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DEVELOPMENT OF MORE EFFICACIOUS Tc-99m ORGAN IMAGING AGENTS FOR USE IN NUCLEAR MEDICINE BY ANALYTICAL CHARACTERIZATION OF RADIOPHARMACEUTICAL MIXTURES

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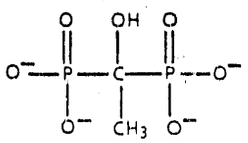
Introduction

Skeletal imaging agents are of major clinical importance in the early diagnosis of bone cancer and in the planning and evaluation of therapy for such patients. This use is particularly important since many types of primary cancer ultimately result in bone metastases. Comparative studies of different Tc-diphosphonate radiopharmaceuticals demonstrate that they exhibit different tumor/bone ratios in animal models, and thus there is considerable potential for improving the clinical utility of these agents.

Skeletal imaging agents are typically prepared by reducing $^{99m}TcO_4^-$ with Sn^{2+} in the presence of phosphonate-containing ligands such as HEDP, MDP, HMDP, DPD, and DMAD.



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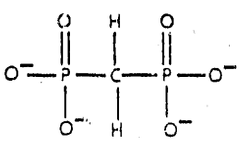
HEDP



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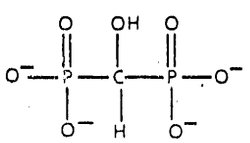
MDP



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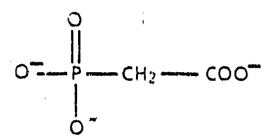

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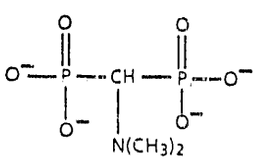

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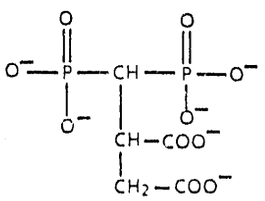
DMAD



dicarboxypropane diphosphonate
DPD

Technetium is reduced by Sn^{2+} to lower oxidation state species which then complex with the ligand. The phosphonate groups of the resulting ^{99m}Tc-ligand complex cause the accumulation of technetium at calcium-containing sites such as the bone.

In 1980 we reported the HPLC (high performance liquid chromatography) separation of a $^{99m}Tc(NaBH_4)$-HEDP radiopharmaceutical analogue into 7 component Tc-HEDP complexes (1). Individual Tc-HEDP complexes could then be collected in pure form as they eluted from the chromatograpny column and their

biodistribution characteristics evaluated by injection into test rats. *In vivo* distributions among femur, kidney, liver, blood and femur muscle were distinctly different for the different Tc-HEDP complexes (2). Thus, skeletal imaging can be improved by using the specific Tc-diphosphonate complex that offers the best imaging properties.

Objectives

The long-range objective of this research program is the development of more efficacious technetium-99m radiopharmaceuticals for use as imaging agents in diagnostic nuclear medicine. Within this overall goal, we seek to isolate and develop distinct site imaging agents, each of which has properties optimized to provide diagnostic information concerning a given pathological condition. These objectives are being accomplished by the development of analytical techniques which are capable of separating clinically used mixtures into their component technetium complexes. Each complex can then be characterized in pure form by techniques such as EXAFS, NMR, and mass spectrometry. Administration of the isolated, single most effective imaging complex, rather than a mixture of technetium-containing complexes, will minimize radiation exposure to the patient and maximize diagnostic information available to the clinician.

The specific objectives during the present funding period (9/1/89 to 8/31/92) have been as follows:

(1) Development of strategies for improving yields of specific Tc-diphosphonate complexes with optimum imaging properties.

(2) Development of electrodes for rapid *in situ* electrochemical generation of skeletal imaging agents.

(3) Development of electrochemical sensors for Tc and Re imaging agents.

(4) Characterization of stable Tc- and Re-diphosphonate complexes obtainable in high yield by structural studies with techniques such as NMR, EXAFS, and Raman spectroscopy.

