FUNCTIONALIZED ISONITRILE COMPLEXES OF TECHNETIUM

AS RADIOPHARMACEUTICALS

by

JAMES FREDRIC KRONAUGE JR.

B.S., University of Cincinnati (1979)

SUBMITTED IN PARTIAL FULFILLMENT

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This doctoral thesis has been examined by a committee of the Department of Chemistry as follows:

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Professor Greg Petsko
To Mom and Dad with my love.
"everything is for the best in this the best of worlds"

Voltaire's Candide
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JAMES FREDRIC KRONAUGE, JR.

Submitted to the Department of Chemistry in May, 1987 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

ABSTRACT

Various carboxy, carboalkoxy and aminocarbonyl substituted isonitrile ligands and their respective hexakis(isonitrile)technetium(I) complexes have been synthesized with the long lived isotope $^{99m}$Tc. These compounds were characterized by IR, FAB mass spectrometry, RP-HPLC, and $^1$H, $^{13}$C, $^{99m}$Tc NMR. Synthesis was extended to the "no carrier added" concentration of $10^{-9}$M for production of complexes with the isotope $^{99m}$Tc and their subsequent evaluation as radiopharmaceuticals. Radiochemical identity, yield, and purity of all compounds were established by reverse-phase HPLC. Initial screening by dynamic Anger scintillation camera studies identified one complex, hexakis(2-carbomethoxyisopropyl isocyanide)technetium(I), Tc(CPI)$_6^{+}$, as a prospective myocardial imaging agent. Subsequent biodistribution studies confirmed this agent's biological properties, culminating in successful human clinical trials.

To evaluate in vivo integrity of this potential radiopharmaceutical, synthesis and characterization of the possible metabolic hydrolysis products was performed. Identification of these products allowed HPLC analysis of in vitro enzymatic hydrolysis in plasma of various animal species. Differences in enzyme systems was established as the source of interspecies biodistribution variation. These studies also confirm the rabbit as an optimistic animal model for screening these ester isonitrile complexes.

Cellular kinetics and binding characteristics of Tc(CPI)$_6^{+}$ were evaluated in chick embryo heart cells grown in monolayer cultures. Myocytes showed uptake of Tc(CPI)$_6^{+}$ to a plateau level with a half-time ($t_{1/2}$) of 4.1 ± 0.7 min; $t_{1/2}$ appeared independent of extracellular Tc(CPI)$_6^{+}$ concentration. Plateau level Tc(CPI)$_6^{+}$ uptakes were a linear function of extracellular Tc(CPI)$_6^{+}$ concentration. Tracer $^{99m}$Tc(CPI)$_6^{+}$ uptake (binding) was not competitively displaced by carrier $^{99}$Tc(CPI)$_6^{+}$. Uptake was temperature-sensitive; however, several inhibitors of cationic membrane transport showed no significant effect. The data do not demonstrate a specific mechanism for uptake of Tc(CPI)$_6^{+}$, however, results show preferential binding to myocytes in a manner proportional to the delivery of the complex to the extracellular spaces. Such properties would be desirable for a myocardial perfusion imaging agent.
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CHAPTER 1

AN INTRODUCTION TO THE PROBLEM
Introduction:

The first recorded use of a metal for medical purposes was the use of gold in China around 2500 BC.(1) Even through the Dark Ages precious metals were used to ward off disease because of their "magical" properties. More recently medicine has discovered numerous applications where metals produce beneficial effects.(2) The "magic" that metals possess, at least to the chemist, is their individual preferences for the specific geometries and distances to their first "inner" coordination sphere of surrounding atoms or ligands. It is usually the nature, size, and shape of potentially coordinating molecules that produces the specificity of metals in enzymes or metabolic events like ion transport. Only recently has the study of metal "essential" compounds focused on the relationship of biological distribution or activity with molecular structure.(1,3)

