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# Zn Complex of Diaminedithiol Tetradentate Ligand as a Stable Precursor for $^{99m}$Tc-Labeled Compounds

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**Abstract:** The diaminedithiol ($N_2S_2$) tetradentate ligand constitutes a useful chelating molecule for preparing $^{99m}$Tc-labeled compounds of high in vivo stability in high radiochemical yields. However, since the thiol groups in the $N_2S_2$ ligand are easy to be oxidized to disulfide bonds, they need to be protected with an appropriate protecting group, which hinders the broad applications of the $N_2S_2$ ligand for radiopharmaceuticals. In this study, a Zn chelate of $N_2S_2$ was evaluated as a precursor for purification-free $^{99m}$Tc-labeled $N_2S_2$ under the mild and simple procedure. Zn-$N_2S_2$ was prepared by reacting Zn acetate with $N_2S_2$, and the Zn-$N_2S_2$ remained stable under aerobic conditions at room temperature. $^{99m}$Tc-$N_2S_2$ was obtained over 90% radiochemical yields at room temperature by a one-pot reaction, consisting of Zn-$N_2S_2$ ($10^{-5}$ M), $^{99m}TcO_4^-$, ethylenediaminetetraacetic acid (EDTA), and a reducing agent ($Sn^{2+}$) at pH = 5.5 to 7.5. $^{99m}$Tc-$N_2S_2$ was also obtained over 90% radiochemical yields when the reaction was conducted in the presence of an equimolar amount of IgG antibody. These findings indicate the Zn complex of $N_2S_2$ ligand constitutes a stable and useful precursor to prepare $^{99m}$Tc-labeled $N_2S_2$ compounds in high yields under the mild and simple procedure.

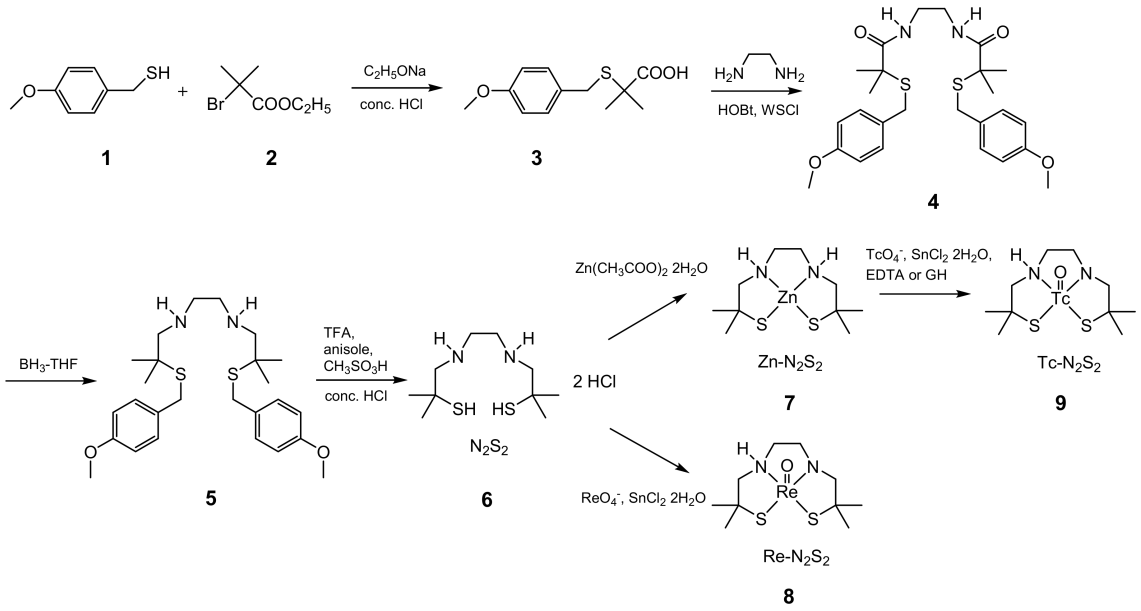
**Keywords:** $^{99m}$Tc; radiopharmaceutical; Zn complex; diaminedithiol; $N_2S_2$

