Stable and Lipophilic Technetium-99m Dithiosemicarbazone Complexes with 5-6-5 Membered Chelate Ring Structure

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Modification of the chelate ring structure of technetium-99m (^{99m}Tc) dithiosemicarbazone (DTS) chelate was carried out in pursuit of a more stable and lipophilic compound. A new DTS chelating molecule, pentane-2,4-dione bis(*N*-methylthiosemicarbazone) (PETS), with a 5-6-5 membered chelate ring structure, was synthesized and labeled with ^{99m}Tc. PETS generated two ^{99m}Tc compounds as major products. Both had much higher stability and lipophilicity than a 5-5-5 membered ^{99m}Tc DTS compound, as well as great stability in plasma. Both ^{99m}Tc-PETS compounds were rapidly extracted by the brain and heart when injected into mice. Thus, the modified chelate ring structure afforded a preferable characteristics to DTS chelate as for the chelating site for technetium radiopharmaceuticals.

Keywords technetium-99m; radiopharmaceutical; dithiosemicarbazone; brain; heart

Introduction

Development of technetium-99m (^{99m}Tc) radiopharmaceuticals reflecting physiological or biochemical changes in various tissues is actively being investigated in diagnostic nuclear medicine. Design and synthesis of ^{99m}Tc-dithiosemicarbazone (DTS) complexes containing various functional groups have constituted our approach to this problem.[1,2] Each of the previously studied DTS derivatives, which formed a 5-5-5 membered chelating ring structure with ^{99m}Tc, closely reflected the predicted physiological or biochemical behavior of the functional group attached.[3] We have continued our studies in pursuit of more functional or target-specific ^{99m}Tc radiopharmaceuticals, and along with consideration of the attached organic functional group, reconsideration of the technetium chelating molecule became inevitable.

It was thought that the use of a new DTS ligand, pentane-2,4-dione bis(*N*-methylthiosemicarbazone) (PETS) (Fig. 1), enabling a 5-6-5 membered chelating ring structure with technetium, would provide the desired degree of stability and lipophilicity. Moreover, in the technetium chelation process, creation of a resonating molecule might also contribute to the predicted enhancement of stability and lipophilicity.[4] The synthesis of PETS and ^{99m}Tc labeling using the stannous reduction method are described in this article. The stability, lipophilicity, and biodistribution in mice of ^{99m}Tc-PETS were compared with a 5-5-5 membered ^{99m}Tc-diacetyl bis(*N*-methylthiosemicarbazone) (DA-α-DTS) complex. The newly designed PETS ligand was found to offer the stability and lipophilicity predicted, and shows promise for use in further development of ^{99m}Tc radiopharmaceuticals.

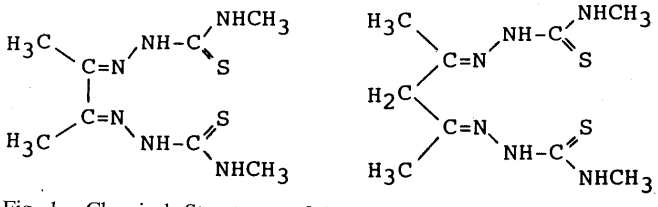

Fig. 1. Chemical Structures of Pentane-2,4-dione Bis(*N*-methylthiosemicarbazone) (PETS) (Right) and Diacetyl Bis(*N*-methylthiosemicarbazone) (DA-α-DTS) (Left)

Materials and Methods

All reagents were purchased from Nacalai Tesqu Inc. (Kyoto, Japan) and were used as received. Proton nuclear magnetic resonance (^{1}H-NMR) was carried out with a Brucker AC-300 apparatus, using tetramethylsilane as an internal standard. Analysis of ^{99m}Tc labeling reactions was performed using either thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) (Shimadzu LC-5A, Japan). TLC was performed on Silica gel 60 plates (Merck Art. 5553) using acetone as an eluent. HPLC was carried out utilizing a reverse phase column (Cosmosil C_{18}, 4 × 150 mm, Nacalai Tesqu Inc.) and an inflow system with a radiodetector, and eluted with a mixture of CH_3CN and 5 mM ammonium acetate (1 : 1) at a flow rate of 1 ml/min. [^{99m}Tc]Pertechnetate ($^{99m}TcO_4^-$) was eluted from Ultra TechneKow (Daiichi Radioisotope Labs. Inc., Japan). Ultrafiltration was carried out using the micropartition system (MSP-3) supplied by Amicon Corp.