A favorable difference in biological distribution is the first principle for selectivity of a drug.(4) In the area of diagnostic agents this is the single most important factor for determining sensitivity of a test. For the field of nuclear medicine technetium-99m has been the radioactive tracer of choice for over 85 percent of all clinical studies because of its ideal physical properties.(5) The variety of applications are due to the central location of technetium in the periodic table and thus its rich chemistry. The accessibility of 8 oxidation states and numerous coordination geometries allows the wide variation of structural properties.(6) Modifying the technetium metal center to localize in normal heart muscle was the goal of this work. To achieve high target organ to background ratios these complexes were derivatized with metabolizable functional groups to facilitate clearance from nontarget organs. The described technetium complexes should provide an improvement for diagnosing heart disease in patients.
Background:

The first man-made element, technetium, has achieved widespread use in the field of diagnostic nuclear medicine due to the physical properties of the isotope $^{99m}$Tc. These characteristics of a 6.02 hour half-life with almost complete decay to the daughter $^{99}$Tc by emission of a single 140 KeV photon are optimal for detection by an Anger camera with low radiation exposure to the patient. In nuclear medicine coordination compounds of this short-lived isotope are injected intravenously into the bloodstream of a patient. The compound will distribute in the body and localization of the drug is visualized and monitored dynamically, by virtue of the gamma photon given off during decay.

The challenge to the chemist is to modify the technetium metal atom to produce an organ specific radiopharmaceutical. Since the longest lived isotope of technetium ($^{97}$Tc) has a half-life of $2.6 \times 10^6$ years, virtually all traces of the element decayed millions of years before life on this planet began. Consequently biological organisms, particularly humans, have not had the opportunity to utilize or adapt to the presence of this element in the environment. The biochemistry of the neighboring element manganese is not instructive for designing these radiopharmaceuticals, because the most stable oxidation state, Mn(II), is not readily accessible for technetium. The technique used to adjust the biological fate of the technetium has been to complex the metal, in various oxidation states, with ligands of various charge and lipophilicity. Generally the effect has been to target one of the two major routes of excretion, renal or hepatobiliary, because the body usually eliminates chemicals it does not need. One of the more difficult problems is to design technetium-based radiopharmaceuticals that localize
in nonexcretory organs such as the heart or brain. The ability to access such organs is essential for determining the extent of disease.

Heart disease is the leading cause of death in America today, accounting for sixty percent of all fatalities. The successful treatment of these patients is contingent upon early detection and monitoring during therapy. The most prevalent form of heart disease is coronary artery disease (CAD). CAD is the loss or reduced perfusion of blood and thus nutrients and oxygen to the heart muscle (myocardium). Typically CAD occurs in a localized region of the myocardium which becomes fatigued, resulting in wall motion defects and reduced cardiac output. The reduced performance of the heart increases hypoxia in the myocardium and the extremities which produces the associated pain or angina.

The first screen for patients suspected of coronary artery disease is the electrocardiogram (EKG), which measures the electric currents that traverse the heart. It does not measure the mechanical events of cardiac contraction or differences in blood flow in coronary arteries. In addition, evaluation of an abnormal EKG is often complicated if nerve impulses pass through an under-perfused (ischemic) zone and do not induce depolarization of the muscle, a condition known as heart block. Also no true positional lead for the EKG exists to analyze the posterior wall of the myocardium. Of primary importance for the evaluation of a patient with CAD is to establish the extent of blood flow down to the arteriolar level in the myocardial tissue. Particularly important is to know if the regional myocardial perfusion is simply reduced or completely diminished and if permanent damage has occurred (infarction) or the condition is transient, i.e. stress-induced ischemia.
The current nuclear medicine procedure for evaluating CAD patients is the use of radioactive $^{201}$Thallium chloride. Patients suspected of transient heart disease are given an injection of $^{201}$TlCl while under physical exertion, such as on a treadmill, and images are obtained of the isotope localized in the myocardium. Thallium(I) is apparently distributed as a potassium analogue due to its similar size and charge, thus it is actively accumulated in contractile muscle through the Na$^+$/K$^+$ pump. This uptake is to a good approximation, at least initially, proportional to blood flow in the myocardium. Several disadvantages, however, exist for the use of this agent. The relatively low energy of the emitted X-ray photon (68-80 KeV) results in substantial absorption by the body's soft tissue and lowers detector efficiency. Also the long physical half-life (73 hours) and biological half-life (57 hours) limits the dosage that can be given to a patient. Consequently this lower photon flux produces a lower resolution image that requires about ten minutes to acquire.