## 1. Introduction

The growth and broad applications of diagnostic nuclear medicine have been mainly driven by the artificial radionuclide, technetium-99m ($^{99m}$Tc), due to its availability from a portable $^{99}$Mo-$^{99m}$Tc generator system and its almost ideal physical properties for external imaging. Although recent efforts are being made to develop radiopharmaceuticals derived from positron emitters such as $^{11}$C and $^{18}$F, over 70% of diagnostic practices are still conducted with $^{99m}$Tc. The cost-effectiveness of $^{99m}$Tc-based radiopharmaceuticals contributes to the medical economy in both developed and developing countries. Thus, $^{99m}$Tc will continue to be used as one of the essential radionuclides for diagnostic nuclear medicine. Radioactive rhenium ($^{186}$Re and $^{188}$Re), the congener of Tc, emits beta rays appropriate to targeted radiotherapy to form radiotheranostic pairs with their $^{99m}$Tc counterparts due to the chemical analogy between Tc and Re. Thus, further development of $^{99m}$Tc-radiopharmaceuticals constitutes a crucial issue for cost-effective and successful patient management.

A tetradentate chelating agent containing two amines and two thiol groups, referred to as a diaminedithiol (DADT) or a bis(amino ethanethiol: BAT), forms a neutral, lipophilic, and stable complex with the pentavalent oxo-Tc and oxo-Re ($TcO^{3+}$ and $ReO^{3+}$) [1,2]. Such characteristics render DADT attractive as a coordination molecule for $^{99m}$Tc- and $^{186/188}$Re-labeled compounds [3–6]. A presentative structure of DADT ligand is shown in Scheme 1, compound **6** ($N_2S_2$). Indeed, $^{99m}$Tc-labeled

*N*,*N*′-ethylene bis (L-cysteine ethyl ester) has been used as a radiopharmaceutical for measuring cerebral blood flow [7]. However, further applications of DADT ligands are hindered due to the inherent easily oxidized property of the thiol groups during the storage.




**Scheme 1.** Synthetic procedure for $N_2S_2$ and its Zn, Re, and $^{99m}$Tc complexes.

Some acylating agents have been used to protect the thiol groups in $N_2S_2$- or $N_3S_1$-type ligands such as mercaptoacetyl glycyl-glycyl-glycine. These include benzoyl [8], acetyl [9], and *m*-phthalic acid [10]. The deprotection of the benzoyl group is usually conducted at an elevated temperature (e.g., in boiling water) under alkaline conditions (pH > 10) [8,11]. The acetyl protecting group can be removed under milder conditions, whereas a long reaction time (1 h) is needed at room temperature [9]. The *m*-phthalic acid protecting group can be removed at room temperature in the presence of 1 M of $NH_2OH$ [10], which may necessitate post-labeling removal of $NH_2OH$ before injection to subjects. Thus, a protecting group that provides a precursor of $N_2S_2$ stable against oxidation and generates $^{99m}$Tc-$N_2S_2$ in high radiochemical yields under mild conditions without post- or pre-labeling purification is highly useful for developing new $^{99m}$Tc-radiopharmaceuticals.

Meanwhile, a transmetallation reaction between Zn chelate of dithiocarbamates (DTCs) and rhenium tricarbonyl has been reported. In this reaction, the thiol groups in the DTCs were stabilized upon complexation with Zn ion, and the direct reaction of the Zn complex with $Re(CO)_3Br_3$ provided $Re(CO)_3$-DTCs in high radiochemical yields [12]. These results suggested that Zn ion would also be applicable as a protecting agent for the thiol groups in $N_2S_2$ ligands removable during the complexation reaction with $^{99m}$Tc and Re. Indeed, a prior study showed the formation of Zn-$N_2S_2$ chelate [13]. Zn is classified as the least toxic group of all metals by the ICH Harmonized Guideline [14], which is advantageous to clinical applications.