(5) Development of improved separation techniques for the characterization of diphosphonate skeletal imaging agents.

(6) Evaluation of the effect of the biological milieu on Tc-diphosphonate complexes.

(7) Electrochemical studies of technetium and rhenium complexes synthesized by Professor Deutsch's research group for heart and brain imaging.

The results of our research in these areas are summarized in the following sections.

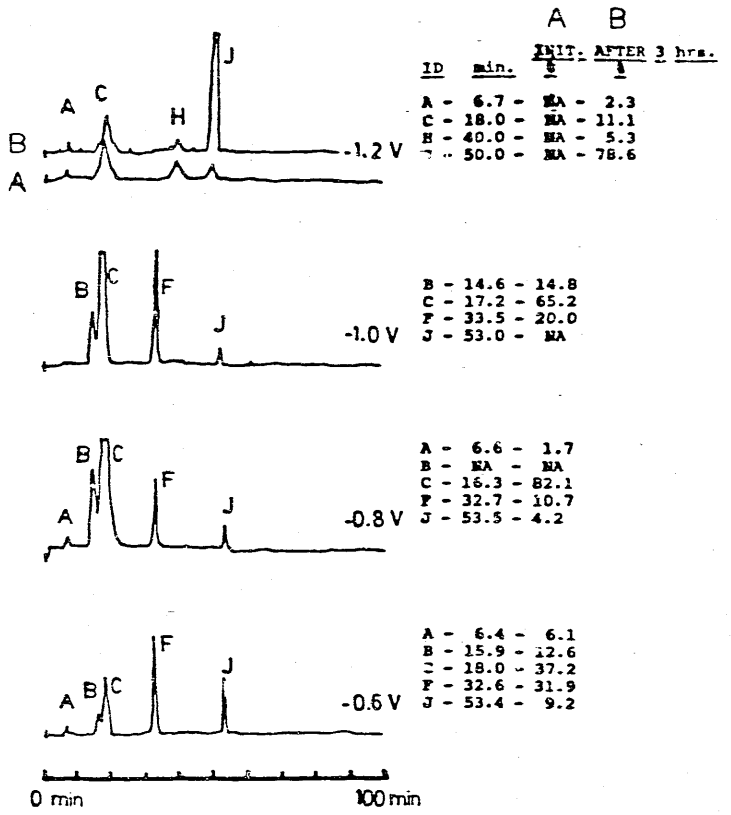
Strategies for Improving Yields of Specific Tc-Diphosphonate Complexes with Optimum Imaging Properties

We have demonstrated that numerous Tc-complexes can be formed for each diphosphonate ligand system, depending on the reaction conditions, and that the imaging efficacies of the individual complexes are variable. In order to obtain optimum imaging properties, it is important to develop procedures that enable the optimum Tc complex to be formulated in high yield, rapidly and easily. We have shown that formulation pH, aerobicity vs. anaerobicity and ligand to metal ratio can influence yield. This was demonstrated in a systematic study of the reduction of TcO_4^- by $NaBH_4$ in the presence of the following diphosphonate ligands: HEDP (3), MDP (4), DMAD (5), PAA (6), and DPD (7).

Electrochemical reduction of TcO_4^- in the presence of a diphosphonate has been demonstrated as an effective means by which to form Tc-diphosphonate complexes. Of special significance is the dramatic effect that electrode potential, in concert with pH, has on the resulting distribution of the Tc-diphosphonate complexes that are electrogenerated. We demonstrated this in the previous funding period (DOE/ER/60487-3) for HEDP (8) and then for MDP (9) ligand systems. Thus, this electrochemical procedure shows promise as a means by which high yields of specific Tc-diphosphonate complexes can be formed in order to generate the optimum agent for skeletal imaging.

