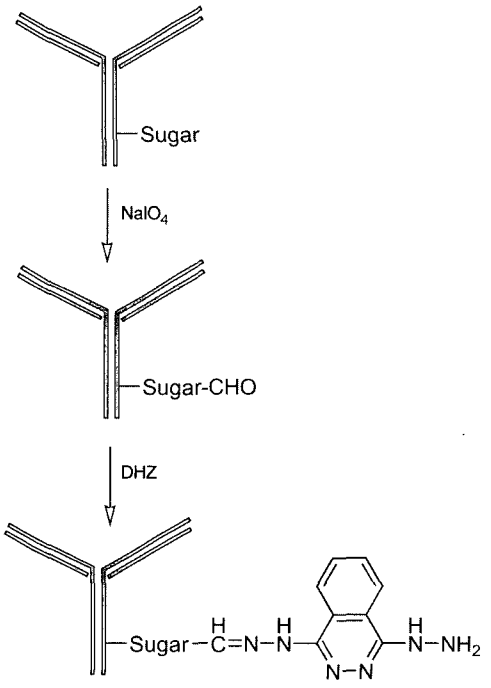

Fig. 2. Conjugation of DHZ to carbohydrate side chain of antibody. Sugar moiety of Fc region was oxidized to aldehyde and then the DHZ was conjugated to the antibody by formation of hydrazone.

fraction of the antibody-DHZ solution was dispensed into Cryotubes and stored in a freezer (-70°C).

Determination of the number of conjugated DHZ

Each of the DHZ conjugated antibody was diluted to 0.5 mg/mL with PBS (pH 5.5). A 90 μL sample of each antibody solution and 10 μL of 10 mM 2-hydroxy-1-naphthaldehyde (HNA) solution were mixed and incubated for 1 h at room temperature. The number of conjugated DHZ was obtained by the difference between the absorbance of the control HNA at 408 nm and that of the reactant. The molar absorbance value 4×10^4 L·mol^{-1}·cm^{-1} of 1-[2-hydroxy-1-naphtyl]methylenhydrazino]-phthalazine was taken from Mari-Buigues *et al.* (1991).

Labeling antibody with ^{99m}Tc

The mixture of 120 μL of 0.4-M sodium gluconate and 60 μL of 1.5 mg/mL stannous chloride in 0.02 M HCl was labeled by ^{99m}Tc by adding 0.2 mL (about 5 mCi) of generator eluted pertechnetate solution in normal saline. The absence of pertechnetate and colloid in the labeled ^{99m}Tc-gluconate was confirmed with ITLC (1 cm×10 cm) eluted by acetone and normal saline, respectively.

Three types of antibody preparation procedures were performed. They were direct labeling, non-specific labeling, and indirect labeling. For direct labeling, 10 μL of 14 mg/mL RPAb or 16 μL of 8 mg/mL T101 was reduced at 37°C for 30 min by 5 μL of 0.3 M b-mercaptoethanol in the presence of 5 μL of 0.1 M sodium bicarbonate buffer (pH 8.3) and 5 μL of 0.03 M EDTA solution. As control, 10 μL of 14 mg/mL antibody or 15 μL of 8 mg/mL T101 was treated in the same condition above except that the β-mercaptoethanol solution was replaced with distilled water. Concerning the indirect labeling, the antibody-DHZ conjugate, prepared as described in the previous section was used.

A 50 μL volume of ^{99m}Tc-gluconate was added to each antibody sample and was incubated for 1 h at 37°C. The labeling efficiencies and colloid formations were checked with ITLC (1×10 cm, eluted with 50% ethanol in 10% ammonium acetate) and paper chromatography (Whatman No.1, 1×10 cm, impregnated with 5% bovine serum albumin, eluted with normal saline). No colloid formation was detected by paper chromatography in the preparations of all ^{99m}Tc labeled antibodies. All other unlabeled radioactivities, such as ^{99m}Tc-gluconate and ^{99m}Tc-pertechnetate, moved to solvent front on the ITLC. The labeled antibodies were further purified with a size exclusion HPLC equipped with a TSK-3000 (7.5 mm×30 cm) column.

Stability test in cysteine solution

10 μL of 10 mM L-cysteine solution was added to 90 μL

of ^{99m}Tc labeled antibody solutions, and incubated for 5 h. During the incubation, 1 μL aliquots of each sample were taken and spotted to an ITLC (1×10 cm, eluted by 50% ethanol in 10% ammonium acetate) at 0, 1, 2, 3, and 5 h. The ITLC plates were cut at the center and the radioactivities were detected by gamma scintillation counter. All the radioactivities released from antibodies moved to solvent front and those bound to antibodies remained at the origin by the itlc described above condition.