In the present study, the Zn ion was evaluated as a protecting agent for the thiol groups in $N_2S_2$ ligands using compound 6. After preparing the Zn complex of 6, the reaction parameters that affected the radiochemical yields of the $^{99m}$Tc-$N_2S_2$ were investigated. The formation of $^{99m}$Tc-$N_2S_2$ from Zn-$N_2S_2$ was also evaluated in the presence of an equimolar amount of IgG antibody to estimate the applicability to Zn-$N_2S_2$ as a chelating moiety of a bifunctional chelating agent for $^{99m}$Tc-labeled polypeptides.

## 2. Results

### *2.1. Synthesis*

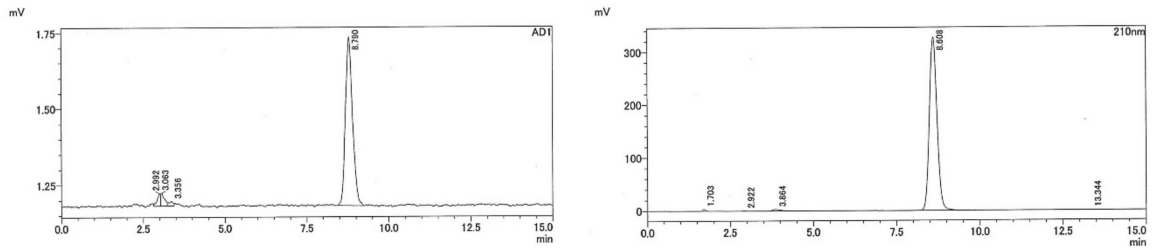
The $N_2S_2$ ligand 6 was synthesized according to the procedure of Ohmomo et al. as shown in Scheme 1. 4-(Methoxyphyenyl)methanethiol 1 was reacted with ethyl 2-bromo-2-methyl propanoate 2 to prepare 3, followed by the condensation with ethylenediamine to provide 4. After reducing the amide bonds in 4 with $BH_3$, the thiol protecting group in the resulting compound 5 was removed with TFA/anisole/methanesulfonic acid to obtain 6.

The Zn complex of the $N_2S_2$ was prepared by mixing Zn acetate with compound 6 in an aqueous solution at neutral pH. The Zn-$N_2S_2$ was obtained by extracting the reaction solution with dichloromethane, followed by recrystallization from the mixture of chloroform and hexane in 89% yield. The Zn-$N_2S_2$ remained stable at room temperature under aerobic conditions over several days.

The non-radioactive $^{185/187}$Re-$N_2S_2$ complex was synthesized by reacting 6 with $ReO_4^-$ in aqueous ethanol using $Sn^{2+}$ as a reducing agent. After silica-gel column chromatography, the oxorhenium complex of $N_2S_2$ was obtained in ca. 20% yield. The structure of this complex was confirmed by mass spectrometry (MS) and infrared spectroscopy (IR).

### *2.2. $^{99m}$Tc Complexation Reaction*

The reaction of Zn-$N_2S_2$ with $^{99m}$Tc was evaluated at room temperature for future applications as a bifunctional chelating agent for labeling heat-sensitive polypeptides. The reaction parameters for preparing $^{99m}$Tc-$N_2S_2$ from Zn-$N_2S_2$, which included the reaction pH, Zn-$N_2S_2$ concentration, presence or absence of ethylenediaminetetraacetic acid (EDTA) or glucoheptonate (GH), and the reaction time, were considered. Figures 1 and 2 show typical reversed-phase high-performance liquid chromatography (RP-HPLC) and thin-layer radiochromatography (TLC) of $^{99m}$Tc-$N_2S_2$ and Re-$N_2S_2$. The RP-HPLC retention time of $^{99m}$Tc-$N_2S_2$ (8.8 min) was similar to that of $^{185/187}$Re-$N_2S_2$ (8.6 min) verified by MS, IR, elemental analysis, and proton nuclear magnetic resonance ($^1$H-NMR). These results, along with previous studies [15,16], supported that the $^{99m}$Tc-$N_2S_2$ would possess the chemical structure shown in Scheme 1.




(**a**) (**b**)

**Figure 1.** Reversed-phase high-performance liquid chromatography (HPLC) radio-ultraviolet (UV) chromatograms: (**a**) $^{99m}$Tc-$N_2S_2$; (**b**) Re-$N_2S_2$.