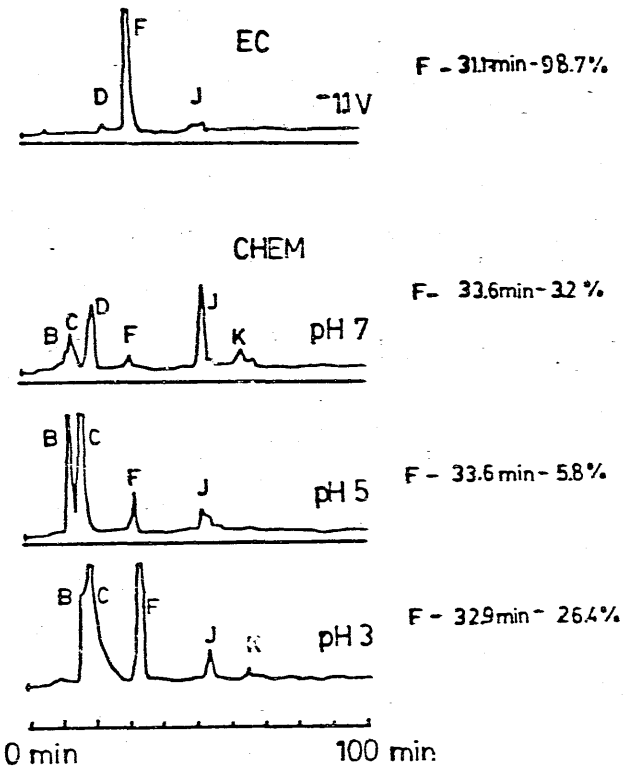
As a part of our systematic study of the electrochemical generation of skeletal imaging agents, we have also studied the DMAD ligand system (8). The effects of variables such as pH, concentration of TcO_4^- and ligand, and especially electrode potential were evaluated. One objective of this study was to determine if different Tc-DMAD complexes are generated by electrochemical reduction than are formed by chemical reduction. A second objective was to determine if electrochemistry is an effective technique for optimizing the yield of the specific complex that is the most efficacious imaging agent. HPLC was used to analyze each solution after complete electrolysis at a specified potential. The electrochemically-formed complexes were then identified by chromatographic rention time.

The results obtained on the DMAD system lead to three observations. (a) The potential applied to the electrochemical cell influences the yield of individual components as shown by the chromatograms Fig. 1 for pH 5 preparations that were electrolyzed at different potentials. (b) The same Tc-DMAD complexes are formed by electrochemical preparation and by chemical ($NaBH_4$) preparation. (c) The control of both potential and pH enables a specific Tc-DMAD complex to be produced in high yields more easily by electrochemical generation than by chemical generation. This is illustrated for component F in Fig. 2 which is prepared in almost pure form by electrochemical (EC) reduction compared to chemical reduction. These results are consistent



Tc-EC-DMAD, CA, Chromatography Obtained at pH 5, and at Selected Potentials

Figure 1



Chromatographic Comparison of Tc-DMAD, CA, Peak F, Prepared by EC (1 pH, 1 Potential) and CHEM, at Preparation pH of 3, 5, and 7

Figure 2

with our results for the other diphosphonate systems studied thus far and are supportive of electrochemical reduction as a means of facilely generating in pure form a high yield of a particular diphosphonate imaging agent.

Electrodes for Rapid *in situ* Electrochemical Generation of Skeletal Imaging Agents

That the nature of the electrode surface influences the course of the chemical reaction to yield different concentrations of products is not surprising since the electron transfer that causes the reduction of TcO_4^- occurs at the electrode-solution interface. Thus, we have good evidence that the nature of the electrode surface can be used in conjunction with electrode potential and formulation pH as a means of controlling the electrode reaction to improve yields of specific components.

