The Chemical Identity of Pentavalent Technetium-99m-Dimercaptosuccinic Acid

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The tumor-targeting radiopharmaceutical known as pentavalent technetium-99m-meso dimercaptosuccinic acid (DMSA) has been studied by a variety of techniques in order to elucidate its structure and chemical behavior. The radiopharmaceutical is identical with a chemically characterized sample of $[^{99}TcO(DMSA)_2]^-$ when studied by mobility methods including thin-layer chromatography, reversed-phase high-performance liquid chromatography, gel filtration, and electrophoresis. The technetium is pentavalent and coordinated by an oxo-ligand and four thiolate sulfurs of two DMSA ligands. No-carrier-added preparations consist of mixtures of three stereoisomers of the square pyramidal, mononuclear complex. The isomers arise from differing orientations of the carboxylate groups in the DMSA ligands and may be designated *syn-endo*, *syn-exo*, and *anti*. All three isomers are significant components of the radiopharmaceutical, raising the question of which are tumor-specific. The carboxylate groups in the complex are almost completely ionized at pH 7, thus the average charge on the complex at this pH approaches −5.

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Pentavalent technetium-99m-meso-dimercaptosuccinic acid (^{99m}Tc(V)DMSA) recently has become established as a useful radiopharmaceutical for imaging a number of tumors (*1–7*). It is particularly avidly accumulated by medullary thyroid carcinoma (*4,5*) and bone metastases (*6*) in a wider range of cancers. Despite its widespread routine use, the mechanism of its uptake in tumors has not yet been identified, neither has its common description as "pentavalent" been experimentally justified. The radiopharmaceutical was originally designed as a metabolic mimic of phosphate, able to localize in cancer cells by virtue of a supposed hydrolysis of the pentavalent DMSA complex within cancer cells to yield the phosphate-like ion TcO_4^{3-} (*8–11*). The pentavalent technetium-DMSA core was selected as offering sufficient stability to survive in plasma until delivered to the target site at which the hydrolysis to TcO_4^{3-} and subsequent metabolism could occur. The significance of this or other possible mechanisms cannot realistically be assessed without further characterization of the radiopharmaceutical and its chemical behavior.

It is quite plausible to suggest that the radiopharmaceutical does indeed contain technetium in the pentavalent state, because complexes containing pentavalent technetium coordinated by 1,2-dithiolate ligands are stable and well-characterized structurally and spectroscopically using the isotope ^{99}Tc (*12–25*). The ^{99}Tc complexes exist as $[^{99}TcO(dithiolate)_2]^-$ with pentavalent technetium coordinated by four thiolate groups and an oxo-ligand in a square pyramidal configuration. 1,2-Dithiols shown to form such complexes include 1,2-ethanedithiol (*12,14,19,20*), dithioglycollic acid (*12,13*), dithiooxalic acid (*12*), maleonitrile-2,3-dithiol (*12,16,19*), toluene-3,4-dithiol (*12,19*), propane-1,2-dithiol (*20*), DMSA (*17,18,20*), *rac*-dimercaptosuccinic acid (*18,19*), DMSA dimethylester (*19,22, 23,25*), *rac*-dimercaptosuccinic acid dimethylester (*19,23*), 2,3-dimercaptopropanol (*17*), 1,2-dimercaptoethylenetrithiocarbonate (*19*), and 1,3-dimercaptopropanesulfonic acid (*24*).

However, DMSA contains, as well as thiol groups, carboxylate groups which are also potential donors, so there may be alternative coordination modes and oxidation states for its complexes, depending on the preparative conditions. Indeed, stannous reduction of [^{99m}Tc]pertechnetate in the presence of DMSA gives under different conditions a number of different complexes (*26*), none of which have been explicitly identified. One of these is the tumor-targeting complex referred to here as ^{99m}Tc(V)DMSA and is osteotropic in rodents, while another, believed to contain technetium in an oxidation state below five and referred to here as "^{99m}Tc(III)DMSA," is the basis of the widely used renal imaging agent (*26*). Studies with ^{99}Tc indicate that one of these [the osteotropic agent, according to Ikeda et al. (*26*) or the renal agent according to Vanlic-Razumenic et al. (*27*)] has an absorption maximum close to 400 nm. Because of the multiplicity of possible complexes it is necessary to exercise caution in extrapolating structural conclusions obtained from macroscopic samples of ^{99}Tc complexes to the no-carrier-added ^{99m}Tc radiopharmaceutical. Despite the extensive studies of ^{99}Tc(V) dithiolate complexes, including partial charac-