The biological properties of a potassium(I) analogue also cause some confusion in interpreting the images. After the initial uptake, $^{201}$Tl$^+$ is not trapped in the cell but exists in a dynamic state of uptake and release by the muscle cells to the intercellular space. The net effect is a gradual washout of activity back into the bloodstream, which in normal myocardial tissue follows a monoexponential rate. In the case of the transient ischemic patient, once stress has been removed, regional perfusion returns to normal, and the defect will disappear due to redistribution of the radioactive thallium. Physicians have used this property of redistribution to their advantage in diagnosing patients with transient heart disease. However, the rate at which the images return to
normal varies greatly and any delay in imaging immediately after TlCl injection may result in a failure to visualize the perfusion defect.

The more attractive physical decay properties of technetium-99m, its greater availability and lower cost, have prompted the drive for a $^{99mTc}$-based myocardial perfusion agent to replace thallium-201. An ideal heart agent should have the properties of high myocardial accumulation as well as rapid background clearance or low blood, lungs, and liver activity. The uptake of the agent in the myocardial tissue should be directly proportional to blood flow, and the washout from the heart should be slow, so that sufficient time is available for systematic studies and redistribution does not occur. Several researchers have synthesized technetium compounds that demonstrate myocardial localization in numerous animal species.\(^{13,14}\) However, when brought to the ultimate test, i.e., humans, they produced poor results.\(^{12}\) All of these agents were monocations of rather high lipophilicity. They demonstrated a wide interspecies variation in biodistribution and reportedly "did not work in humans" for various reasons including ease of metal reduction or binding to blood components.\(^{12}\)

It is obvious that the success of an agent is due to a number of complex interactions, not just lipophilicity. The single exception has been the complex hexakis(t-butyl isocyanide)technetium(I), Tc(TBI)$_6^+$, as shown by Davison, Abrams and Jones.\(^{9}\) Although this compound fared well in clinical trials, two artifacts of the biodistribution diminished the image and diagnostic quality of the agent. Due to the lipophilic nature of the complex, a significant amount of activity accumulated in the lungs and liver, obscuring the heart; see Figure 1.
Consequently a relatively long delay time was required between injection and imaging to allow for clearance of lung activity. An added disadvantage of this slow clearance is the effective diluting of the injection bolus. Rapid accumulation of the tracer into the myocardium is preferred in stress tests where transient ischemia begins to reverse immediately after the stress is removed. The substantial accumulation and retention of activity in the liver also caused image interference. Since the heart rests on top of the diaphragm immediately above the liver, the apex and inferior wall of the left ventricle are flooded by scatter from the very hot right lobe of the liver. This is particularly bad because both of these areas, being distal to the aorta, are usually involved in coronary artery disease.

The object of this work was to determine if functionalizing the isonitrile ligand would yield complexes that exhibit more favorable biodistribution properties. The initial approach was to produce compounds with terminal groups that could be metabolized in vivo, thus yielding a route of excretion from nontarget organs, i.e., the blood, lungs, and liver. Ligands were synthesized that contained various ester and amide groups distal to the isonitrile function. It was felt that nonspecific esterases or proteinases in the liver and blood could hydrolyze these terminal groups and yield the more hydrophilic anionic species. The addition of different alkyl groups afforded the design of a large number of similar compounds varying in lipophilicity, shape and fluctuation. These ligands provided a system to study the effect on their biological activity of subtle changes in molecular structure. The ligands and respective technetium-99 complexes were synthesized and characterized. Correlation of the identified compounds with the
radiopharmaceuticals made with the gamma emitting $^{99m}$Tc isotope was accomplished with RP-HPLC using a combination of UV absorption and gamma ray scintigraphic detection. This step was necessary to confirm the chemical identity of the species produced in the reaction at the no carrier added (NCA) $^{99m}$Tc generator eluant concentration ($\sim 10^{-8}$ M).(6)