We have initiated a comprehensive study of the electrochemical generation of Tc-MDP complexes at reticulated vitreous carbon (RVC). RVC is a type of carbon foam that can be used as a porous electrode with a large surface area. We are interested in this material for the rapid electrochemical generation, by means of the proposed electrochemical syringe, of specific complexes that exhibit optimum imaging properties. The objective of this study was to determine what effect the carbon surface of RVC has on the generation products as compared with chemical reduction procedures and electrochemical generation at the mercury electrodes that we have used in the past. If we are able to obtain the same control over product distribution with RVC as we have demonstrated with mercury electrodes, then RVC should be useful for the proposed application.

This study involved three types of experiments (11). First cyclic voltammetry is used to determine the reduction potential of TcO_4^- in the presence of MDP ligand. Tc-MDP complexes are then generated electrochemically at an appropriate potential applied to the RVC electrode. The resulting products are analyzed by HPLC to determine the distribution of Tc-MDP complexes. This experiment is repeated at several potentials and then at several pHs to determine the influence of these experimental paramenters. We have performed these experiments at pH 2, 5, 7, 9, and 11 at four potentials for each pH.

The results of these experiments can be summarized as follows: (a) The reduction potential of TcO4- shifts to more negative values as the solution pH is increased. (b) Electrode potential and solution pH can be used in concert to control yields of specific Tc-MDP complexes. (c) Higher yields of complexes are obtained at RVC than at mercury electrodes. (d) The same complexes, as determined by chromatographic retention time, are formed at RVC as we have found at mercury electrodes and by chemical reduction with $NaBH_4$.

In conclusion, RVC is a viable electrode material for the proposed electrochemical syringe. It offers the advantage of a flow-through electrode with a large surface area for the rapid generation of Tc-MDP complexes at very low cost. RVC also avoids the toxicity problems of mercury electrodes.

Electrochemical Sensors for Tc and Re Imaging Agents

In the field of electrochemical sensor development, charged polymer films on electrode surfaces have demonstrated their utility for the selective extraction and preconcentration of electroactive analytes, most notably metal ions such as $Fe^{2+/3+}$, Cu^{2+}, Co^{2+}, Cr(VI), and charge transition metal complexes such as $Fe(CN)_6^{3-}$. The success of these sensors is based on the extraction of the component of interest from the sample by the polymer film and the resulting enhancement in concentration at the electrode surface, which improves sensitivity and detection limit.

Sensor for TcO_4^-. Since TcO_4^- is both electroactive and negatively charged, an electrochemical sensor based on an electrode that is modified with a positively charged polymer film should enable the direct detection of TcO_4^- at very low concentrations with a single electrode sensing device. We are exploring this concept with the objective of making a sensor for pertechnetate that could be used for its determination in radiopharmaceutical preparations, technetium generators, and in the environment.

The polymer poly(dimethyldiallylammonium chloride), DMDAAC, has been demonstrated to enhance surface concentrations of negatively charged species at electrode surfaces (12). Thus, we are evaluating this polymer first as a means of developing the pertechnetate sensor. We have developed a procedure for immobilizing the polymer film on a graphite electrode by crosslinking. These electrodes are rugged and the polymer is attached tenaciouly because it soaks into the slightly porous electrode and is thereby anchored to the substrate. The general experimental approach is to prepare a polymer coated electrode, immerse it in a solution of pertechnetate, and determine if the TcO_4^- is loaded into the polymer by its voltammetric reduction.

For preliminary experiments we used ferricyanide as a representative electroactive anion (11). Ferricyanide is a reversible electrochemical system, which enables us to perform multiple experiments on a sensor without affecting its properties. It also enables us to develop the sensor while avoiding unneccessary handling of radioactive material. Our initial studies in which we are detecting ferricyanide that partitions into the polymer film with cyclic voltammetry and differential pulse voltammetry have shown an enhancement of the detector signal for ferricyanide at the polymer modified electrode of x10 as compared to that at a bare electrode.

Similar experiments have been performed on the prototype sensor with samples of TcO_4^-, and the same 10-fold enhancement of signal occurs at the polymer-coated electrodes. The following aspects of the TcO_4^- sensor are being evaluated: response time, detection limit, operating range, reproducibility, lifetime, and interferences from other components of real samples. The effect of polymer film thickness and degree of crosslinking on these performance factors is also being investigated.