Immunoreactivity test

Human serum albumin (HSA) was conjugated to CNBr activated Sepharose CL-4B to 6 mg/mL-settled volume. The HSA conjugated bead was diluted 100 times and used as solid phase antigen for immunoreactivity tests. 100 μL of ^{99m}Tc-RPAb (0.26 μCi, 50 ng) or ^{99m}Tc-DHZ-RPAb (0.36 μCi, 50 ng), 100 μL of PBS (pH 7.4) (or 2 mg/mL RPAb for non-specific binding), and 200 μL of HSA conjugated Sepharose (100 μL settled volume, 6 μg of conjugated HSA) were mixed and incubated for 1 h while shaking at room temperature. The test tubes were centrifuged at 3,000 rpm for 30 min and the supernatants were aspirated. The beads were washed twice with PBS (pH 7.4), and their radioactivities were counted using a gamma counter.

75 μL of ^{99m}Tc-T101 (0.14 μCi, 24.8 ng) or ^{99m}Tc-DHZ-T101 (0.13 μCi, 25.0 ng), 25 μL of PBS (or 1 mg/mL T101 for non-specific binding), and 100 μL of serially diluted cultured CCRF cells (12.9×10^7/mL – 2.5×10^6/mL) were incubated for 1 h at 4°C with shaking. The test tubes were centrifuged and the supernatants were aspirated. The radioactivities of the precipitated cells were counted using a gamma counter.

Biodistribution study in mice

Balb/c mice (Harlan Sprague-Dawley, Indianapolis, IN, female, 5~8 week) were injected intravenously through a tail vein either with directly labeled ^{99m}Tc-T101 or indirectly labeled ^{99m}Tc-DHZ-T101. The animals (n = 3~5) were killed by exsanguinations at 2, 7, 19, and 43 h after injection. The organs (liver, kidney, thyroid) were excised, blotted with gauze and weighed. The radioactivity was measured using a gamma counter. Organ uptakes were expressed as the percent of the injected dose (% ID/organ) and normalized to a 20 g body weight.

RESULTS

The carbohydrate chains of RPAb and T101 were oxidized by sodium periodate to generate aldehyde groups on the antibody. The DHZ was conjugated to the aldehyde groups through the formation of hydrazone (Fig. 2). After the reaction, the antibody-DHZ conjugates were purified using a size-exclusion HPLC, and it was found that 1.7 DHZ were bound to each antibody by the HNA method.

The purified monomeric antibodies were labeled with ^{99m}Tc by isotope exchange from ^{99m}Tc-gluconate, and the labeling yield varied from 67% to 87% for RPAb (Fig. 3A) and from 46% to 85% for T101. DHZ-RPAb showed 75% labeling yield when incubated with ^{99m}Tc-gluconate (Fig. 3B). The labeling yield decreased to 38% for RPAb, when