Figure 3 shows the results from a preliminary experiment as a function of reaction parameters. $^{99m}$Tc-$N_2S_2$ was obtained in high radiochemical yields only when the reaction was conducted in the presence of EDTA. The reaction did not proceed without EDTA, while the presence of GH in place of EDTA resulted in low radiochemical yields (Figure 3a). The increase in EDTA concentration in the reaction mixture increased the radiochemical yields of $^{99m}$Tc-$N_2S_2$ (Figure 3b). The radiochemical yields of $^{99m}$Tc-$N_2S_2$ decreased as the reaction pH increased from pH = 5.5 to 7.5. However, the radiochemical yields reached similar to one another at 30 min (Figure 3c).

Figure 4 shows the radiochemical yields of $^{99m}$Tc-$N_2S_2$ under the optimal conditions in the presence or absence of an IgG equimolar amount to that of Zn-$N_2S_2$. No significant differences were observed in the radiochemical yields of $^{99m}$Tc-$N_2S_2$ between the two experimental conditions.

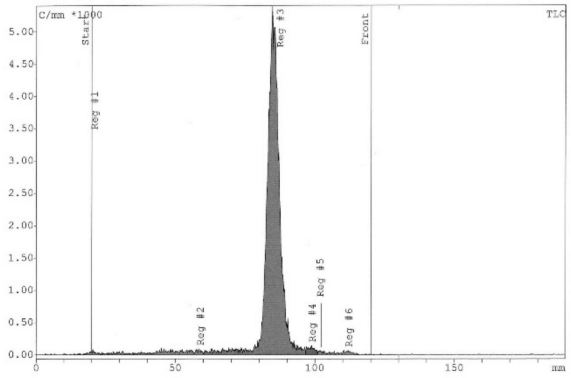
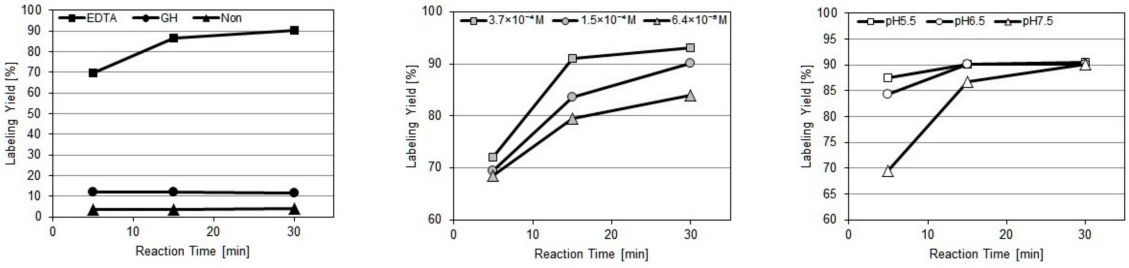



**Figure 2.** Thin-layer radiochromatography (TLC) of $^{99m}$Tc-$N_2S_2$.




(**a**) (**b**) (**c**)

**Figure 3.** The radiochemical yields of $^{99m}$Tc-$N_2S_2$ as a function of (**a**) ethylenediaminetetraacetic acid (EDTA) or glucoheptonate (GH) (pH = 7.5, Zn-$N_2S_2$: $1.0 \times 10^{-5}$ M, EDTA: $1.5 \times 10^{-4}$ M, GH: $1.5 \times 10^{-4}$ M); (**b**) EDTA concentration (pH = 7.5, Zn-$N_2S_2$: $1.0 \times 10^{-5}$ M); (**c**) reaction pH (Zn-$N_2S_2$: $1.0 \times 10^{-5}$ M, EDTA: $1.5 \times 10^{-4}$ M).

