Characterization of Technetium-99m Complexes of Pentane-2,4-dione Bis(N-methylthiosemicarbazone)

Yasushi Arano,1, Masashi Yabuki,1 Alun G. Jones,2 and Akira Yokoyama3,4

Department of Radiopharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University,6 Sakyo-ku, Kyoto 606, Japan and Department of Radiology, Harvard Medical School,6 Boston, 02115, MA, U.S.A. Received June 19, 1990

Further characterization of the two neutral technetium-99m (99mTc) complexes of pentane-2,4-dione bis(N-methylthiosemicarbazone) (PETS) was carried out using a new dianionic PETS derivative, 3,3-dimethyl-pentane-2,4-dione bis(N-methylthiosemicarbazone) (DM-PETS), and the well characterized 99mTc complex of 2,2,9,9-tetramethyl-4,7-di-a-1,10-decanedithiol (DADT) as references. While PETS generated two neutral 99mTc complexes, 99mTc-PETS-L1, and 99mTc-PETS-L2, by both the stannous reduction method and the ligand exchange reaction with six-coordinated 99mTc(V) complex of N,N′-ethylenebis(acetylacetonio)imine, DM-PETS formed only one neutral 99mTc complex. 99mTc-DM-PETS-L2, the more lipophilic complex of the two 99mTc-PETS, was obtained with a much higher yield than 99mTc-PETS-L1 by the ligand exchange reaction of PETS with the five-coordinated 99mTc(V) complex of glucoheptonate. In addition, while 99mTc-PETS-L2 and 99mTc-DADT remained unchanged in the presence of CN− anions, a breakdown of the original complexes was observed in 99mTc-PETS-L1, and 99mTc-DM-PETS. All four 99mTc complexes exhibited similar brain, heart and pancreas extraction when injected into mice. These cumulative results imply that 99mTc-PETS-L1 and 99mTc-DM-PETS are six-coordinated mononuclear 99mTc(V) complexes and that 99mTc-PETS-L2 is a five-coordinated mononuclear 99mTc(V) complex. These results also suggest that while the chelate ring structure of the 99mTc-dithiosemicarbazone (DTS) chelate played a significant role in its stability, ionization of the third proton of the PETS molecule and the subsequent resonating structure afforded further stability to the 99mTc-PETS complex. Markedly high lipophilicity of the 99mTc-PETS-L2 may also be explained by assuming that the chelate ring of the 99mTc-PETS-L2 is the five-coordinated resonating structure.

Keywords Technetium-99m; radiopharmaceutical; dithiosemicarbazone; octahedral; square-pyramidal; stability; lipophilicity

Introduction

We previously reported that a new dithiosemicarbazone (DTS) ligand, pentane-2,4-dione bis(N-methylthiosemicarbazone) (PETS), holding a 5–6–5 membered chelate ring structure, yielded two neutral technetium-99m (99mTc) complexes of different lipophilicity. Both complexes exhibited much higher stability and lipophilicity than those of the 5–5–5 membered 99mTc-DTS chelate, as well as rapid extraction in the brain and heart, when injected into mice.11 In the present study, the PETS derivative, 3,3-dimethylpentane-2,4-dione bis(N-methylthiosemicarbazone) (DM-PETS) was synthesized. While PETS can ionize up to three protons upon technetium coordination due to its diverse resonating structure, DM-PETS was designed to release only two protons in spite of having the same coordination geometry as that of PETS. DM-PETS also differs from diacetyl bis(N-methylthiosemicarbazone) (DA-DS-DTS) in its chelate ring size, although it has the same number of ionized protons upon technetium coordination. Typical resonating structures of the three ligands are shown in Fig. 1. After the 99mTc labeling of PETS and DM-PETS, in vitro characteristics and in vivo behaviors of the 99mTc complexes were investigated using the well characterized 99mTc complex of 2,2,9,9-tetramethyl-4,7-di-a-1,10-decanedithiol (DADT) (Fig. 2) as a reference. Factors influencing the stability of 99mTc-DTS complexes and the relation between the two 99mTc-PETS complexes were discussed.