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terization of $[^{99}TcO(DMSA)_2]^-$ (*17,18,21,25*), no direct evidence has been presented to identify the latter complex with the radiopharmaceutical ^{99m}Tc(V)DMSA prepared on the no-carrier-added scale. Indeed, a number of differing stoichiometries and structures for ^{99m}Tc(V)DMSA have been proposed in the literature. Horiuchi et al. (*8*) propose a polynuclear complex (on the basis of gel filtration behavior) containing pentavalent technetium, while Ikeda et al. (*26*) conclude from radiometric redox titration experiments using ^{99}Tc that the complex contains tetravalent technetium. Other authors (*22*) assume for discussion purposes a mononuclear pentavalent technetium core $[TcO(DMSA)_2]^-$ on the basis of the recognized stability of thiolate-coordinated ^{99}Tc(V). On the other hand, elsewhere the latter structure has been assigned (*28*) to the renal agent, again by analogy with pentavalent ^{99}Tc complexes. There is thus considerable uncertainty as to the structure of carrier-free technetium-DMSA radiopharmaceuticals. In this paper, we attempt to shed light on the structure of the tumor agent by comparing ^{99m}Tc(V)DMSA with the spectroscopically characterized complex $[^{99}TcO(DMSA)_2]^-$ using a variety of chromatographic and electrophoretic techniques. The results will assist in proposing plausible mechanisms for accumulation in tumors and in the future design of tumor-targeting radiopharmaceuticals for imaging and perhaps therapy of cancer.

MATERIALS AND METHODS

Technetium-99, as ammonium pertechnetate, and DMSA kits for renal imaging were purchased from Amersham International plc, UK. Other chemicals were obtained from Sigma or Aldrich Chemical Co., UK, and used as received. Technetium-99m was obtained as pertechnetate by elution of a Mallinckrodt Diagnostica generator.

Chemical Analysis and Spectroscopy

Microanalysis (C, H, N) was carried out by Butterworth Laboratories, UK. IR spectra were of Nujol mulls between sodium chloride windows. Optical spectra were determined in aqueous solution. Spectra (270 MHz ^1H NMR) were obtained with use of d^6-dimethylsulfoxide as solvent and tetramethylsilane as internal reference.

Preparation of $[Bu_4N][^{99}TcO(DMSA)_2]$

Sodium dithionite (0.08 g, 0.46 mmol) was dissolved in degassed water (25 ml). To this solution were added DMSA (0.151 g, 0.83 mmol) and ammonium pertechnetate (0.48 ml of 0.35 *M* aqueous solution, 0.17 mmol). The solution was made alkaline by addition of a few drops of aqueous sodium hydroxide and stirred at room temperature. An orange coloration developed. After 3 hr, the solution was acidified with dilute hydrochloric acid, filtered to remove the resulting precipitate of DMSA acid, and treated with an excess of tetrabutylammonium bromide. After three days, orange-red crystals of the complex were collected by filtration and washed with water. Microanalytical data: found: C 40.5%, H 6.0%, N 1.9%; calculated for $C_{24}H_{44}NO_9S_4Tc$, C 40.2%, H 6.2%, N 2.0%.

Preparation of ^{99m}Tc(V)DMSA

Amersham DMSA kits were used to prepare no-carrier-added solutions of the radiopharmaceutical using a literature method (*29*).

Thin-layer chromatography (TLC)

Merck $60F_{254}$ silica gel TLC plates were used with various solvent systems to check the purity of ^{99m}Tc(V)DMSA and to compare its behavior with that of $[^{99}TcO(DMSA)_2]^-$. Solvent systems in which ^{99m}Tc(V)DMSA has an R_f value of zero were rejected. Plates were developed a distance of 5 cm from the origin. The ^{99m}Tc compounds were visualized by scanning with a gamma camera.