Synthesis of Pentane-2,4-dione Bis(*N*-methylthiosemicarbazone) (PETS) PETS was synthesized according to the procedure of O'Callaghan and Twomey,[5] with modifications as follows: pentane-2,4-dione (2.5 g, 25 mmol) and *N*-methylthiosemicarbazide (5.26 g, 50 mmol) were dissolved in 50% aqueous methanol (50 ml). To this solution ethylenediamine (1.5 g, 25 mmol) was added and the reaction mixture was refluxed with stirring for 2 h. After cooling to room temperature, the separated crystals were collected and recrystallized from methanol. In this way, 1.4 g (20.4%) of PETS was obtained as white crystals, mp 187—188 °C (uncorr.). *Anal.* Calcd for $C_9H_{18}N_6S_2$: C, 39.39; H, 6.61; N, 30.63. Found: C, 39.51; H, 6.62; N, 30.89. ^{1}H-NMR (DMSO-d_6) ppm: 1.74 (3H, s, C$\underline{H}_3$), 1.97 (3H, s, C$\underline{H}_3$), 2.90 (6H, d, NHC$\underline{H}_3$), 6.41 (1H, s, N$\underline{H}$), 7.75 (1H, s, N$\underline{H}$), 8.19 (2H, d, N$\underline{H}$CH$_3$).

Synthesis of Diacetyl Bis(*N*-methylthiosemicarbazone) (DA-α-DTS) Diacetyl (0.86 g, 10 mmol) dissolved in ethanol (25 ml) was added to a solution of *N*-methylthiosemicarbazide (2.31 g, 22 mmol) in 0.1 N HCl (20 ml) at 60 °C, and the reaction mixture was stirred for an additional hour at this temperature. After cooling to room temperature, the separated pale yellow crystals were collected. It this way, 1.2 g (46.1%) of crude DA-α-DTS was obtained. Recrystallization from 50% aqueous acetic acid afforded the pure product with mp of 220 °C (uncorr.). *Anal.* Calcd for $C_8H_{16}N_6S_2$: C, 36.90; H, 6.19; N, 32.28. Found: C, 36.85; H, 6.22; N, 32.31.

^{99m}Tc Labeling The ^{99m}Tc labeling of PETS was carried out according to the following procedure: to PETS solution (1 ml, 1×10^{-3} M) prepared in 0.1 M phosphate buffer (pH 8.0) were added $^{99m}TcO_4^-$ eluate (0.5 ml, 18.5—37 MBq) and freshly prepared stannous chloride (10 μl, 2.2×10^{-2} M in 0.1 N HCl). The mixture was then heated at 85 °C for 30 min in a sealed vial, followed by extraction with 5 ml of hexane. After evaporating the organic solvent under a stream of N_2, the residue was dissolved in a mixture of CH_3CN and 5 mM ammonium acetate (1 : 1), and purified by HPLC to obtain the two major radioactive components. Each component was collected and then extracted with 3 ml of ethyl acetate; after evaporation of ethyl acetate under a N_2 stream, each component was dissolved in saline to give a ligand concentration of 2.4×10^{-4} M. Hereafter, the first eluted component is abbreviated as ^{99m}Tc-PETS-L_1, and the second as ^{99m}Tc-PETS-L_2.