Sensor for Heart Imaging Agents. Our objective in this project is to develop sensors for *in vivo* monitoring of technetium and rhenium compounds for imaging the heart. Our strategy for development of an *in vivo* sensor for 99m-Tc heart agents is a microelectrode sensor that would rely on a polymer coating into which the agent would partition and thereby enhance its detection in the biological matrix. Experiments performed thus far have been planned to answer questions regarding sensor design and selection of materials for key components. These experiments have been performed on a Re analog, $[Re(diars)_2Cl_2]Cl$, of the 99m-Tc agents to avoid unnecessary handling of radioactive material.

The search for a polymer that will extract the imaging agent and thereby concentrate it at the probe has begun. Since the agent is electroactive, extraction into a film that is immobilized on an electrode surface is easily measured by an electrochemical technique such as cyclic voltammetry in which the peak current is proportional to the concentration of the agent in the film. Experiments are being conducted on a series of polymers with varying lipophilicity and charge in order to determine the extraction characteristics of the agent. The polymers are poly(N-vinylpyrrolidone), poly(vinyl alcohol), poly(acrylic acid), and poly(dimethyldiallylammonium chloride). We have shown that these polymers can be immobilized on graphite electrodes as a network by exposure of dry coatings of the polymer on the electrode surface to gamma radiation as the cross-linking agent (12,13). Once a suitable polymer is found, it will be evaluated as a coating on a carbon fiber ultramicroelectrode (diameter, ca. 5um) for an electrochemical agent sensor. Results to date indicate that $[Re(diars)_2Cl_2]^+$ partitions most strongly into a poly(acrylic acid) film at a solution pH at which the acid is dissociated to give an anionic polymer network.

Structural Characterization of Tc and Re Diphosphonates

Radiopharmaceuticals prepared by reduction of $^{99m}TcO_4^-$ in the presence of excess MDP or HEDP have been shown by HPLC analysis to be a mixture of Tc-MDP or Tc-HEDP complexes. We have chromatographically isolated a pure Tc-MDP complex at sufficiently high concentrations for structural analysis by EXAFS and nuclear magnetic resonance (NMR) spectroscopy. Two Re-HEDP complexes have also been prepared. The first complex was made via a ligand substitution method, with trans-

[(pyridine)$_4$(O)$_2$Re]Cl*xH_2O as the starting material. The second complex was prepared by reduction of ReO_4^- with stannous ion in the presence of excess HEDP.

^{1}H NMR studies on all of these samples show resonances that are attributable to protons on MDP or HEDP. However with these spectra we are unable to unambiguously distinguish between protons on free excess ligand and ligand coordinated to Tc or Re. When the ^{99}Tc nucleus of the Tc-MDP sample was examined, no resonances were observed. This could be caused by ^{99}Tc being in a paramagnetic oxidation state. ^{31}P NMR and ^{187}Re NMR experiments are planned to provide more information about these systems.

EXAFS studies on the Tc-MDP sample have shown that there are 5.9 oxygen atoms at 1.99 A and 1.5 Tc atoms at 2.99 A (14). These results were interpreted in terms of a possible tetrameric structure. EXAFS spectra on the Re-HEDP complexes show 4-5 Re-O bonds at 2.02 A and 1 Re-O bond at 1.69 A. Two peaks between 3.0 and 3.8 A were also found in the spectrum for the tin reduced sample, but not for the substitution product. We have tentatively assigned these two peaks to a Re-Re and a Re-Sn interaction. The presence of this interaction suggests an oligomeric structure similar to that found for the Tc-MDP complex (14).