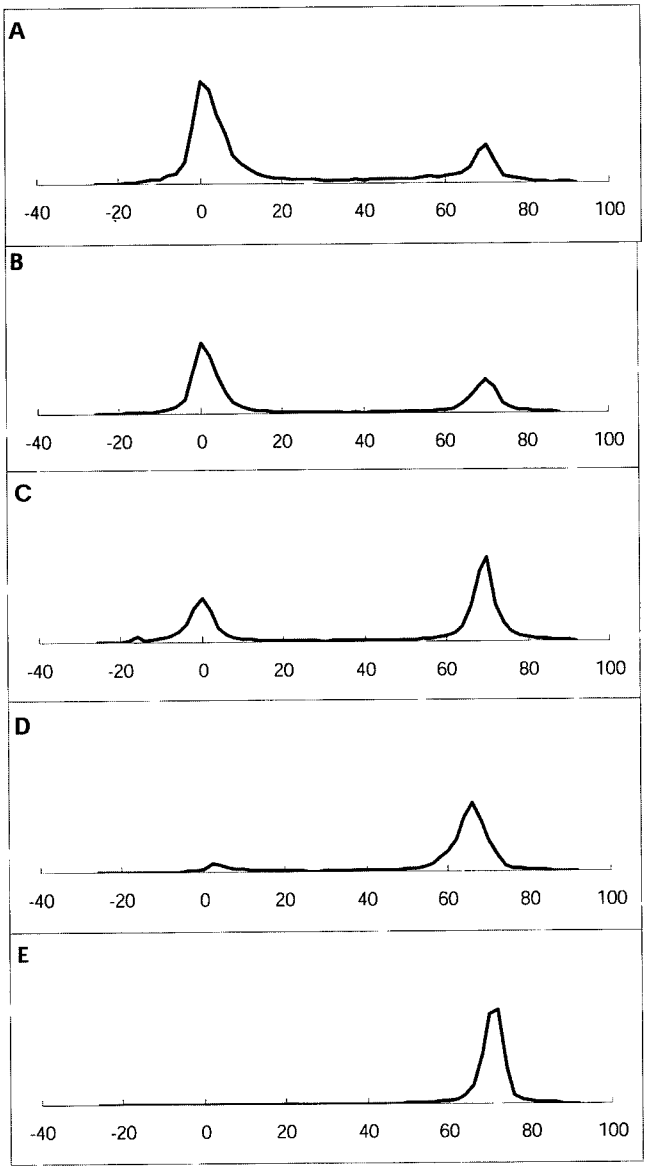

Fig. 3. ITLC profile of ^{99m}Tc labeled antibodies. The X-axis shows the distance from sample loading position in millimeter. Antibody bound activities remained at the loading position and non-bound activities moved to 70 mm of the solvent front. A: Direct labeled RPAb, B: DHZ conjugated RPAb labeled by isotope exchange from ^{99m}Tc-gluconate, C: DHZ conjugated RPAb labeled by isotope exchange from ^{99m}Tc-glucarate, D: intact RPAb reacted with ^{99m}Tc-gluconate, and E: intact RPAb reacted with ^{99m}Tc-gluconate in the presence of free DHZ.

0.4-M ^{99m}Tc-glucarate was used instead of 0.4-M ^{99m}Tc-gluconate (Fig. 3C). The non-modified RPAb had a 7% labeling yield (Fig. 3D), but no radioactivity was incorporated to RPAb in the presence of 1 mM DHZ with same condition (Fig. 3E).

The stability test in the 1 mM cysteine solution showed that indirectly labeling using DHZ conjugated antibody showed higher stability than directly labeled antibody, and that RPAb was more stable than T101 (Fig. 4).

The immunoreactivities of ^{99m}Tc labeled RPAb's were obtained by binding to HSA-conjugated, Sepharose beads in the presence and absence of excess cold antibody. Immunoreactivities could be obtained by the difference between total binding and non-specific binding, and was detected for 91.4% and 90.0% for ^{99m}Tc-RPAb and ^{99m}Tc-DHZ-RPAb, respectively. Immunoreactivities of ^{99m}Tc labeled T101 were obtained by drawing a saturation curve after the cell binding assay. It was found to be 78.4% and 72.7% for ^{99m}Tc-T101 and ^{99m}Tc-DHZ-T101, respectively.

According to a biodistribution study, ^{99m}Tc-T101 showed higher blood activity (27.5% ID/organ) than ^{99m}Tc-DHZ-T101 (21% ID/organ) at early time (2 h) and similar activities at later time (7~43 h). ^{99m}Tc-T101 had lower liver activities than ^{99m}Tc-DHZ-T101 at later time (7~43 h) (Fig. 5). No significantly different biodistribution was observed in the other organs and time.

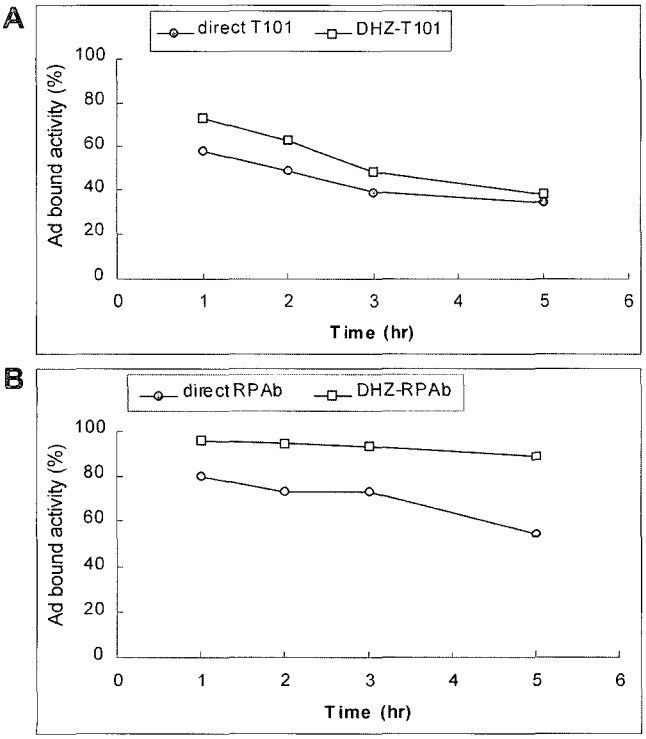

Fig. 4. Result of stability test in 1 mM cysteine solution. The analysis was performed using ITLC (50% ethanol in 10% ammonium acetate). A: T101, B: RPAb.