High-performance Liquid Chromatography (HPLC)

A 15-cm Hamilton PRP-1 column was used, in conjunction with several linear gradient systems with differing pH, including acetate buffer (pH 4)/propan-2-ol, tetrabutylammonium chloride (pH 2.5 – 6)/acetonitrile, tetrabutylammonium phosphate (pH 7.4)/acetonitrile, and 0.1% trifluoroacetic acid in water/0.1% trifluoroacetic acid in acetonitrile. Samples of ^{99m}Tc(V)DMSA were filtered through silica gel sample preparation columns (Jones Chromatography, UK) before injection onto the HPLC column. The effluent from the column was monitored by UV absorbance at 420 nm and by radiometric detection using a gamma flow cell with Na(Tl)I crystal detector constructed in-house. A dual-pen strip chart recorder was used to record simultaneously the output from the gamma and UV detectors using mixed samples.

Electrophoresis

Electrophoresis experiments were carried out using Whatman no. 1 paper, 46 × 20 cm, in a Shandon electrophoresis apparatus for 1 hr at a constant voltage of 300 V. A range of buffer solutions was used (KCl/HCl, citrate, phosphate, bicarbonate), varying the pH from 2.0 to 9.2. Spots were visualized as described for TLC plates.

RESULTS

Reaction of aqueous [^{99}Tc]ammonium pertechnetate with sodium dithionite and DMSA at alkaline pH affords a yellow solution from which an orange-red crystalline tetrabutylammonium salt can be isolated following acidification and addition of tetrabutylammonium bromide. This material was characterized by microanalysis and infra-red, ultra-violet/visible, and proton magnetic resonance spectroscopy. The combined data suggest, and are fully consistent with, a mixture of three isomers of the compound $[Bu_4N][TcO(DMSA)_2]$ (Fig. 1).

The infra-red spectrum shows bands attributable to the tetrabutylammonium ion and also a sharp band at 958 cm^{-1} assignable to a terminal Tc=O bond. This value is comparable with those of related $[TcO(dithiolate)_2]^-$ complexes (range 925–980 cm^{-1}) (*12,13,16,19,20*), but differs from the value quoted by Byrne and Smith for $[^{99}TcO(DMSA)_2]^-$ of 940 cm^{-1} (*20*). This difference may be a result of solid-state/counter ion effects (the counter ion was not named in the study). Also present are bands at 3,000 cm^{-1} (broad) and 1,700 cm^{-1} assigned to the O-H and C=O bonds of free (uncoordinated) carboxylic acid

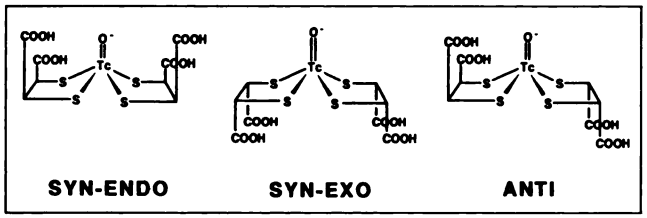

FIGURE 1. Geometrical isomers of $[TcO(DMSA)_2]^-$.

groups. There is no evidence of absorptions characteristic of S-H bonds.

The ^1H NMR spectrum shows resonances due to the counter-ion protons and the protons of the DMSA ligand (12 ppm, acidic protons; 4.19–4.34 ppm, protons bound to carbon), although again there is no evidence of S-H bonds. The C-H signal is split into four peaks (Fig. 2), comprising a strong singlet (4.19 ppm, 2.9H), a weak singlet (4.34 ppm, 0.2H) and two singlets of 1:1 integral ratio (4.25 and 4.31 ppm, 0.45H each) of intensity intermediate between the other two. This pattern is consistent with the presence of three isomers (Fig. 1).