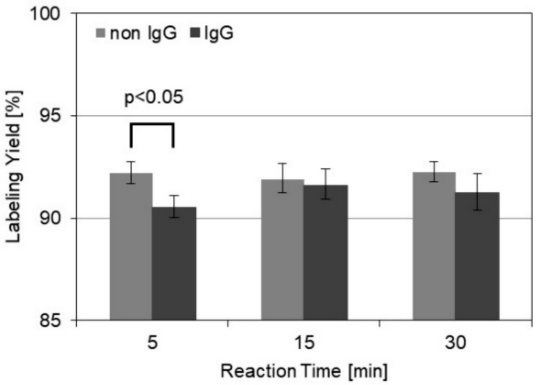



**Figure 4.** The radiochemical yields of $^{99m}$Tc-$N_2S_2$ in the presence or absence of an equimolar amount of IgG (EDTA: $1.5 \times 10^{-4}$ M, pH = 5.5, Zn-$N_2S_2$: $1.0 \times 10^{-5}$ M, IgG: 0, or $1.0 \times 10^{-5}$ M).

## 3. Discussion

$^{99m}$Tc radiopharmaceuticals are usually prepared under sterile conditions by mixing a solution of $^{99m}TcO_4^-$ with a kit formulation consisting of a ligand and a reducing agent. The in situ deprotection of $N_2S_2$ ligand and subsequent $^{99m}$Tc complexation reaction is preferable for clinical applications. In this study, the one-pot synthesis of $^{99m}$Tc-$N_2S_2$ from Zn-$N_2S_2$ was investigated at room temperature for the applications to heat-sensitive molecules. The Zn-$N_2S_2$ concentration of $1.0 \times 10^{-5}$ M was selected for future applications to imaging the saturable systems of the body [16].

Since the direct reaction of $^{99m}TcO_4^-$ and Zn-$N_2S_2$ in the presence of a reducing agent, $Sn^{2+}$, failed to produce $^{99m}$Tc-$N_2S_2$ (Figure 3a), GH was added to the reaction mixture to stabilize the

pentavalent $^{99m}TcO^{3+}$ against hydrolysis [16]. Under the conditions, small amounts of $^{99m}$Tc-$N_2S_2$ (ca. 10%) were obtained with unchanged radiochemical yields with the reaction time (Figure 3a). These results suggested that GH might rather act as a weak demetallation agent to produce $N_2S_2$ from Zn-$N_2S_2$. The concentration of GH was 15-times higher than that of Zn-$N_2S_2$. The stability constant for Zn-GH was assumed to be close to the stability constant for Zn-gluconic acid (1.70) [17], considering the similar chemical structures and acid deprotonation constants between GH and gluconic acid [18]. Thus, a deprotection agent of higher stability constant with Zn ion was then investigated to assess the working hypothesis.

To facilitate the removal of Zn ion from Zn-$N_2S_2$, EDTA was selected due to its high stability constant of 16.44 with Zn ion [19]. As shown in Figure 3a, over 90% radiochemical yields were achieved under the Zn-$N_2S_2$ concentration of $10^{-5}$ M at room temperature for 30 min. The radiochemical yields of $^{99m}$Tc-$N_2S_2$ increased with an increase in the EDTA concentration (Figure 3b), indicating that the demetallation of Zn from Zn-$N_2S_2$ constituted the rate-determining step for the synthesis of $^{99m}$Tc-$N_2S_2$ from Zn-$N_2S_2$. It should be noted that EDTA also forms a complex with the trivalent Tc [20,21]. However, since much higher EDTA concentrations (ca. $>10^{-3}$ M) are needed to prepare $^{99m}$Tc-EDTA [20,21], EDTA acted as a demetallation agent to generate $N_2S_2$ from Zn-$N_2S_2$ under the present reaction conditions. These results also indicated that $N_2S_2$ preferentially provided its $^{99m}$Tc complex in high radiochemical yields under the presence of higher concentrations of labile chelating molecules. Indeed, $^{99m}$Tc-$N_2S_2$ was obtained in high radiochemical yields in the presence of an equimolar amount of IgG, as shown in Figure 4. The formation of $^{99m}$Tc-$N_2S_2$ under a wide range of reaction pH rendered Zn-$N_2S_2$ applicable to a variety of biomolecules of interest (Figure 3c). The gathered findings indicate that the Zn-$N_2S_2$ constitutes a useful precursor to prepare a variety of $^{99m}$Tc-$N_2S_2$-based radiopharmaceuticals at low Zn-$N_2S_2$ concentrations under mild reaction conditions by a simple procedure.