Raman spectroscopy is also planned to detect the presence of Tc=O and Re=O bonds in the complexes. Preliminary Raman experiments reveal an intense band at 1025 cm^{-1} in the trans-[pyridine)$_4$(O)$_2$Re]Cl*H_2O starting material, which is attributed to a Re=O bond. Spectra of the Re-HEDP sample prepared by reduction with $SnCl_2$ gave an intense band at 1125 cm^{-1}, which we attribute to the a mono-oxo species.

Further experimentation on these complexes should give additional structural information.

Development of Capillary Electrophoresis as a Separation Technique for Nuclear Medicine

In the past ten years capillary electrophoresis (CE) has developed into a powerful tool in the field of separation science (15). CE gives separations similar to more traditional bed electrophoresis techniques, but with dramatically improved separations and in much shorter times.

In this technique, a sample is introduced into one end of a fused silica capillary, typically 50-100 um i.d. and 10-100 cm in length, and each end of the capillary is immersed into an electrolyte solution. The sample components migrate through the capillary under an electric field of typically 300 V/cm, and are detected as they move past an in-line detector. The separation mechanism is based largely on charge-to-size ratio. Perhaps the greatest advantage of this technique compared to traditional

liquid chromatography is the very high separation efficiency possible, with literature reports of up to 1 million theoretical plates, as compared to typically 10,000 plates for reversed phase liquid chromatography. This separation efficiency dramatically increases peak capacity and resulting confidence in the purity of single peaks.

CE instruments became commercially available about four years ago, and the technique is currently undergoing rapid growth. New applications in the separation of biological molecules such as DNA and proteins, modifications of the capillary walls to induce different separation selectivities, and new detectors such as electrochemical, mass spectrometric and radioisotopic are being reported. The growth of this technique is witnessed by the inclusion for the first time, of a capillary electrophoresis section in the 1990 biannual Fundamental Review issue of *Analytical Chemistry* (16).

CE has the potential to be a valuable separation technique for the characterization and development of improved radiopharmaceuticals. Both the greater peak capacity and the orthogonal retention mechanism compared to liquid chromatography will prove beneficial in the separation of mixtures of imaging agents.

Using borrowed instrumentation, we have been able to perform some experiments to evaluate the feasibility of using CE for the separation of Tc and Re diphosphonate skeletal imaging agents. (We have used the chemical similarities of the Re analogues to Tc complexes to our advantage and to avoid unnecessary radioactive contamination of instrumentation.) Electropherograms have been obtained on samples of pure Na_2HEDP ligand, pure ReO_4^-, and a Re-HEDP mixture of complexes prepared by reducing ReO_4^- with stannous ion in the presence of excess HEDP and gentisic acid stabilizer.

Fig. 3 shows an electropherogram of the Re-HEDP sample. Detection was by UV absorption at 214 nm at which ReO_4^-, gentistic acid, and complexes of Re-HEDP are known to absorb. Experiments on pure ReO_4^- and pure gentistic acid showed that the sharp peak at 6.8 min is excess ReO_4^- in the sample and the large peak at 2.6 min is gentistic acid. The broad response in the region 3.7 to 5.7 min is attributed to Re-HEDP complexes in the sample. Electropherograms with detection at 200 nm at which both Re-HEDP complexes and free HEDP ligand absorb showed a separation of the free ligand in the sample from the Re-HEDP complexes.

Closer examination of the broad response region in Fig. 3 reveals that at least 2 components are partially resolved. Whether these represent different Re complexes or oligomers remains to be investigated. How many more components lie within this broad region remains to be answered by our proposed work. We anticipate very similar behavior for the Tc based complexes.

