A New Electrophoretic Method for Determining Ligand: Technetium Stoichiometry in Carrier Free $^{99m}$Tc-Radiopharmaceuticals

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An electrophoretic procedure is outlined for the determination of the number of ligands bound to technetium-99m radiopharmaceuticals. The approach involves use of ligands that will complex technetium in a similar fashion but that differ in charge. This approach was applied experimentally to dimercapto ligands in which the ligating sulfur atoms are separated by a flexible three-carbon chain (1,3-dimercapto compounds). Two such ligands studied are 1,3-dimercaptopropane (DMP) and dihydrothioctic acid (DHTA). The Tc compound of DHTA migrates much farther on electrophoresis than the Tc complex of DMP. However, when TcO$_4^-$ is reduced by SnCl$_2$ or NaBH$_4$ in the presence of equimolar quantities of DHTA and DMP, a new compound is formed being twice as abundant as either the TcDMP or the TcDHTA compound and migrating an intermediate distance. The formation of this new complex and the 1:2:1 distribution indicates that two 1,3-dimercapto compounds are attached to the Tc-center in all three compounds.


The absence of a well-defined and predictable chemistry for technetium coupled with the difficulty of establishing the structure of Tc-99m radiopharmaceuticals has hindered the full utilization of this radionuclide in nuclear medicine (1). For this reason we have been investigating methods that will provide useful structural information about Tc-99m radiopharmaceuticals and can be applied widely. In this report we wish to outline an approach that, although it provided only partial structural information, has distinct advantages with regard to ease of application and yields some of the most important information necessary to appreciate the nature of Tc-99m radiopharmaceuticals. Although this approach is versatile, and we have already applied it to several radiopharmaceuticals, we will introduce and illustrate our method with dimercapto ligands.

These ligands were selected because Tc compounds of different stability are formed. The ligands that can yield five-membered chelate rings (Fig. 1) form more stable complexes than ligands yielding only six-membered rings (Fig. 2). This difference has allowed us to evaluate factors controlling formation from stannous chloride reduction of per-technetate in the presence of competing ligands. The technetium complex of one of these ligands, dihydrothioctic acid (DHTA, Fig. 3) was originally prepared by Jackson and Bolles (3) and has undergone clinical trials as a potential radiopharmaceutical for the evaluation of hepatobiliary function.
S-0 pertechnetate was eluted from a generator with normal saline.

Preparation of Bis(dimercapto) complexes of Tc using SnCl₂. The dimercapto ligand (1 μl) was added to anhydrous ethanol (0.1 ml) in a vial. The vial was sealed and the contents purged with N₂. Bicarbonate buffer was injected into the reaction vial. Next, 0.3 ml of NaTcO₄ in N₂-purged saline was added to the reaction vial, followed by 0.05 ml of a SnCl₂ solution (200 μg/ml of anhydrous ethanol). Reduction and complex formation were complete in 10 min. The carbonate buffer was prepared by N₂-purging an aqueous solution that contained 4.9 mg of NaHCO₃/ml in a stoppered vial. Next, sufficient 0.1 N HCl was added to adjust the pH to 6.5. The CO₂ produced was not vented since it is part of the buffer system.

Preparation of mixed-ligand dimercapto complexes using SnCl₂. The procedure was identical to that described above except that 1 μl of DHTA and 0.5 μl of other dimercapto ligands were used. This volume ratio gives approximately 1:1 molar ratios of ligands. Complex formation was complete in 10 min and the product distribution remains unchanged for 2 hr.

Preparation of bis-dimercapto and mixed-ligand complexes of Tc using NaBH₄. The dimercapto ligand (2 μl) was dissolved in 0.1 ml of anhydrous ethanol in a reaction vial, which was then sealed with a serum stopper and purged with N₂ gas. To the sealed vial was added 0.3 ml of 0.75 M NaHCO₃ dissolved in N₂-purged water, followed by 0.3 ml of N₂-purged NaTcO₄ solution. After addition of 0.1 ml of N HCl (the CO₂ gas generated by the acid was not vented as it is part of the buffer system), 0.1 ml of 0.5 M NaBH₄ in anhydrous ethanol was added. Reduction was complete within 10 min. The mixed complex of DHTA and 1,3-dimercaptopropane was prepared similarly except that 1 ml of DHTA and 0.5 ml of 1,3-dimercaptopropane were used as ligands. Note that since the NaBH₄ procedure results in the thermodynamic product distribution, no mixed 5- and 6-membered chelate ring complexes may be prepared using this reducing agent.