Materials and Methods

Experimental Chemistry All chemicals were of reagent grade and were used as received. Organic compounds were characterized by their melting point, proton nuclear magnetic resonance (H-NMR) and elemental analysis. Nuclear magnetic resonance (NMR) spectra were taken by Bruker AC-300 using tetramethylsilane as an internal standard. DADT was synthesized according to the procedure of Chiari et al.21 Glucosac kits, containing glucoheptonate (200 mg) and SnCl2 (0.06 mg), were purchased from NEN/DuPont. Analysis of 99mTc labeling reactions was performed using high performance liquid chromatography (HPLC) utilizing a reverse phase column (Cosmosil C 18, 4.6×150 mm, Nacalai Tesque, Japan) and an inflow system comprised of a radiodetector.

Fig. 1. Typical Resonating Structures of PETS (Top), DM-PETS (Bottom, Left) and DA-DS-DTS (Bottom, Right)

While PETS can ionize up to three protons, DM-PETS and DA-DS-DTS can ionize two protons. PETS and DM-PETS form a 5–6–5 membered chelate and DA-DS-DTS forms a 5–5–5 membered chelate with technetium.

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Elution was carried out with a mixture of CH$_3$CN and 5mM NH$_4$OAc (1:1) at a flow rate of 1ml/min.

Synthesis of 3,3-Dimethyl-pentane-2,4-diene Bis(N-methylthiosemicarbazone) (DM-PETS) The precursor, 3,3-dimethyl-pentane-2,4-diene was synthesized according to the procedure of Bloomfield in a 41% yield (bp 168-172°C, 0.3 mmHg). 3,3-Dimethyl-pentane-2,4-diene (0.64 g, 5 mmol) in ethanol (80 ml) was added dropwise to a solution of N-methylthiosemicarbazide (1.16 g, 11 mmol) in 0.1N HCl (80 ml) at room temperature then kept stirring overnight. The precipitated crystals were collected and washed with H$_2$O and cold ethanol. The product (0.15 g) was obtained as a white crystal (99% yield), mp 163-165°C. Anal. Calc for C$_7$H$_{12}$N$_2$S$_2$: C, 43.68; H, 7.33; N, 27.79. Found: C, 43.52; H, 7.45; N, 27.59. 1H-NMR (DMSO-d$_6$): ppm: 1.06 (6H, d, CH$_3$), 1.34 (3H, s, CH$_3$), 1.93 (3H, s, CH$_3$), 2.94 (6H, d, NH$_2$CH$_3$), 5.78 (1H, s, NH), 7.88 (1H, s, NH), 8.38 (2H, d, NH$_2$CH$_3$).

Prepulized 99mTc Complexes 99mTc-PETS complexes were synthesized by the stannous reduction method according to the procedure as previously described. Two isolated 99mTc-PETS complexes with HPLC retention times of 9.7 and 11.6 min were abbreviated as 99mTc-PETS-L$_1$ and 99mTc-PETS-L$_2$, respectively. 99mTc-DM-PETS was synthesized by the stannous reduction method as follows: to a 1mM solution of DM-PETS (10$^{-3}$ M) prepared in 0.1M phosphate buffer (pH 8.0) was added 0.5mL of 99mTcO$_4^-$ (18.5-37 MBq) and 10µL of freshly prepared stannous chloride (2.2 x 10$^{-2}$ M in 0.1N HCl). The reaction mixture was heated at 85°C for 100 min. The product was then separated by HPLC, followed by extraction with 3mL of ethyl acetate. After evaporating the organic solvent under a stream of N$_2$, the residue dissolved in saline to give a DM-PETS concentration of 2.4 x 10$^{-4}$ M.

99mTc-DADT was synthesized according to the procedure of Lever et al. as follows: to DADT ligand (2µg) dissolved in 1mM of 0.1M phosphate buffer (pH 7.0) was added 0.5mL of 99mTcO$_4^-$ (12.1-18.5 MBq), followed by the addition of 10µL of SnCl$_2$ (1.33 x 10$^{-4}$ M) in ethanol. After stirring at room temperature for 10 min, the reaction mixture was extracted with 5mL of CHCl$_3$, and the CHCl$_3$ layer was dried over anhydrous sodium sulfate. The filtered solution was condensed to dryness under a stream of N$_2$. The residue was then dissolved in saline to give a DADT concentration of 2.4 x 10$^{-4}$ M.

Ligand Exchange Reaction To a 1mM solution of PETS, DM-PETS (1 x 10$^{-3}$ M) in 0.1M phosphate buffer, pH 8.0 (or DADT 3 x 10$^{-3}$ M in the same buffer), was added a 1mL solution of 99mTc complex of N,N'-ethylenebis(acetylaceetone) (99mTc-en) in CH$_2$Cl$_2$, prepared according to the procedure of Deutsch et al. After evaporating CH$_2$Cl$_2$ under a stream of N$_2$, the reaction mixture was heated at 85°C for 15 min, extracted with hexane, then analyzed by HPLC.