## 4. Materials and Methods

### *4.1. Materials*

All chemicals and an antibody were reagent grade and used without further purification. The pertechnetate-99m solution was obtained from a commercial $^{99}$Mo-$^{99m}$Tc Generator (Ultra-Techne Kow, FUJIFILM Toyama Chemical Co., Ltd., Tokyo, Japan).

### *4.2. Equipment*

Radiochemical purities were determined with a Radio-Thin Layer Chromatography (TLC) Analyzer (GITA-STAR, Elysia-RAYTEST, Straubenhardt, Germany). High-performance liquid chromatography (HPLC) analyses were performed using a SHIMADZU model LC-20AD (Kyoto, Japan).

### *4.3. Syntheses*

The $N_2S_2$ ligand, 1,1′-(ethane-1,2-diylbis(azanediyl))bis(2-methylpropane-2-thiol) dihydro-chloride, was synthesized according to the procedure of Ohmomo et al. [22].

Zn complex of $N_2S_2$ ligand: Under an argon atmosphere, 1,1′-(ethane-1,2-diylbis-(azanediyl))bis(2-methylpropane-2-thiol) dihydrochloride 50.0 mg (0.162 mmol) and anhydrous sodium acetate 19.1 mg (0.233 mmol) were dissolved in $H_2O$ (1.3 mL) at room temperature. A mixture of Zn(II) aceteate dehydrate 59.1 mg (0.269 mmol) and anhydrous sodium acetate 19.0 mg (0.232 mmol) in 1.3 mL of $H_2O$ was added dropwise to the solution. The reaction mixture was stirred at room temperature for 1.5 h. The solution was extracted with 10 mL of dichloromethane three times. The organic solution was dried over $Na_2SO_4$. After filtration, the filtrate was evaporated, and the residue was recrystallized from chloroform and hexane to afford Zn-$N_2S_2$ as a white powder. Yield 43.4 mg (89.4%). ESI-MS, $C_{10}H_{22}N_2S_2Zn$ $[M + H]^+$ *m/z* 299.06, found: 299.11. Anal. $C_{10}H_{22}N_2S_2Zn{\cdot}0.6Na_2SO_4$: C, 31.19; H, 5.76; N, 7.28%, found: C, 31.59; H, 5.75; N, 7.09%.

Oxorhenium(V) complex of $N_2S_2$ ligand: The $N_2S_2$ ligand 62.3 mg (2.01 mmol) in 7 mL of 50% (*v*/*v*) ethanol/water was added to a solution of potassium perrhenate 58.2 mg (0.201 mmol) in 30 mL of 50% (*v*/*v*) ethanol/water. Tin(II) chloride dehydrate 45.5 mg (0.202 mmol) in 7 mL of 50% (*v*/*v*) ethanol/water was then added to the mixture, and the reaction mixture was stirred at room temperature for 2 h. After removing ethanol by evaporation, an aqueous solution of 1 M potassium carbonate was added to bring the reaction pH neutral. The solution was extracted with 100 mL of ethyl acetate three times. The organic solution was dried over $Na_2SO_4$. After filtration, the filtrate was evaporated to dryness. The residue was purified by silica gel chromatography eluted with a mixture of dichloromethane and methanol (95:5) to provide a purple crystal of ReO-$N_2S_2$. Yield 16.8 mg (19.2%). ESI-MS, Mass $C_{10}H_{21}N_2OReS_2$ $[M + H]^+$ *m*/*z* 437.07, found 437.19. Anal. $C_{10}H_{21}N_2OReS_2$: C, 27.57; H, 4.86; N, 6.43%, found: C, 27.34; H, 4.73; N, 6.22%. IR (KBr) 920 $cm^{-1}$ (Re=O). $^1$H-NMR (400 MHz, $CD_3OD$) δ1.46 (s, 6H), 1.60 (s, 3H), 1.80 (s, 3H), 2.99 (br, 2H), 3.37 (br, 2H), 3.76 (br, 4H).