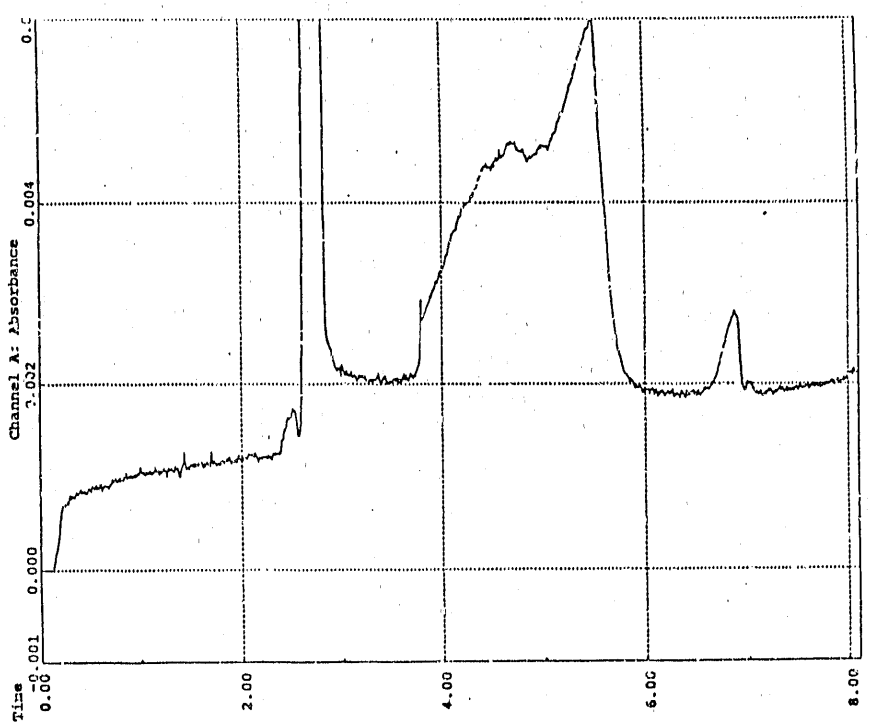


Fig. 3. Electropherogram of Re HEDP sample.

The results of these preliminary experiments with CE show promise for the analysis of radiopharmaceuticals consisting of diphosphonate metal complexes. The UV-VIS detector exhibited adequate sensitivity for the samples investigated thus far. The addition of a radiochemical detector would improve sensitivity and give added specificity for radioactive components in the sample. Further work is required to develop the full resolving power of CE as applied to this system so that all of the Re-HEDP components in the broad peak in the electropherogram in Fig. 3 can be resolved. An understanding of the interaction of diphosphonate complexes with the capillary wall will be required to fully exploit the resolving power of the technique.

Effects of Biological Milieu on Tc-PAA Complexes

Although ^{99m}Tc-diphosphonate radiopharmaceuticals have been widely used in clinical settings for the last 15 years, there is

little information about how these complexes are affected by physiological and pathological processes in the body. It is known that complexation of Tc with a diphosphonate ligand results in a mixture of complexes that has a variable composition. This composition is sensitive to formulation conditions such as pH, temperature, ligand to reductant ratio, Tc concentration, presence of oxygen, and the reducing agent used (3). Given this sensitivity, it is possible that the complex that is injected into the patient is altered by the biological milieu, and is not the chemical species actually responsible for the imaging. For this reason we have investigated the effect of the biological milieu on a Tc-phosphonoacetic acid (Tc-PAA) mixture using anion exchange HPLC with gamma detection to monitor the chemical identities of the Tc-PAA complexes in the radiopharmaceutical mixture.

The Tc-PAA radiopharmaceutical was prepared by reducing $^{99m}TcO_4^-$ with sodium borohydride in the presence of excess PAA. Analysis of this mixture by HPLC showed two major components and one minor component. Initially, *in vitro* experiments were performed to evaluate the effect of saline and urine on the three Tc-PAA complexes in the radiopharmaceutical. Chromatograms of the radiopharmaceutical diluted with saline and urine indicated that saline has no significant effect on the composition of the mixture, as there was only a slight shifting of retention times. However, one of the major components of the mixture reacted slowly in urine to yield a new Tc-PAA complex.