Protein Binding The relative plasma protein binding of 99mTc-PETS-L$_1$, 99mTc-PETS-L$_2$, 99mTc-DM-PETS and 99mTc-DADT was compared by the trichloroacetic acid (TCA) precipitation method. Fifty microliter solutions of 99mTc complexes were added to 500µL of mouse plasma, and incubated at 37°C for 10 min, after which 1mL of saline and 1mL of 5% TCA were added. After centrifugation at 3000g for 15 min, supernatant was aspirated, and the residue was washed with 1mL of saline and 1mL of 5% TCA, then centrifuged. The relative plasma protein binding of each 99mTc complex was determined by calculating the ratio of counts per minute of precipitate to that of total radioactivity.

Mice Distribution Studies Biodistribution of 99mTc-PETS-L$_1$ and 99mTc-PETS-L$_2$ in mice was compared with that of 99mTc-DM-PETS and 99mTc-DADT. Fifty microliter of 99mTc complexes were injected into ddY male mice (5 weeks old) from the lateral tail vein. At appropriate times after injection, the mice were decapitated and blood samples were collected. The organs of interest were excised, weighed, and the radioactivity was counted.

Results

Preparation of DM-PETS DM-PETS was synthesized by the alkylation of the precursor, pentane-2,4-diene, in the presence of sodium hydride, followed by the condensation reaction of the resulting 3,3-dimethylpentane-2,4-diene with N-methylthiosemicarbazide in the presence of an acid catalyst.

Preparation of 99mTc Complexes Radiochemical purities of 99mTc-PETS-L$_1$, 99mTc-PETS-L$_2$, 99mTc-DM-PETS and 99mTc-DADT were determined to be over 98% by HPLC analysis. The HPLC retention time of the four 99mTc complexes is shown in Table I.

In Vitro Studies Cellulose acetate electrophoresis of 99mTc-PETS-L$_1$, 99mTc-PETS-L$_2$, 99mTc-DM-PETS and 99mTc-DADT showed a single peak at the origin, and no further 99mTc radioactivity was observed.

The stability of 99mTc-DM-PETS in mouse plasma is shown in Fig. 3. HPLC analysis of the 5min incubation sample showed a slight decrease of the original radioactivity. After this, however, 99mTc-DM-PETS remained unchanged during the following 3h incubation as well as after the addition of fresh plasma.

In Fig. 4, HPLC radioactivity profiles of the lipid exchange reactions of PETS with 99mTc-(en) and 99mTc-GH are shown. When PETS was reacted with 99mTc-(en), two radioactive peaks with the same retention time as those of 99mTc-PETS-L$_1$ and 99mTc-PETS-L$_2$ were observed with almost the same radiochemical yields. On the other hand, when 99mTc-GH was reacted with PETS, radioactivity corresponding to that of 99mTc-PETS-L$_2$ was obtained as a major component and that of 99mTc-PETS-L$_1$ was observed as only a minor peak (Fig. 4). Radioactivity peaks corresponding to those of the stannous reduction method were also observed by the exchange reaction of DM-PETS and DADT with both 99mTc-(en) and 99mTc-GH.

HPLC profiles of the four 99mTc complexes in the presence of CN$^-$ anions are shown in Fig. 5. The monodentate ligand, CN$^-$, altered the HPLC radioactivity traces of 99mTc-PETS-L$_1$ and 99mTc-DM-PETS. On the other hand, HPLC radioactivity traces of 99mTc-PETS-L$_2$ and 99mTc-
TABLE I. Comparative Lipophilicity of the Four $^{99m}$Tc Complexes

<table>
<thead>
<tr>
<th></th>
<th>PC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protein binding&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HPLC retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{99m}$Tc-PETS-L&lt;sub&gt;1&lt;/sub&gt;</td>
<td>284.4 (41.9)</td>
<td>259.1 (32.5)</td>
<td>269.3 (49.9)</td>
</tr>
<tr>
<td>$^{99m}$Tc-PETS-L&lt;sub&gt;2&lt;/sub&gt;</td>
<td>841.2 (82.0)</td>
<td>820.0 (63.1)</td>
<td>791.7 (79.5)</td>
</tr>
<tr>
<td>$^{99m}$Tc-DM-PETS</td>
<td>321.6 (19.1)</td>
<td>327.2 (16.4)</td>
<td>321.7 (7.9)</td>
</tr>
<tr>
<td>$^{99m}$Tc-DADT</td>
<td>82.1 (3.5)</td>
<td>81.2 (3.2)</td>
<td>82.6 (5.6)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Partition coefficient: octanol/0.1 M phosphate buffer. Mean (S.D.) for three experiments.  
<sup>b</sup> % serum protein binding. Mean (S.D.) for three experiments.