The subsequent complexes of the short-lived gamma emitting $^{99m}$Tc isotope were screened for biodistribution kinetics in an animal model system. Initial studies involved intravenous injection of the complex into rabbits and the acquisition of sequential images on an Anger camera. By comparing counts in specific regions over time, the uptake and clearance in different organs could be studied. These experiments indicated several compounds of potential interest which were further evaluated in mice, rats, and guinea pigs. Table II lists the names and formulas of the ligands synthesized for these studies. (The inorganic nomenclature has been used to avoid confusion in later chapters.) Of the thirty compounds tested, one in particular hexakis(2-carbomethoxyisopropyl isocyanide)technetium(I), $\text{Tc(}\text{CPI})_6^+$, demonstrates a significant improvement over $\text{Tc(TBI})_6^+$ as a myocardial perfusion agent. In vitro base-catalyzed hydrolysis studies employing HPLC-FAB(+) mass spectrometry techniques allowed the identification of hydrolysis products of this agent. In vivo and in vitro enzyme kinetics experiments with blood plasma and liver homogenate showed a large interspecies variation in hydrolysis rate that correlated with biodistribution studies. The demonstrated ability of this compound to undergo enzymatic hydrolysis as well as its more hydrophilic character appear to increase its rate of nontarget organ clearance. Chick heart cell culture studies were also initiated to study the uptake mechanism
and the origin of myocyte specificity. Subsequent human studies have confirmed the improved imaging properties of $\text{Tc(CPI)}_6^+$ as predicted by the animal modeling studies.
Figure 1.1 Whole body images, at stress and at rest, of two normal volunteers six hours after intravenous injection of $^{99m}$Tc(t-butyl isocyanide)$_6^+$. 
References:


CHAPTER 2

ORGANIC SYNTHESIS OF FUNCTIONALIZED ISONITRILE LIGANDS
Introduction:

Numerous synthetic routes are known for the production of isonitriles as outlined by Ugi.\(^{(1)}\) The most direct and efficient path to the free compounds involves the dehydration of an N-monosubstituted formamide.\(^{(2,4)}\) A wide variety of acylating agents dehydrate formamides in the presence of bases.\(^{(1-3)}\) The most generally applicable and efficient is the combination of phosgene and trialkylamines.\(^{(2)*+}\) However, due to the necessary handling precautions involved in the use of phosgene, a similar procedure using trichloromethylchloroformate (diphosgene) was adapted from work by Efray et al.\(^{(3,5)}\)

The general synthesis consisted of esterification of the carboxylic end of an amino acid followed by acylation of the free amine\(^{(4)}\) and subsequent dehydration with diphosgene (Figure 1). The resulting ester isonitrile was then converted to the corresponding amide isonitrile by nucleophilic attack at the carbonyl by the desired amine (Figure 2).\(^{(8)}\) This reaction sequence worked well, yielding all the desired ligands from the corresponding available amino acids. Presented here is a detailed description of the synthesis of one of the most interesting ligands tested. It is followed by a list of the characterized functionalized isonitriles synthesized. Characterization was accomplished by IR, \(^{1}\)H and \(^{13}\)C NMR. Isonitriles exhibit a characteristic IR absorption frequency at \(\nu_{CN} = 2100\text{ cm}^{-1}\) and have a distinctive stench. These along with NMR data provided confirmation of the functional groups present and their conformation. All the synthesized esters were viscous liquids at room temperature\(^{(7,8)}\) while the amides were white solids at room temperature.

\* Phosgene HCl content must be low.
\+ Trialkylamine must be free of H\(_2\)O and amines that can undergo acylation.
Experimental:

Amino acid starting materials were obtained from Aldrich Chemical Company, Milwaukee WI or Fluka AG, W. Germany, as the free acids, reagent grade, and were used as received. Solvents used in the esterification step were spectrographic grade (Omnisolv) from EM Science, Gibbstown NJ. Infrared spectra were obtained on a Perkin-Elmer 1420 spectrophotometer as the neat ligand on sodium chloride plates or in chloroform solution. Fourier transform $^1$H NMR spectra were obtained on a Bruker WM 250 MHz instrument with CDCl$_3$ as solvent and TMS as the internal standard. $^{13}$C NMR spectra were obtained on a Varian 400 MHz instrument with CDCl$_3$ as the standard.