Substitution reactions.

1. In the presence of SnCl₂—A bis-dimercapto alkane complex was prepared by the standard SnCl₂ reduction procedure using 2 μl of DHTA. This product was analyzed via electrophoresis to confirm absence of both TcO₂ and TcO₄⁻. Exchange conditions were initiated by the introduction of a tenfold excess of a second ligand (10 μl). The appearance upon electrophoresis of the mixed ligand and the completely exchanged bis-ligand complex were taken as evidence for exchange. Product dis-
tributions were determined at various times and the half-time of the original complex was estimated.

2. In the presence of NaBH$_4$—The original complex may be prepared either by the standard SnCl$_2$ or NaBH$_4$ reduction procedure. When NaBH$_4$ is used, exchange conditions are again initiated simply by the addition of a tenfold excess of a second ligand. When the standard SnCl$_2$ preparation is used, additional NaHCO$_3$ must be added in order to stabilize the pH to the subsequent addition of NaBH$_4$. Therefore, to the standard SnCl$_2$ reaction vessel is added a tenfold excess of a second ligand followed by 0.3 ml of 0.75 M NaHCO$_3$ and 0.1 ml of N HCl and finally by 0.1 ml of 0.5 M NaBH$_4$ dissolved in ethanol.

Under these conditions, exchange takes place in the presence of both SnCl$_2$ and NaBH$_4$. When short time periods were being examined, exchange conditions in both cases were terminated by addition of 0.2 ml of N HCl. Addition of HCl to the complexes under these conditions had no effect on the stability of the complexes. Exchange was not promoted by addition of the additional buffer alone when the SnCl$_2$ reduction was used.

3. Exchange in the absence of reducing agent—The original Tc complex may be prepared by either reduction procedure. A sample of the reaction is analyzed electrophoretically using the condition previously described. The paper containing the complex is cut out and extracted into a solution mixture containing 0.15 ml of ethanol, 0.2 ml of the carbonate buffer used in the standard Sn(II) reduction procedure, and 0.3 ml of saline. To this solution is added 1 µl of the original dimercapto alkane ligand and a tenfold excess of a second ligand. Exchange is followed by electrophoresis.

Electrophoretic procedures. Electrophoregrams were obtained, using the system described below, at pH 7.5 in 0.01 M NaHCO$_3$ on Whatman No. 1 paper at a field strength of 100 v/cm. This buffer system was selected to minimize complications that can result from reaction of Tc complexes with buffer anions. The 1,3-dimercapto complexes in particular underwent reaction with common buffers such as phosphate, citrate, and acetate in both basic and acidic solutions.

The bicarbonate buffer has a low buffering capacity at or near neutrality, and, therefore, the following system was devised to increase the effective buffer capacity. Around each electrode, two dialysis tubes were placed coaxially. The first, ¼ in. in diameter, contained glass beads of large enough diameter (3 mm) to permit free gas flow. The second tube, 1 in. in diameter, contained a mixture of ion exchange resins (see below). The dialysis tubes were tied coaxially at both ends to lengths of plastic tubing. Gas produced at the electrodes accumulates within the inner bag and escapes through the plastic tubing.

At the cathode (where H$_2$ gas and hydroxyl ions are produced) the anion (Bio-Rad AG2-X8) and cation (Dowex 50W-X8) resins are charged with bicarbonate and hydroxyl ions, respectively. Before the hydroxyl ions produced can enter the bulk solution in the buffer trough, they must pass the dialysis tube containing the resins. Interaction with the anion resin produces HCO$_3^-$ ion whereas interaction with the cation resin produces hydroxyl ions. Equivalent capacity of each resin was used at each electrode based on information provided by the supplier. In our system, 75 g of resin was used at each electrode.

A pertechnetate standard was used with all samples, and the distance each complex migrated was determined by scanning the dried paper with a chromatogram scanner equipped with a one-inch sodium iodide detector and collimator. The latter consisted of 0.635 cm of lead with a slit 2.54 cm x 3.0 mm. When more than one peak was found on the electrophoregram, the relative activity in each peak was determined by cutting the electrophoregram into 2.5 mm strips and counting in a well counter. The ratio of activity in each peak were determined by summing the counts in each peak and normalizing the activity to the smallest peak, which was assigned a value of 1. If only one peak was observed on scanning, no further analysis was performed. Calibration of the scanning system with known quantities of radioactivity demonstrated that peaks containing < 1% of the activity in the major peak were easily detected. Therefore, observation of one peak on scanning indicates that any other peaks that may be present represent less than one percent of the activity in the major peak.