The electronic spectrum shows peaks at 223 nm (9850 $cm^{-1}mol^{-1}l$) and 418 nm (1830 $cm^{-1}mol^{-1}l$) with shoulders at 240 and 277 nm, which is very similar in the visible region to the spectrum (*12,20*) of the crystallographically characterized (*14*) complex $[^{99}TcO(ethanedithiolate)_2]^-$ and further confirms the presence of the $Tc(V)OS_4$ core. These absorptions are tentatively assigned to sulfur-to-metal charge transfer transitions.

Preparation of no-carrier-added $^{99m}Tc(V)DMSA$ under alkaline conditions by the method of Sampson (*29*) consistently gave a radiopharmaceutical that was distinct from the renal agent $^{99m}Tc(III)DMSA$, which is prepared under acid conditions. The latter was shown to be absent (i.e., <1%) from the preparation by silica-gel TLC. Since the concentration of complex in these solutions is so low, spectroscopic methods of characterization are impractical and the material was therefore characterized by various types of mobility studies, including TLC, HPLC, and electrophoresis. The spectroscopically characterized sample of $[Bu_4N][^{99}TcO(DMSA)_2]$ was subjected to the same analyses, usually simultaneously on the same plate, column, or paper.

When studied by silica gel TLC with various solvent systems, $^{99m}Tc(V)DMSA$, $^{99m}Tc(III)DMSA$, and pertechnetate were readily distinguished (e.g., n-butanol/acetic acid/water 3:2:3 gave R_f values of 0.56, 0.07, and 0.88, respectively). However, no systems were found to separate $^{99m}Tc(V)DMSA$ from $[^{99}TcO(DMSA)_2]^-$.

All of the HPLC gradient systems used in this study separated $^{99m}Tc(V)DMSA$, $^{99m}Tc(III)DMSA$, and $^{99m}TcO_4^-$. Technetium-99m(III)DMSA failed to elute from the column. (Following this observation, all samples of $^{99m}Tc(V)DMSA$ were passed through a silica-gel sample preparation column prior to HPLC analysis to avoid contaminating the HPLC column.) Pentavalent technetium and $[Bu_4N][^{99}TcO(DMSA)_2]$ were compared both separately and by analyzing mixtures of the two. Under all conditions, elution times for $[^{99}TcO(DMSA)_2]^-$ were identical to those of $^{99m}Tc(V)DMSA$ when recorded on a dual-pen recorder. However, only systems capable of separating the three isomers of $[^{99}TcO(DMSA)_2]^-$ were regarded as sufficiently discriminating to prove that $^{99m}Tc(V)DMSA$ and $[^{99}TcO(DMSA)_2]^-$ share the same structure. This separation was achieved with the Hamilton PRP-1 column and a gradient consisting of 0.1% trifluoroacetic acid in water/0.1% trifluoroacetic acid in acetonitrile. The results with this system (Fig. 3) confirm the identity of $^{99m}Tc(V)DMSA$ and $[^{99}TcO(DMSA)_2]^-$ and show that all three isomers are present in significant relative concentration in both.

Paper electrophoresis clearly demonstrated that $^{99m}Tc(V)DMSA$ is anionic over a range of pH values and confirmed its similarity to $[^{99}TcO(DMSA)_2]^-$. Migrations of the two complexes (relative to pertechnetate on the same paper, i.e., migration distance of complex/migration distance of pertechnetate) for a range of buffers are plotted in Figure 4. The relative migration rises steeply between pH 2 and pH 7, indicating a dramatic increase in negative charge on the complex as pH is raised (the charge on the pertechnetate anion is unaffected over this range), but then

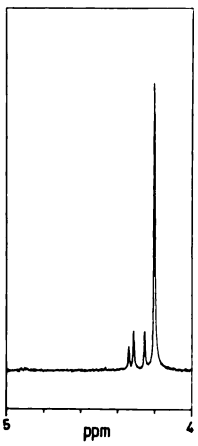

FIGURE 2. ^1H NMR spectrum of C-H proton region (4–5 ppm) of $[^{99}TcO(DMSA)_2]^-$.