### *4.4. Technetuim-99m Complexatoin Reaction*

All the solutions were bubbled with a stream of $N_2$ before use. A 1-mL solution of pertechnetate-99m ($^{99m}TcO_4^-$, 581 ± 185 MBq) was added to 0.5 mL of 0.2 M of sodium phosphate buffer (pH = 5.5, 6.5 and 7.5) and 0.1 mL of Zn-$N_2S_2$ (6.39, 38.3 and 230 μg) ethanol solution. Then, 0.5 mL of $SnCl_2{\cdot}2H_2O$ ($2.1 \times 10^{-4}$ M) solution or $SnCl_2{\cdot}2H_2O$ ($2.1 \times 10^{-4}$ M) containing a transfer ligand (EDTA·2Na·$2H_2O$ $6.4 \times 10^{-4}$ M or GH sodium salt $6.4 \times 10^{-4}$ M) aqueous solution was added to the mixture. The mixture was stood at room temperature for 5, 15, and 30 min. The effect of EDTA concentrations (1.0, 6.1, and $37 \times 10^{-5}$ M) on the radiochemical yields was also evaluated.

The radiochemical yields of $^{99m}$Tc-$N_2S_2$ were also determined in the presence of an equimolar amount of IgG at pH = 5.5 in the presence of $1.5 \times 10^{-4}$ M of EDTA. The concentration of Zn-$N_2S_2$ and the IgG were $1.0 \times 10^{-5}$ M.

### *4.5. Measurement of Radiochemical Yields of $^{99m}$Tc-$N_2S_2$*

Radiochemical yields of $^{99m}$Tc-$N_2S_2$ were determined by TLC method with the C18 reversed-phase TLC plate (NAGEL RP-18W/UV254) eluted with a mixture of acetone and 0.5 M of ammonium acetate (65:35). Under the conditions, $^{99m}$Tc-$N_2S_2$ had a Rf value of 0.6–0.7, while the Rf values of $^{99m}TcO_4^-$ and $^{99m}TcO_2{\cdot}nH_2O$ were 0.9 to 1.0 and 0, respectively.

### *4.6. Characterization of $^{99m}$Tc-$N_2S_2$ and Re-$N_2S_2$*

The HPLC retention time of $^{99m}$Tc-$N_2S_2$ and Re-$N_2S_2$ was compared with SHISEIDO CAPCELL PAK UG120 (5 μm, 4.6 × 150 mm) eluted with 45% (*v*/*v*) aqueous methanol at 40 °C under a flow rate of 1 mL/min.

### *4.7. Statistical Analysis*

Results were statistically analyzed using EXSUS Version 8.1. Differences were considered statistically significant when $p$ was $< 0.05$. A Shapiro–Wilk test was used to determine normality. When data were normally distributed for two groups, a student's *t*-test was used. If that was not the case, the nonparametric Wilcoxon's rank sum test was used.

## 5. Conclusions

The Zn-$N_2S_2$ was easily synthesized, remained stable under aerobic conditions, and provided $^{99m}$Tc-$N_2S_2$ in high radiochemical yields under a mild one-pot reaction at the Zn-$N_2S_2$ concentration of $10^{-5}$ M. The formation of $^{99m}$Tc-$N_2S_2$ was not hindered by the presence of labile chelators, such as EDTA and an IgG antibody. The gathered findings would facilitate the development of cost-effective kit formulations for $^{99m}$Tc-radiopharmaceuticals using the $N_2S_2$ ligand as the chelating moiety.

**Author Contributions:** S.O. conceived, designed, performed experiments on syntheses of Zn, Re-$N_2S_2$ and $^{99m}$Tc labeling, wrote paper; T.U. performed experiments on synthesis of $N_2S_2$ and Zn-$N_2S_2$, revised the paper; H.S. performed experiments on synthesis of $N_2S_2$; M.K.-S. performed statistical analysis on $^{99m}$Tc-$N_2S_2$ labeling with non IgG and IgG; A.H. designed, revised the paper; Y.A. participated in the design and execution of the studies, and critically revised the paper. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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**Sample Availability:** Samples of the compounds are not available from the authors.