Mixed-ligand experiment—the statistical approach. The basis for our method of characterization of the Tc:dimercapto stoichiometry relies on the expected statistical distribution of complexes formed in solutions equimolar in two ligands that have essentially identical coordinating abilities. If this ratio were 1:1, then equal concentrations of two complexes are expected. Alternatively, if the metal to bound-ligand ratio were 1:2, one would expect to observe three complexes. Two of these are the complexes formed in the presence of each of the ligands separately. The third and new "mixed-ligand" complex, which is statistically more probable, contains both ligands. The expected ratio of complexes is 1:2:1. Extending this argument, one expects to find that, for a metal-bound
ligand ratio of three, four complexes are formed in the ratios of 1:3:3:1. The two new mixed-ligand complexes formed will be more abundant. These arguments could be extended to higher ratios, but since most Tc-99m radiopharmaceuticals contain chelate ligands, such an extension is unnecessary for our purposes.

Several potential difficulties could arise in reducing the above theoretical arguments to practice in the study of Tc-99m radiopharmaceuticals. First, since a charged group must be introduced into the complex, it is possible that the new ligand will use this group in coordination with the metal. However, for N and S donor ligands, this is unlikely to be a problem if the new group is an oxygen donor and also if a favorable chelate ring (five- or six-membered) cannot be formed using the additional charged group (2). It should be emphasized that the chelating N or S groups should be designed to form five- or six-membered chelate rings. Furthermore, both ligands should be capable of forming identical chelate rings. It would be best to design the ligand so that the charge-carrying group cannot possibly form a chelate ring in conjunction with the N or S donor. The best experimental criteron for excluding this altered method of chelation is to evaluate the statistical distribution of complexes formed. If this distribution is close to that expected, it is unlikely that a new means of coordination has been introduced with the charge-carrying group.

The failure of the system to attain true thermodynamic equilibrium is a second complication. However, the statistical arguments are based on the relative probability that each of the ligands will coordinate to the metal center. As long as this probability is essentially the same for both competing ligands, the above statistical arguments are still valid. In fact, systems in which thermodynamic equilibrium is reached and maintained through rapid reactions pose a third potential problem. Thus, as electrophoresis is performed, the uncoordinated ligands will migrate at different speeds both relative to each other and also relative to the radiopharmaceuticals, and well-resolved distinct peaks will not be observed. Such distinct peaks also will not be observed if the buffer or the support reacts with the complex. This occurrence is unlikely to be encountered very often, since most useful radiopharmaceuticals will either be slow to undergo substitution or will become attached to the paper. For our purposes, we can say that, if after preparation of the radiopharmaceutical, an excess of a competing ligand is added and, after an hour, there is incomplete conversion to a new complex, the radiopharmaceutical can be considered inert to substitution. The concept of inertness in transition-metal chemistry is complicated, and the above operational definition should not be considered to be general.

Additionally, it should be pointed out that the presence or absence of tin in the radiopharmaceutical will not invalidate the above arguments. The ratios discussed would then be simply Tc-Sn : bound ligand ratios. The Tc and Sn would be in some fixed ratio, probably 1:1. Details of the results would be slightly different, however, if the manner of binding of the two ligands were not the same. Thus, whether or not Sn were also incorporated into the radiopharmaceutical two-mixed ligand complexes would be formed, if two sites of different type were available for the two ligands. The distribution would then be 1:1:1:1. Another potential problem is that the complexes formed could be dimeric (contain two Tc-99m), but this possibility is statistically very improbable. However, again our arguments would still hold, but now the complexes would be formulated as containing two technetium centers. We emphasize that the presence of two technetium atoms in the complex is highly unlikely. Furthermore, Loberg (5) has performed quantitative calculations, based on several reasonable assumptions, that clearly show such dimeric technetium compounds are unlikely to be formed.