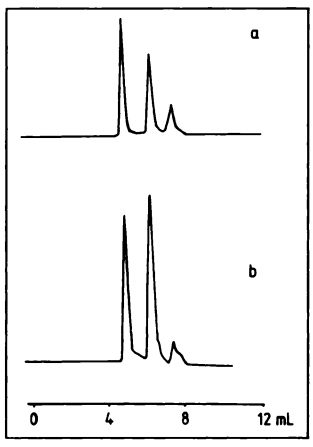

FIGURE 3. HPLC chromatograms of complexes showing separation of isomers. (A) $^{99m}Tc(V)DMSA$, no-carrier-added, detected by gamma radiation monitoring. (B) $[^{99}TcO(DMSA)_2]^-$ detected by UV monitoring at 420 nm. Solvent A: 0.1% trifluoroacetic acid in water. Solvent B: 0.1% trifluoroacetic acid in acetonitrile; flow rate 2 ml min^{-1}. Gradient (t, %B): 0, 0; 1 min, 0; 20 min, 50; 24 min, 50.

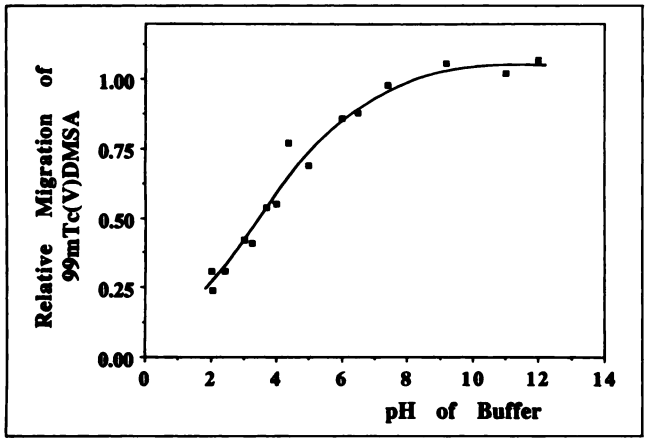

FIGURE 4. Plot of ratio of migration distance of ^{99m}Tc(V)DMSA to that of $^{99m}TcO_4^-$ versus pH upon paper electrophoresis.

levels off. No evidence of separation of isomers was found by electrophoresis.

DISCUSSION

The spectroscopic properties of the crystalline ^{99}Tc complex show that it contains the complex ion $[^{99}TcO(DMSA)_2]^-$ with the isomeric structures shown in Figure 1. The existence of $[^{99}TcO(DMSA)_2]^-$ gives credence to the suggestion that one of the complexes formed by stannous reduction of carrier-free $^{99m}TcO_4^-$ contains pentavalent technetium. The question of whether either ^{99m}Tc(V)DMSA or ^{99m}Tc(III)DMSA can be identified with this complex was addressed by exhaustive comparison of the complexes by a range of mobility studies as discussed below.

Thin-layer chromatography under various conditions, although clearly distinguishing ^{99m}Tc(III)DMSA, ^{99m}Tc(V)DMSA, free DMSA, and $^{99m}TcO_4^-$, failed to distinguish ^{99m}Tc(V)DMSA and $[^{99}TcO(DMSA)_2]^-$. These observations are substantiated by the more discriminating HPLC methods over a wide pH range. Similarly, electrophoresis over a wide pH range failed to distinguish the two complexes. The electrophoresis results imply that they share the same ionizable groups (i.e., carboxylic acid) with the same pK_a values and the same charge even when the carboxylate groups are fully associated (-1) and fully dissociated (-5). The mobility of the complex (in ratio to that of pertechnetate) plateaus at pH values above 7, implying that at physiologic pH the complex is fully ionized with an average overall charge approaching -5.