**Synthesis of Methyl 2-aminoisobutyrate hydrochloride.** 2-aminoisobutyric acid (25 g, 0.24 mol) was placed in a 500-mL three-neck flask and the system purged with argon. Dry methanol (200 mL) was added via cannula and the mixture was stirred with a magnetic stir bar. Anhydrous hydrogen chloride was bubbled carefully through a medium porosity glass fritted dispersion tube and the addition continued for 30 minutes until all the amino acid had dissolved. The clear solution was stirred for another 30 minutes at room temperature, then transferred to a 1-L round-bottom flask and the volume reduced under vacuum to obtain a viscous oil. The ester hydrochloride salt was crystallized from a methanol/hexane/diethyl ether mixture as a white crystalline hygroscopic solid which was filtered on a medium glass frit and dried in a vacuum oven at 40 °C, 10 mmHg, for 24 hours. The yield was 33 g (90%): mp 157-158 °C.

**Synthesis of methyl 2-formamidoisobutyrate.** To a solution of methyl
2-aminoisobutyrate hydrochloride (10 g, 0.07 mol) in formic acid (95%, 30 mL) was added a solution of sodium formate (5.5 g, 0.07 mol) in formic acid (10 mL). The solution was heated to dissolve the salts and then stirred for 2 hours at 25 °C. The white precipitate (NaCl) was removed by filtration through Celite 545. To the filtrate in a 300-mL round-bottomed flask equipped with a condenser and magnetic stirrer was added acetic anhydride (30 mL) and formic acid (50 mL). This mixture was stirred for 2 hours until the initial reaction had subsided and was then refluxed for another 20 hours. Another aliquot (20 mL) of the acetic anhydride/formic acid mixture (1:2 v/v) was added and the solution refluxed for 24 hours. The solvents were then removed in vacuo at room temperature and the product, methyl 2-formamidoisobutyrate, was separated from the residue by vacuum distillation (86-89 °C, 0.8 mmHg). Yield (60%) 5.3 g. ¹H NMR (CDCl₃) δ 1.6 (s, 6H, β-CH₃); δ 3.73 (s, 3H, OCH₃); δ 7.00 (bs, 1H, NH); δ 8.28 (s, 1H, CHO).

**Synthesis of carbomethoxyisopropyl isocyanide.** A solution of methyl 2-formamidoisobutyrate, vide supra, (5.1 g, 0.04 mol) in CH₂Cl₂ (100 mL) in a 500-mL four-neck round-bottom flask was purged with argon and cooled to -30 °C. Trimethylamine (35 mL) dissolved in CH₂Cl₂ (75 mL) was added. With stirring and continued cooling, a solution of trichloromethylchloroformate (diphosgene) (4.01 g, 0.02 mol) in CH₂Cl₂ (50 mL) was added dropwise. Upon addition, the colorless solution changed to a heterogeneous brown mixture. The reaction was allowed to warm slowly to room temperature and then heated to reflux for 30 minutes. The mixture was then treated with ammonium hydroxide (30%, 100 mL) and the CH₂Cl₂ layer separated. The aqueous layer was extracted with CH₂Cl₂ (5 x 50 mL) and the extracts combined. The CH₂Cl₂ extract was then dried
over sodium sulfate and the volume was reduced under vacuum. The product, carbomethoxyisopropyl isocyanide, was isolated by vacuum distillation (70-71 °C, 26 mmHg). Yield 2.8 g (55%): IR (CHCl₃) νₙₐₖ = 2141, νₓ = 1752 cm⁻¹. ¹H NMR (CDCl₃) δ 1.68 (s, 6H, β-CH₃); δ 3.83 (s, 3H, OCH₃). ¹³C NMR (CDCl₃) δ 169.2 (CO); δ 157.4 (CN); δ 59.0 (t, J = 6 Hz, α-C); δ 52.9 (OCH₃); δ 27.0 (CH₃).