RESULTS AND DISCUSSION

Reduction of [\(^{99}\text{Tc}\)]pertechnetate with either SnCl\(_2\) or NaBH\(_4\) in the presence of the dimercapto ligands, 1,2 or 1,3-dimercaptopropane, yield products that have nearly identical electrophoretic behavior (Table 1). This finding strongly suggests that the compounds are identical in structure except for the formation of five- and six-membered chelate rings, respectively. Moreover, Loherg (5) has performed quantitative calculations, based on several reasonable assumptions, that clearly show such dimeric technetium compounds are unlikely to be formed.

<table>
<thead>
<tr>
<th>TABLE 1. ELECTROPHORETIC MOBILITIES IN cm(^2)/volt-min</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Tc(DHTA)})_n(^{(+n)})</td>
</tr>
<tr>
<td>(\text{Tc(1,3DMPane)})_n(^{(+n)})</td>
</tr>
<tr>
<td>(\text{Tc(1,2DMPane)})_n(^{(+n)})</td>
</tr>
<tr>
<td>(\text{Tc(1,2DME)})_n(^{(+n)})</td>
</tr>
<tr>
<td>(\text{Tc(DHTA)(1,2DMPane)})(^{(+n)})</td>
</tr>
<tr>
<td>(\text{Tc(DHTA)(1,3DMPane)})(^{(+n)})</td>
</tr>
<tr>
<td>(\text{Tc(DHTA)(1,3DME)})(^{(+n)})</td>
</tr>
<tr>
<td>(\text{Tc(1,2DME)(1,3DMPane)})(^{(+n)})</td>
</tr>
</tbody>
</table>
TABLE 2. PRODUCT DISTRIBUTIONS (DHTA)2, (DHTA)3, L, IN MIXED-LIGAND EXPERIMENTS WITH EQUIMOLAR QUANTITIES OF COMPETING LIGANDS

<table>
<thead>
<tr>
<th>Reductant/L-2</th>
<th>1,3DMP</th>
<th>1,2DMP</th>
<th>1,2DME</th>
</tr>
</thead>
<tbody>
<tr>
<td>SnCl2</td>
<td>1.13:2.03:1.00</td>
<td>1.00:5.05:3.92</td>
<td>1.00:4.91:4.03</td>
</tr>
<tr>
<td>NaBH4</td>
<td>1.05:1.98:1.00</td>
<td>0.00*:0.00:1.00</td>
<td>0.00:0.00:1.00</td>
</tr>
</tbody>
</table>

* Value of 0.00 indicates that no peak was observed for complex on scanning electrophoregram.

FIG. 4. Electrophoregram obtained from mixed ligand experiment using 1,3-DMP and DHTA.

those of the complexes formed by the dimercapto propane ligands. However, the carboxyl group of DHTA is deprotonated, which would lead to an overall higher negative charge if, as expected, the oxidation state and general coordination sphere of the technetium is identical in all the compounds.

The results of carrying out the reduction with either SnCl2 or NaBH4, in the presence of DHTA and an equimolar amount of a second dimercapto compound, are given in Table 2; the electrophoretic mobilities are presented in Table 1. Several features emerge. First, when both competing ligands are 1,3-dimercapto compounds, the distribution obtained is exactly that expected if the complexes formed are bis complexes (Fig. 4)—that is, if there are two dimercapto ligands in each complex. Moreover, this result was obtained regardless of whether SnCl2 or NaBH4 was used as the reductant. These findings suggest that both kinetic and thermodynamic distributions are similar. The charge on the bis DHTA complex is two units greater than that on the bis complexes of other ligands used here.

When a 1,2-dimercapto ligand was used with DHTA, there were still three compounds formed with SnCl2 as reductant. The mixed-ligand compound is still more abundant, but now the bis 1,2-dimercapto compound is formed to a greater extent than Tc(DHTA)2. Under the conditions of the experiment, the much more stable bis 1,2-dimercapto complexes are thermodynamically favored, as was found when NaBH4 was used as the reductant. The observation of any of the bis-DHTA and mixed-ligand complexes suggests that reduction by SnCl2 is a kinetically controlled process during at least one of the reduction steps.