The ^{99m}Tc labeling of DA-α-DTS was carried out in ethanol as previously

described.[1] In brief, DA-α-DTS in 95% ethanol (2 ml, 1×10^{-3} M) was mixed with $^{99m}TcO_4^-$ (0.5 ml, 12.1—18.5 Bq), followed by the addition of freshly prepared stannous chloride (20 μl, 5×10^{-4} M in 0.1 N HCl) in an atmosphere of N_2. After stirring for 5 min, the sample was diluted with saline to contain 50% ethanol. The labeling efficiency was determined by TLC.[1]

***In Vitro* Studies** Electrophoresis: Cellulose acetate electrophoresis was carried out in 0.1 M phosphate buffer (pH 7.0) in an electrostatic field of 0.8 mA/cm for 30 min using presoaked strips (1 × 11 cm). Under these conditions, $^{99m}TcO_4^-$ migrated 2.5 to 3.0 cm towards the anode.

Stability Determination: Freshly separated solutions of ^{99m}Tc-PETS-L_1 and ^{99m}Tc-PETS-L_2 in saline and freshly prepared ^{99m}Tc-DA-α-DTS solution were heated to 37 °C. At various time intervals, samples were withdrawn, and the percent radioactivity remaining as intact chelate in each preparation was calculated by taking the original radiochemical purities as 100% and using HPLC for both ^{99m}Tc-PETS compounds and TLC for ^{99m}Tc-DA-α-DTS.

The stability of the two ^{99m}Tc-PETS preparations in plasma was also determined; 1 ml of each preparation was mixed with 3 ml of mouse plasma and incubated at 37 °C. At various post-incubation intervals, 500 μl of each sample was withdrawn, deproteinized by ultrafiltration at 1500*g* for 30 min, and the filtrate was analyzed by HPLC. After 3 h of incubation, fresh plasma was added, incubated for 10 min, and the above procedure was repeated.

Partition Coefficient (PC) Determination: The PC of the three ^{99m}Tc compounds was measured according to the procedure of Volkert *et al.*,[6] with slight modifications as follows: 100 μl of each ^{99m}Tc compound was mixed with 3 ml each of octanol and 0.1 M phosphate buffer (pH 7.0, 7.4, and 8.0) in a test tube. After being vortexed for 30 s, each tube was centrifuged at 500*g* for 5 min. Then, 50 μl samples from each of the octanol and buffer layers were counted in a well counter. The PC value was determined by calculating the ratio of the cpm/50 μl of octanol to the cpm/50 μl of buffer. A sample from the octanol layer was repartitioned with the same volume of buffer until a constant PC value was obtained.

***In Vivo* Studies** Each ^{99m}Tc compound (50 μl) was injected into the lateral tail vein of ddY male mice (5 weeks old). At appropriate times after injection, the mice were decapitated and blood samples were collected. Organs of interest were then excised, weighed, and their radioactivity was determined.

Results

^{99m}Tc Labeling The HPLC analysis of the reaction between ^{99m}Tc and PETS detected two major ^{99m}Tc compounds, totalling about 60% yield after being extracted with hexane. Each of these compounds, ^{99m}Tc-PETS-L_1 and ^{99m}Tc-PETS-L_2 showed an identical retention time with freshly separated ^{99m}Tc-PETS (Fig. 2), even after standing at room temperature for more than 24 h. The radiochemical purity of each compound was greater than 98%.

The ^{99m}Tc-DA-α-DTS was obtained with over 80% radiochemical purity.

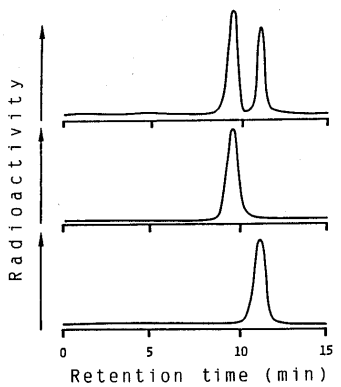

Fig. 2. HPLC Elution Profiles of Radioactivity

The radioactivity profile of ^{99m}Tc-PETS after hexane extraction of the labeled solution (top). The elution profiles of ^{99m}Tc-PETS-L_1 (middle) and ^{99m}Tc-PETS-L_2 (bottom) after purification by HPLC.