Synthesis of 2-aminocarbonylisopropyl isocyanide, CNC(CH₃)₂CONH₂.

2-Carbomethoxyisopropyl isocyanide (0.5 g, 4.0 mmol) was dissolved in dry methanol (30 mL) in a 50-mL 2-neck flask equipped with a condenser and magnetic stir bar. The system was purged with argon for 15 minutes after which anhydrous ammonia was bubbled through the clear solution via cannula with stirring. The reaction was monitored by TLC (silica gel stationary phase, diethyl ether development) which gave a R_f of 0.40 for the product and 0.65 for the starting material. After 10 minutes of ammonia addition, the cannula was removed and the volume of the reaction reduced under vacuum to ~5 mL. The impure product was loaded onto a silica gel column (6.0 g; 3.1 cm x 9.0 cm) (E. Merck, EM Science, Kieselgel 60H, W. Germany) and eluted with diethyl ether. Aliquots containing the pure product were combined and the solvent reduced under vacuum. The white solid was filtered, washed with diethyl ether (20 mL), and dried in a vacuum oven at 41 °C for 24 hours. Yield 0.15 g (35%): mp 118-120 °C. IR (KBr) νₓ = 2147, νₓ = 1693, νamide = 1635 cm⁻¹, ¹H NMR (CDCl₃): δ 1.35 (s, 2H, NH₂); δ 1.65 (t, 6H, β-CH₃). ¹³C NMR (CDCl₃): δ 171.7 (CO); δ 159.7 (t, CN); δ 60.0 (t, α-C); δ 27.4 (β-CH₃).
Results:

The following derivatized isonitrile ligands were synthesized by methods similar to those described, vide infra, starting with the corresponding amino acid and alcohol. The characterizations for the resulting isonitrile ligands are presented here. Notice that compounds are named by the inorganic nomenclature as the functionalized isonitrile to avoid confusion in subsequent chapters.
Characterization of carbomethoxymethyl isocyanide, CHCH₂COOCH₃. A viscous oil, bp 64-65 °C @ 10 mmHg. IR (neat, NaCl plates): νₕ = 2162, νᵣ = 1761 cm⁻¹. ¹H NMR (CDCl₃): δ 3.78 (s, 3H, OCH₃); δ 4.20 (s, 2H, α-CH₂). ¹³C NMR (CDCl₃): δ 164.2 (CO); δ 160.1 (CN); δ 53.0 (OCH₃); δ 43.1 (t, α-CH₂).

Characterization of carboethoxymethyl isocyanide, CNCH₂COOCH₂CH₃. A viscous oil, bp 47-49 °C @ 0.8 mmHg. IR (neat, NaCl plates): νₕ = 2162, νᵣ = 1760 cm⁻¹. ¹H NMR (CDCl₃): δ 1.32 (t, 3H, CH₃); δ 4.24 (s, 2H, α-CH₂); δ 4.30 (q, 2H, OCH₂). ¹³C NMR (CDCl₃): δ 163.6 (CO); δ 160.3 (CN); δ 62.4 (OCH₂); δ 43.3 (t, α-CH₂); δ 13.7 (CH₃).

Characterization of carboxypropoxymethyl isocyanide, CNCH₂COOCH₂CH₂CH₃. A viscous oil, bp 75-80 °C @ 5 mmHg. IR (neat, NaCl plates): νₕ = 2162, νᵣ = 1760 cm⁻¹. ¹H NMR (CDCl₃): δ 0.97 (t, 3H, CH₃); δ 1.71 (hex, 2H, 2-CH₂); δ 4.18 (t, 2H, OCH₂); δ 4.26 (bs, 2H, α-CH₂). ¹³C NMR (CDCl₃): δ 163.8 (CO); δ 161.0 (CN); δ 67.8 (OCH₂); δ 43.2 (t, α-CH₂); δ 21.5 (2-CH₂); δ 9.9 (CH₃).