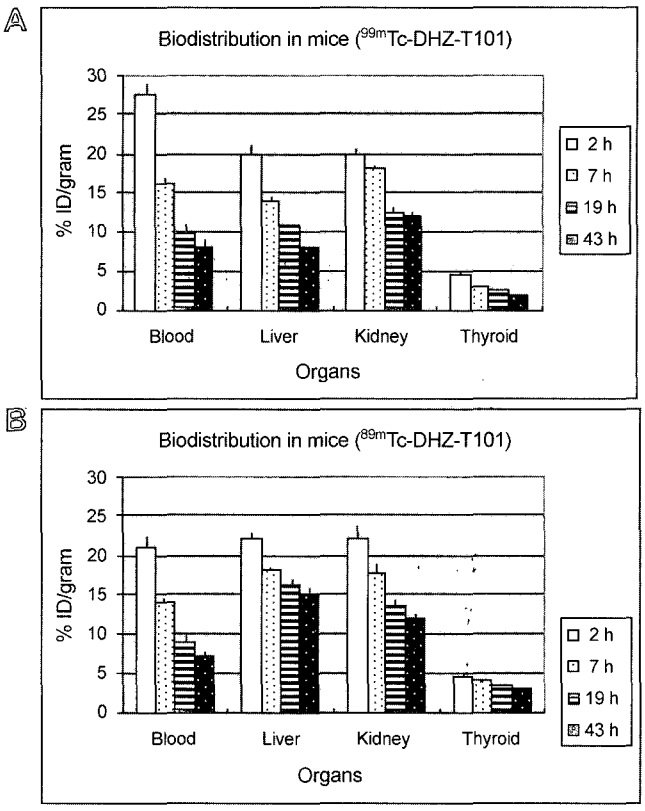

Fig. 5. Biodistribution study of ^{99m}Tc labeled T101. A: T101 was labeled directly; B: T101 was labeled indirectly via DHZ.

DISCUSSION

Although, HYNIC conjugation to antibody is proved to be a promising method for labeling ^{99m}Tc (Abrams *et al.*, 1990; Callahan *et al.*, 1991; Schwartz *et al.*, 1991), it is not an ideal method because the conjugation is not site-specific. A succinimide-activated HYNIC would be conjugated to any amino group of antibody non-specifically by making an amide bond. Because of the lack of site-specificity, significant decrease of immunoreactivity has been reported. For example, the immunoreactivities of C110 was reported to decrease from 91% to 49% in direct compared to the HYNIC method (Hnatowich *et al.*, 1993). In addition, HYNIC is not commercially available and it should be activated to the unstable active ester. Ester can cause problems during long-term storage or in the controlling of the conjugation number. DHZ is a well-known antihypertensive drug along with hydralazine. It is a stable compound in ambient environment. In the present study, DHZ was site-specifically conjugated to antibody, and then labeled with ^{99m}Tc with high yield and without affecting immunoreactivity. Substantially, ^{99m}Tc-DHZ-RPAb showed no difference in the immunoreactivity with that of ^{99m}Tc-RPAb. The small decrease in the immunoreactivity of ^{99m}Tc-DHZ-T101 compared to that of ^{99m}Tc-T101 was

attributed to experimental error.

DHZ-conjugated antibodies showed higher stability in cysteine solution. The data are consistent with reported results in the literature that showed that ^{99m}Tc labeled antibody using HYNIC conjugate was more stable than directly labeled antibody (Hnatowich *et al.*, 1993).

Although, the mechanism or chemical structure of ^{99m}Tc labeled DHZ-conjugated antibody was not thoroughly studied, it can be identified with that of HYNIC conjugated antibody. Both DHZ and HYNIC have hydrazino group and aromatic heterocyclic ring containing nitrogen. Moreover, the chemical structure of ^{99m}Tc-HYNIC-antibody is well known (Fig. 1b). Generally, the chemical structured of a technetium complex is deduced from the chemical structure of the rhenium complex because rhenium has chemical properties very similar to that of technetium. Hydralazine, which has a similar chemical structure with DHZ, has been reported to make an octahedral complex with rhenium $ReCl_2(PPh_3)_2(NNR)$ (R = hydralazine) by reacting with $ReOCl_3(PPh_3)_2$ (Nicholson and Zubieta, 1988). In addition to labeling antibodies, HYNIC has been used for labeling many other peptides with ^{99m}Tc (Babich *et al.*, 1993; Babich and Fischman, 1995; Fichna and Janecka, 2003).

A little high blood activity of the directly labeled antibody at early time points and low liver activity of the indirect labeled antibody using DHZ conjugate in biodistribution studies may not affect its practical use.

In conclusion, DHZ can be used as a site-specific bifunctional chelating agent for labeling antibody with ^{99m}Tc. Moereover, ^{99m}Tc labeled antibody *via* DHZ showed excellent chemical and biological properties for nuclear medicine imaging.

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