***In Vitro* Studies** Cellulose acetate electrophoresis of each ^{99m}Tc compound showed a single peak at the origin; no further ^{99m}Tc-pertechnetate or other ^{99m}Tc compounds were detected.

The stability of both ^{99m}Tc-PETS compounds and that of ^{99m}Tc-DA-α-DTS is shown in Fig. 3. While both ^{99m}Tc-PETS compounds showed unchanged HPLC elution profiles after standing at 37 °C for various time periods. TLC analysis of ^{99m}Tc-DA-α-DTS showed its gradual breakdown with time, and only 65% of the freshly prepared radioactivity remained as the intact form after 3 h.

Then, the stability of the two ^{99m}Tc-PETS compounds in murine plasma was studied, as shown in Fig. 4. HPLC analysis of the 5-min incubation sample after deproteinization showed a slight decrease of the original radioactivity; after this, however, both ^{99m}Tc-PETS compounds remained unchanged not only during the following 3 h of incubation but even after the addition of fresh plasma and subsequent incubation.

Table I shows the PC values of the three ^{99m}Tc compounds. The PC values between pH 7.0 and 8.0 were

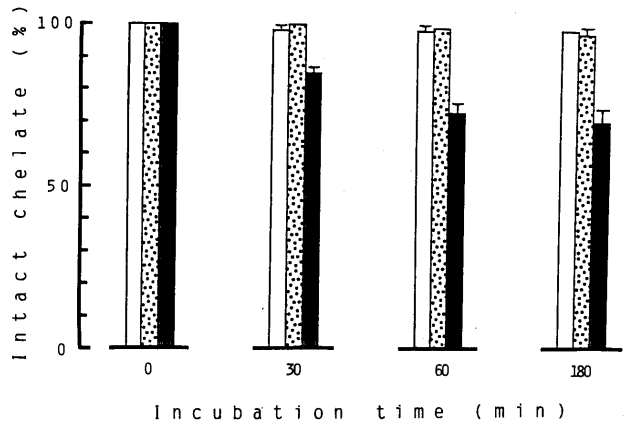

Fig. 3. Comparative Stability of ^{99m}Tc-PETS-L_1 (□), ^{99m}Tc-PETS-L_2 (▨) and ^{99m}Tc-DA-α-DTS (■)

After purification or labeling, each solution was heated to 37 °C. At various intervals, samples were withdrawn and the stability of each of the three ^{99m}Tc-DTS compounds was determined. Each value was calculated by dividing the radioactivity remaining as intact chelate at various intervals by the radiochemical purities of the freshly separated or prepared samples.

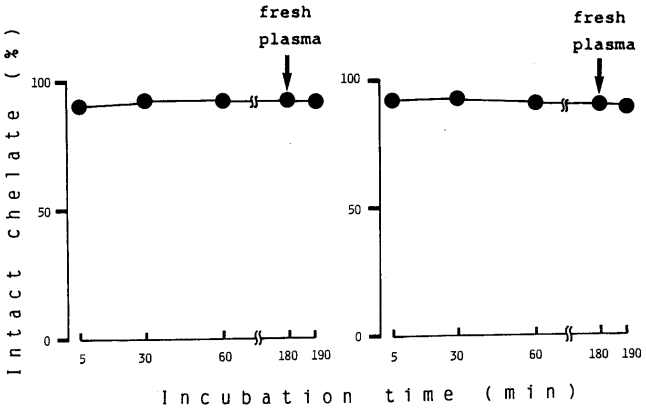

Fig. 4. Stability of ^{99m}Tc-PETS-L_1 and ^{99m}Tc-PETS-L_2 in Plasma

Each preparation was incubated at 37 °C in murine plasma. At various intervals, plasma samples were withdrawn and deproteinized. Then, the percent radioactivity remaining as intact chelate was determined by HPLC. Addition of fresh plasma after 3 h of incubation induced no radioactivity change in the two ^{99m}Tc-PETS preparations.