When NaBH4 is the reductant, only the stable bis 1,2-dimercapto complexes are found to any appreciable extent, which suggests that the less stable complexes are either not formed or are rapidly converted to the stable complexes by ligand exchange. Catalysis of the exchange was demonstrated by preparing the DHTA complex with SnCl2 and NaBH4 reductant (Table 3). Addition of an excess of a second dimercapto ligand, as described in the experimental section, led to complete exchange in less than five min under the NaBH4 reducing conditions, but exchange was much slower when no NaBH4 was present. Similar experiments were performed preparing the Tc-complex of 1,3-DMP followed by addition of an excess of DHTA. Again, exchange was rapid when NaBH4 was present and resulted in formation of Tc(DHTA)2. Two types of experi-

TABLE 3. HALF-LIVES FOR THE SUBSTITUTION REACTION OF Tc COMPLEXES WITH VARIOUS SUBSTITUTING LIGANDS

<table>
<thead>
<tr>
<th>Complex</th>
<th>1,3DMP</th>
<th>1,2DMP</th>
<th>1,2DME</th>
<th>DHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tc(DHTA)2</td>
<td>&gt;6 hr</td>
<td>~1 hr</td>
<td>~1 hr</td>
<td>—</td>
</tr>
<tr>
<td>Tc(1,2DMP)2</td>
<td>—</td>
<td>—</td>
<td>No reaction</td>
<td>&gt;6 hr</td>
</tr>
<tr>
<td>Tc(1,3DMP)2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

B. NaBH4

<table>
<thead>
<tr>
<th>Complex</th>
<th>1,3DMP</th>
<th>1,2DMP</th>
<th>1,2DME</th>
<th>DHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tc(DHTA)2</td>
<td>&lt;1 min</td>
<td>&lt;1 min</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tc(1,2DMP)2</td>
<td>—</td>
<td>—</td>
<td>No reaction</td>
<td>—</td>
</tr>
<tr>
<td>Tc(1,3DMP)2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>&lt;1 min</td>
</tr>
</tbody>
</table>
ments were conducted to determine whether the SnCl₂ was inhibiting exchange. First, NaBH₄ was added to Tc(DHTA)₂ which had been prepared by the SnCl₂ method, and excess ligand was also added. Exchange was observed as for the NaBH₄ preparation. Second, the Tc(DHTA)₂ was purified by electrophoresis. Addition of excess dimercapto ligand led to exchange behavior very similar to that of the unpurified Tc(DHTA)₂.

The mechanism of the catalysis is not clear but could involve the formation of small amounts of Tc complex of a lower oxidation state; this is exchange-labile and readily reoxidized to the stable oxidation state. It would be of interest to investigate the mechanism of ligand exchange reactions in these systems using Tc-99. Since our interests were in establishing ligand Tc stoichiometry, however, we did not investigate these reactions in depth.

The identical electrophoretic behavior of complexes prepared using SnCl₂ or NaBH₄ suggests that tin is not incorporated into these radiopharmaceuticals. From the electrophoretic mobility of the Tc complexes, it is not possible to assign the magnitude of the negative charge on the complex. This point will be pursued in a later paper.

Subsequent to the completion of our study, Byrne and Smith (6) have investigated the synthesis, isolation, and characterization of the Tc-99 complex of dimercaptoethane. Using Raman spectroscopy, elemental analysis, and x-ray diffraction crystallography, they showed that there are two dimercapto ligands and one terminal oxo group bound to the Tc atom, and that the complex has a net charge of −1. They also demonstrated, using electrophoresis and two chromatographic systems (6), that their ⁹⁹mTc(DME)₂ complex is identical to our ⁹⁹Tc(DME)₂ complex. The results of their work, therefore, confirm our finding that dimercapto ligands form negatively charged bis complexes with Tc-99m and support our mixed-ligand approach to assigning ligand:Tc stoichiometry.

The approach we have outlined here has the advantage of ready applicability to a wide range of Tc-99m radiopharmaceuticals in laboratories currently engaged in radiopharmaceutical research. Although the ideal would seem to be the isolation and complete chemical characterization of Tc-99 complexes such as the studies carried out by Byrne and Smith, it is very probable that many actual Tc-99m radiopharmaceuticals will be difficult, if not impossible, to isolate in pure form. However, the mixed-ligand procedure does not require isolation of the complex, and yields some of the most important structural information—ligand:Tc stoichiometry. The principal influence on the biodistribution of the radiopharmaceutical will be that of the larger chelate ligands and the charge that these, in combination with the smaller ligands, impart to the complex. It is imperative that a knowledge of the number of these chelate ligands existing in a complex be established if one is to have a rational understanding of the factors that control the biodistribution of the complexes.

FOOTNOTES

• Aldrich

ACKNOWLEDGMENTS

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REFERENCES


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