In vivo studies were performed by injecting the Tc-PAA mixture into a Sprague-Dawley rat and collecting the urine via a urine catheter. Chromatograms of the urine showed the same components as were in the radiopharmaceutical before injection and approximately the same relative concentrations. Thus, the bioligical milieu of the rat exerts no significant change on the chemical identities of the Tc-PAA complexes that are removed by the kidney. This result is consistant with the results of similar experiments that were performed on a Tc-HEDP complex, which was found to pass through dogs unchanged (7, DOE/ER/60487-3).

These studies clearly establish the feasibility of using HPLC to monitor injected Tc-phosphonate complexes that clear the body in urine. This analytical capability should prove useful in metabolism studies of individual Tc-phosphonate complexes.

Electrochemical Characterization of Thiolato-Technetium Complexes That Are Potential Myocardial Imaging Agents

As part of our program to study the redox properties of technetium and rhenium complexes and the implication that their redox chemistry has on the biodistribution of these complexes, a new series of technetium-thiolato complexes has been studied by electrochemical and spectroelectrochemical techniques.

Technetium(III) Complexes *trans*-$[Tc(SR)_2(DMPE)_2]^+$. The use of a reduction-substitution route to prepare the thiolato-technetium complexes *trans*-$[Tc(SR)_2(DMPE)_2]^+$ from the Tc(V) starting material *trans*-$[Tc(OH)(O)(DMPE)_2]_2^+$ and alkane- or benzenemethanethiols has been investigated (DMPE represents 1,2-bis(dimethylphosphino)ethane). The four newly prepared complexes (R = C_2H_5, n-C_3H_7, $CH_2C_6H_5$, $CH_2C_6H_4$-*p*-OCH_3) were characterized by spectroelectrocnemistry (17). The complexes exhibit a reversible Tc(III/II) couple at about -0.5 V, a reversible Tc(II/I) couple at about -1.8 V, and an irreversible redox process at about +0.9 V that is tentatively assigned as resulting from a metal-based Tc(IV/III) couple (all potentials obtained in 0.5M TEAP/DMF vs Ag/AgCl (3 M NaCl)). The potentials of all three couples are dependent on the nature of the thiolato R group; e.g., the Tc(III/II) couple varies from -0.513 V for R = benzyl to -0.622 V for R = n-propyl. These dependencies are understood on the basis of competition between the sigma donating and pi accepting properties of the various thiolato ligands. The visible spectra of the five thiolato-Tc(II) complexes were obtained by spectroelectrochemical techniques. For both the Tc(III) and Tc(II) complexes the visible spectra are dominated by sulfur-to-technetium charge-transfer transitions, the energies of which depend on the technetium oxidation state but do not depend on the nature of the thiolato R group.

Bis(1,2-bis(dimethylphosphino)ethane)technetium(III) Complexes with Arene-thiolato Ligands. The reaction of *trans*-$[Tc^{V}(OH)(O)(DMPE)_2]_2^+$ with a series of arenethiols in base produces the novel complexes cis-$[Tc^{III}(SC_6H_4\text{-}p\text{-}X)_2(DMPE)_2]^+$ for X = H, Cl, CH_3, OCH_3, $C(CH_3)_3$ (18). Electrochemical and spectrochemical measurements show reversible Tc(III/II) redox couples in the range -0.19 V to -0.38 V vs Ag/AgCl (3 M NaCl). Irreversible couples are exhibited at ca. -1.1 V to -1.2 V for Tc(II/I) and +0.7 V to +0.9 V for Tc(IV/III). The nucleophilicity at sulfur can be modified by changing the nature of the R group. S-alkyl complexes are ca. 300 mV harder to reduce (Tc(III/II)) than the S-arenes. For the Tc(II/I) couple, S-alkyl complexes are ca. 600 mV harder to reduce thatn S-arene complexes. Subtle variations of $E^{o'}$ with R can be tracked throughout the series. These variations can be related to the electron donating or withdrawing power of the substituent and are more profoumd than those resulting from equivalent R changes on the phosphine ligands.

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