TABLE I. Partition Coefficient of Three ^{99m}Tc Compounds[a]

Compound	pH 7.0	7.4	8.0
^{99m}Tc-PETS-L_1	284.4 (41.9)	259.1 (32.5)	269.3 (49.9)
^{99m}Tc-PETS-L_2	841.2 (82.0)	820.0 (63.1)	791.7 (79.5)
^{99m}Tc-DA-α-DTS	5.3 (1.0)	4.7 (1.2)	4.6 (1.2)

a) Octanol/0.1 M phosphate buffer. Mean (S.D.) for three experiments.

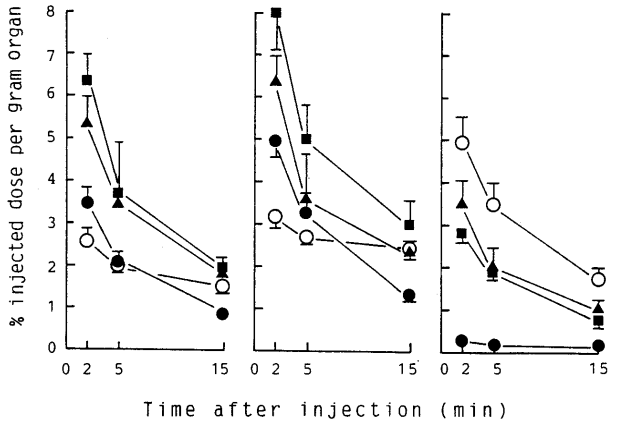

Fig. 5. Biodistribution of Radioactivity in the Brain (●), Heart (▲), Pancreas (■) and Blood (○) after Intravenous Injection of ^{99m}Tc-PETS-L_1 (Left), ^{99m}Tc-PETS-L_2 (Center) and ^{99m}Tc-DA-α-DTS (Right) in Mice

Each value represents mean and S.D. for five animals.

essentially unchanged for all the compounds. The ^{99m}Tc-PETS compounds had higher PC values than did ^{99m}Tc-DA-α-DTS, with ^{99m}Tc-PETS-L_2 showing an extremely high PC value: 3 and 160 times higher than those of ^{99m}Tc-PETS-L_1 and ^{99m}Tc-DA-α-DTS, respectively.

***In Vivo* Studies** In Fig. 5, the biodistribution of the three ^{99m}Tc compounds in the mice is presented. The amount of radioactivity accumulated in the brain, heart and pancreas followed the order of the PC data, *i.e.*, ^{99m}Tc-PETS-L_2 had the highest, followed by ^{99m}Tc-PETS-L_1, and then ^{99m}Tc-DA-α-DTS.

Discussion

Many recent efforts have been made to develop ^{99m}Tc radiopharmaceuticals reflecting physiological or biological changes in the brain and heart.[7] The present study showed that the newly designed and easily synthesized DTS ligand, PETS, had characteristics which were valuable for radiolabeling with ^{99m}Tc. Two neutral ^{99m}Tc complexes were detected and isolated by HPLC. Both compounds, designated respectively as ^{99m}Tc-PETS-L_1 and ^{99m}Tc-PETS-L_2, were stable even after being left to stand for more than 24 h at room temperature. Moreover, both exhibited much higher stability and lipophilicity than the 5-5-5 membered ^{99m}Tc-DA-α-DTS (Fig. 3, Table I), as well as great stability in plasma (Fig. 4).

These favorable properties of the PETS ligand could also be visualized in the biodistribution in mice, particularly in the high extraction detected in the brain and heart. The highest accumulation in the brain and heart was achieved by the most lipophilic compound, ^{99m}Tc-PETS-L_2 (Fig. 5). The rapid clearance from these organs suggests the biological stability and inertness of the compounds.