TABLE II. Biodistribution of the Four $^{99m}$Tc Complexes in Mice<sup>c</sup>

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>$^{99m}$Tc-PETS-L&lt;sub&gt;1&lt;/sub&gt;</th>
<th>$^{99m}$Tc-PETS-L&lt;sub&gt;2&lt;/sub&gt;</th>
<th>$^{99m}$Tc-DM-PETS</th>
<th>$^{99m}$Tc-DADT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5</td>
<td>15</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Brain</td>
<td>3.44 (0.45)</td>
<td>2.07 (0.26)</td>
<td>0.84 (0.09)</td>
<td>4.92 (0.35)</td>
</tr>
<tr>
<td>Heart</td>
<td>5.32 (0.66)</td>
<td>3.43 (0.34)</td>
<td>1.80 (0.49)</td>
<td>6.35 (0.62)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>6.35 (0.63)</td>
<td>3.69 (1.15)</td>
<td>1.96 (0.24)</td>
<td>7.93 (0.82)</td>
</tr>
<tr>
<td>Blood</td>
<td>2.57 (0.29)</td>
<td>1.98 (0.16)</td>
<td>1.52 (0.21)</td>
<td>3.22 (0.31)</td>
</tr>
</tbody>
</table>

<sup>c</sup> % injected dose per gram tissue. Mean (S.D.) for five mice.

Fig. 3. Plasma Stability of $^{99m}$Tc-DM-PETS
$^{99m}$Tc-DM-PETS was incubated at 37°C in murine plasma. At various intervals, a plasma sample was withdrawn and deproteinized. The percent of radioactivity remaining as intact chelate was determined by HPLC.

Fig. 4. HPLC Elution Profiles of Radioactivity
The profile after hexane extraction of the reaction mixture of PETS and $^{99m}$Tc-(en) (left), and PETS and $^{99m}$Tc-GH (right). Formation of $^{99m}$Tc-PETS-L<sub>1</sub> and $^{99m}$Tc-PETS-L<sub>2</sub> was demonstrated by HPLC analysis.

DADT remained unchanged under the same reaction conditions.

The PC value, relative plasma protein binding and HPLC retention time of the four $^{99m}$Tc complexes are summarized in Table I. PCs between pH 7.0 and pH 8.0 were essentially the same for all four $^{99m}$Tc complexes. $^{99m}$Tc-PETS-L<sub>2</sub> showed extremely high lipophilicity; the PC value of this complex was 3 times higher than that of $^{99m}$Tc-PETS-L<sub>1</sub> and $^{99m}$Tc-DM-PETS, and 10 times higher than that of $^{99m}$Tc-DADT. The plasma protein binding of these complexes was proportional to their PC values.

Biodistribution in Mice In Table II, biodistribution of the four $^{99m}$Tc complexes in mice is shown. All $^{99m}$Tc complexes exhibited rapid radioactivity accumulation in
the brain, heart and pancreas with gradual clearance over time. The amount of radioactivity accumulated in these organs was virtually proportional to the levels found in the blood.

Discussion
Since our development of a $^{99m}$Tc complex that crosses the intact blood-brain-barrier (BBB), synthesis and characterization of technetium complexes reflecting cerebral blood flow have been carried out by many research groups.\(^{2,7-9}\) All characterized technetium complexes that cross the BBB are neutral, lipophilic and relatively small (< 500 daltons) mononuclear complexes with a pentavalent TcO$^{5+}$ core.\(^{7,8}\) The $^{99m}$Tc–DADT complex, used as a reference in this study, is in this category (Fig. 2).\(^{7,9}\) Technetium complexes of N$_2$S$_2$ ligands other than DADT have also been characterized; they are also pentavalent mononuclear complexes containing a TcO$^{5+}$ core.\(^{10-12}\) Both PETS and DM-PETS have N$_2$S$_2$ donors for technetium coordination (Fig. 1) and the formation of $^{99m}$Tc–PETS-L$_1$, $^{99m}$Tc–PETS-L$_2$ and $^{99m}$Tc–DM-PETS was indicated by the reaction of PETS or DM-PETS with the pentavalent $^{99m}$Tc complexes of $^{99m}$Tc–(en) and $^{99m}$Tc–GH (Fig. 4).\(^{5,13}\) It is, therefore, most likely that $^{99m}$Tc–PETS-L$_1$, $^{99m}$Tc–PETS-L$_2$ and $^{99m}$Tc–DM-PETS are pentavalent mononuclear technetium complexes. This is supported by the similar in vivo behavior of $^{99m}$Tc–PETS-L$_1$, $^{99m}$Tc–PETS-L$_2$ and $^{99m}$Tc–DM-PETS to that of $^{99m}$Tc–DADT (Table II).