^{1}H NMR spectroscopy on $[^{99}TcO(DMSA)_2]^-$ suggests the presence of all three possible isomers of the square pyramidal complex. The pattern of chelate ring proton resonances observed (Fig. 2) is similar to that observed for $[^{99}TcO(DMSA\text{-dimethylester})_2]^-$ (*23*). The dominant isomer in this sample is clearly one of the symmetrical isomers, probably the *syn-endo* by analogy with the DMSA-dimethylester complex (*22*), although this will require crystallographic confirmation. The presence of three isomers is confirmed by HPLC using the aqueous trifluoroacetic acid/acetonitrile gradient system. The radiopharmaceutical preparations described in this paper appear to give two major isomers and one relatively minor isomer. It is not possible with the present data to assign specific isomers to these HPLC peaks, and the relative concentrations are such that any of the isomers could be tumor-specific. Similarly, any one or two of the isomers may significantly degrade images.

CONCLUSIONS

Technetium forms a stable complex $[^{99}TcO(DMSA)_2]^-$ when pertechnetate is reduced by dithionite in the presence of DMSA in alkaline solution. This complex contains pentavalent technetium coordinated by four thiolates of two DMSA ligands and an apical oxo group. Despite extensive attempts to show that this complex is different to the tumor-imaging agent ^{99m}Tc(V)DMSA using a variety of chromatographic and electrophoretic techniques, we have not been able to demonstrate any differences in behavior. Thus, ^{99m}Tc(V)DMSA has the formula $[TcO(DMSA)_2]^-$. On the other hand, ^{99m}Tc(III)DMSA, the widely used renal agent, clearly does not have this structure despite assumptions to the contrary in the review literature (*28*). There is no evidence for significant interaction of the carboxylic acid groups with the metal center, and at pH values above 7, they approach complete ionization, imparting an average overall charge of up to -5 to the complex. As expected from structural considerations, three geometrical isomers coexist in the present sample of $[^{99}TcO(DMSA)_2]^-$. All three are also present in the no-carrier-added preparations, raising the question of which constituents are tumor-specific. Further work aimed at resolving the in vivo behavior of individual isomers may therefore significantly improve the sensitivity and specificity of the radiopharmaceutical and help illuminate its mode of action.

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EDITORIAL

Small Coordination Complexes in Tumor Imaging

In recent years, a great deal of effort has gone into incorporating various radiometals (e.g., ^{111}In, ^{99m}Tc, ^{90}Y) into macromolecules (particularly monoclonal antibodies and their fragments) for the purposes of tumor diagnosis and treatment (*1–3*). In these conjugates, targeting is accomplished via the recognition of some site on the tumor cell by a specific domain on the labeled macromolecule. A stable coordination site must be provided for the metal ion in such a way as to not interfere with this recognition process. In parallel (and somewhat in the background) to this effort, progress is being made in finding "small" (molecular weight under 2 KD) coordination complexes that localize in various tumors due to the inherent structure and/or reactivity of the complex. Examples of this latter class include ^{67}Ga-citrate (which circulates through the body mostly as the Ga-transferrin complex), ^{57}Co, ^{111}In and ^{99m}Tc-bleomycin, various metalloporphyrin and phthalocyanine derivatives, ^{99m}Tc-Sn-N-pyridoxyl-5-methyltryptophan, ^{99m}Tc-MIBI, metal complexes of phosphonate ligands (e.g., ^{153}Sm-EDTMP), and ^{99m}Tc(V)DMSA (*4–12*).

The tumor-imaging properties of ^{99m}Tc(V)DMSA were first described by Ohta et al. in 1984 and the compound was formulated by Yokoyama et al. as a polynuclear technetium complex on the basis of gel permeation chromatography results. These workers hypothesized that ^{99m}Tc(V)DMSA is metabolized within tumor cells to the Tc(V) anion, TcO_4^{3-}, which presumably mimics the biochemistry of phosphate ion (*13,14*). The polynuclear formulation for ^{99m}Tc(V)DMSA was further supported by Horiuchi et al. who claimed that the thin-layer chromatography behavior of the complex was dilution-dependent (*15*). These results were obtained for Tc(V)DMSA prepared at no-carrier-added levels of technetium.

The great advantage of using macroscopic quantities of the long-lived isotope (^{99}Tc) in the study of radiopharmaceutical preparations is that compounds can be isolated and their chemical composition and structure determined by a variety of methods.

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