Thus, it appears that increasing the chelate ring structure of the DTS ligand from 5-5-5 to 5-6-5 generated a more favorable geometry for the formation of a stable technetium complex with a compact structure. Furthermore, the molecular model approach has indicated the involvement of a rather planar configuration contributed by the four N_2S_2 donors in the 5-6-5 membered PETS in contrast to the distorted 5-5-5 membered DA-α-DTS. Another factor contributing to the observed stability, lipophilicity, and tissue accumulation characteristics might be the presence of conjugated double bonds and the effect of the resulting resonating molecule. Recent studies on technetium chemistry have suggested that the two ^{99m}Tc-PETS compounds could be pentavalent technetium complexes with an octahedral and/or square-pyramidal structure.[8] Further characterization of ^{99m}Tc-PETS will be reported in a separate paper.

The very high lipophilicity and the biological inertness of the newly synthesized ^{99m}Tc-PETS appear to offer a very advantageous ligand to be further exploited as the basic structure of ^{99m}Tc radiopharmaceuticals. Moreover, the presence of a highly reactive methylene group in the PETS synthesis precursor, the pentane-2,4-dione, followed by the subsequent simple thiosemicarbazone condensation reaction involved, gives the PETS derivatives great potential for the future synthesis of ^{99m}Tc radiopharmaceuticals designed to reflect physiological or biological changes in the brain and heart.

References

1) T. Hosotani, A. Yokoyama, Y. Arano, K. Horiuchi, K. Wasaki, H. Saji, and H. Tanaka, *Int. J. Nucl. Med. Biol.*, **12**, 431 (1985).
2) Y. Arano, A. Yokoyama, Y. Magata, K. Horiuchi, H. Saji, and K. Torizuka, *Appl. Radiat. Isot.*, **37**, 587 (1986); Y. Arano, A. Yokoyama, T. Furukawa, K. Horiuchi, T. Yahata, H. Saji, H. Sakahara, T. Nakashima, M. Koizumi, K. Endo, and K. Torizuka, *J. Nucl. Med.*, **28**, 1027 (1987).
3) T. Hosotani, A. Yokoyama, Y. Arano, K. Horiuchi, H. Saji, and K. Torizuka, *Appl. Radiat. Isot.*, **37**, 505 (1986); T. Hosotani, A. Yokoyama, Y. Arano, K. Horiuchi, H. Saji, and K. Torizuka, *Nucl. Med. Biol.*, **13**, 603 (1986).
4) E. Bayer and E. Breitmaier, *Chem. Ber.*, **101**, 1579 (1968).
5) C. N. O'Callaghan and D. J. Twomey, *Chem. Soc.*, (*C*), **1967**, 2400.
6) W. A. Volkert, T. J. Hoffman, R. M. Seger, D. E. Troutner, and R. A. Holmes, *Eur. J. Nucl. Med.*, **9**, 511 (1984).
7) E. Chiotellis, A. D. Varvarigou, Th. Maini, and C. I. Stassinopoulou, *Nucl. Med. Biol.*, **15**, 215 (1988); H. F. Kung, Yu-Zhi Guo, Chi-Chou Yu, J. Billings, V. Subramanyam, and J. C. Calabrese, *J. Med. Chem.*, **32**, 433 (1989); U. Scheffel, H. W. Goldfarb, S. Z. Lever, R. L. Gungon, H. D. Burns, and H. N. Wagner, *J. Nucl. Med.*, **29**, 73 (1988).
8) A. Davison, A. G. Jones, C. Orvig, and M. Sohn, *Inorg. Chem.*, **20**, 1629 (1981); L. A. Epps, H. D. Burns, S. Z. Lever, H. W. Goldfarb, and H. N. Wagner Jr., *Appl. Radiat. Isot.*, **38**, 661 (1987); J. G. H. DuPreez, T. I. A. Ferber, and O. Knoesen, *Inorg. Chim. Acta*, **133** 3 (1987).