DM-PETS generated one neutral $^{99m}$Tc complex with high stability (Fig. 3) and lipophilicity (Table I). $^{99m}$Tc–DM-PETS also exhibited brain extraction when injected into mice (Table II). While DM-PETS and PETS form a 5–6-5 membered chelate with $^{99m}$Tc, DM-PETS acts, as DA-€DTS does, as a diatomic tetradentate ligand (Fig. 1). These results clearly indicate that the increased chelate ring structure from 5–5 to 5–6–5 played a significant role in the improved stability of the $^{99m}$Tc–DTS chelate. Formation of only one neutral $^{99m}$Tc complex of DM-PETS implies the presence of the third ionizable proton in the PETS molecule to be responsible for the two neutral $^{99m}$Tc complex formations.

Two different structures have been reported as being mononuclear pentavalent technetium complexes of N$_2$S$_2$ ligands; a five-coordinated square-pyramidal structure (Fig. 2) and a six-coordinated octahedral structure.\(^{11}\) As shown in Fig. 4, the six-coordinated $^{99m}$Tc–(en) and the five-coordinated $^{99m}$Tc–GH afforded $^{99m}$Tc–PETS-L$_1$ and $^{99m}$Tc–PETS-L$_2$ with different radiochemical yields.\(^{5,13}\) A difference between $^{99m}$Tc–PETS-L$_1$ and $^{99m}$Tc–PETS-L$_2$ was also observed in their lipophilicity and the reactivity toward CN$^-$ ions, as shown in Table I and Fig. 5. These results imply a structural difference between $^{99m}$Tc–PETS-L$_1$ and $^{99m}$Tc–PETS-L$_2$ and suggest $^{99m}$Tc–PETS-L$_2$ is the five-coordinated square-pyramidal structure.

Since DM-PETS acts as a diatomic ligand, coordination of an additional monodentate anion to the $^{99m}$TcO$_{5+}$ core is necessary for the neutral complex formation (Fig. 1). Present studies of $^{99m}$Tc–DM-PETS as well as a report of the technetium complex of pentane-2,4-dione bis(S-methyl-dithiocarbazate) support the hypothesis that $^{99m}$Tc–DM-PETS is the six-coordinated octahedral structure.\(^{11}\) An altered HPLC $^{99m}$Tc–DM-PETS profile by the presence of CN$^-$ anions, when compared with the unchanged HPLC profile of the five-coordinated square-pyramidal structure of $^{99m}$Tc–DADT, also implies a structural difference between $^{99m}$Tc–DM-PETS and $^{99m}$Tc–DADT. Comparative in vitro studies indicate the resemblance of $^{99m}$Tc–DM-PETS to $^{99m}$Tc–PETS-L$_1$, rather than to $^{99m}$Tc–PETS-L$_2$ (Table I, Fig. 5).

The data imply that the structure of $^{99m}$Tc–PETS-L$_1$ is a six-coordinated pentavalent mononuclear $^{99m}$Tc complex and that $^{99m}$Tc–PETS-L$_2$ is a five-coordinated pentavalent mononuclear $^{99m}$Tc complex with a resonating structure, resulting from the deprotonation of the third proton from the PETS molecule.\(^{14}\) In other words, while the chelate ring structure plays a significant role in the stability of the $^{99m}$Tc–DTS chelate, ionization of the third proton of the PETS molecule and subsequent resonating of the five-coordinated structure affords further stability to the $^{99m}$Tc–PETS complex. Extremely high lipophilicity of $^{99m}$Tc–PETS-L$_2$ may be well explained by assuming that $^{99m}$Tc–PETS-L$_2$ is the five-coordinated resonating structure. Further characterization of technetium–PETS complexes is also under way using technetium-99.

References