Characterization of carboisopropoxymethyl isocyanide, CNCH₂COOCH(CH₃)₂. A viscous oil, bp 68-88 °C @ 0.9 mmHg. IR (neat, NaCl plates): νₕ = 2160, νᵣ = 1760 cm⁻¹. ¹H NMR (CDCl₃): δ 1.30 (d, 6H, CH₃); δ 4.20 (bs, 2H, α-CH₂); δ 5.12 (hept, 1H, OCH). ¹³C NMR (CDCl₃): δ 163.3 (CO); δ 161.2 (CN); δ 70.7 (OCH); δ 43.5 (t, α-CH₂); δ 21.4 (CH₃).

Characterization of carbobutoxymethyl isocyanide, CNCH₂COO(CH₂)₃CH₃. A viscous oil, bp 67-92 °C @ 4 mmHg. IR (neat, NaCl plates): νₕ = 2161,
\[ v_{CO} = 1760 \text{ cm}^{-1}. \]  

**1H NMR (CDCl₃):** δ 1.70 (t, 3H, 4-CH₃); δ 2.15 (hex, 2H, 3-CH₂); δ 2.40 (pent, 2H, 2-CH₂); δ 4.95 (t, 2H, OCH₂); δ 5.10 (bs, 2H, α-CH₂).  

**13C NMR (CDCl₃):** δ 164.3 (CO); δ 160.9 (t, CN); δ 66.5 (OCH₂); δ 43.6 (t, α-CH₂); δ 30.4 (2-CH₂); δ 19.0 (3-CH₂); δ 13.7 (4-CH₃).

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**Characterization of carboisobutoxymethyl isocyanide, C₇N₇COOCH₃CH(CH₃)₂.** A viscous oil, bp 54-56 °C @ 0.4 mmHg. IR (neat, NaCl plates): \( v_{CN} = 2161, v_{CO} = 1760 \text{ cm}^{-1}. \)  

**1H NMR (CDCl₃):** δ 0.95 (d, 6H, CH₃); δ 2.00 (m, 1H, CH); δ 4.05 (d, 2H, OCH₂); δ 4.25 (bs, 2H, α-CH₂).  

**13C NMR (CDCl₃):** δ 163.7 (CO); δ 160.3 (t, CN); δ 72.1 (OCH₂); δ 43.2 (t, α-CH₂); δ 27.2 (2-CH₂); δ 18.6 (3-CH₃).

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**Characterization of carbotertbutoxymethyl isocyanide, C₇N₇COOC(CH₃)₃.** A viscous oil, bp 50-52 °C @ 0.4 mmHg. IR (neat, NaCl plates): \( v_{CN} = 2163, v_{CO} = 1763 \text{ cm}^{-1}. \)  

**1H NMR (CDCl₃):** δ 1.53 (s, 9H, CH₃); δ 4.16 (bs, 2H, α-CH₂).  

**13C NMR (CDCl₃):** δ 163.8 (CO); δ 160.3 (t, CN); δ 83.7 (OC); δ 43.8 (t, α-CH₂); δ 27.6 (CH₃).

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**Characterization of 1-carbomethoxyethyl isocyanide, C₇N₇(CH₃)COOCH₃.** A viscous oil, bp 64-65 °C @ 13 mmHg. IR (neat, NaCl plates): \( v_{CN} = 2147, v_{CO} = 1753 \text{ cm}^{-1}. \)  

**1H NMR (CDCl₃):** δ 1.55 (d, 3H, CH₃); δ 3.70 (s, 3H, OCH₃); δ 4.20 (q, 1H, α-CH).  

**13C NMR (CDCl₃):** δ 167.4 (CO); δ 159.3 (t, CN); δ 53.1 (OCH₃); δ 51.4 (t, α-CH₂); δ 19.1 (CH₃).

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**Characterization of 1-carboethoxyethyl isocyanide, C₇N₇(CH₃)COOCH₂CH₃.** A viscous oil, bp 56-57 °C @ 13 mmHg. IR (neat, NaCl plates): \( v_{CN} = 2147, v_{CO} = 1755 \text{ cm}^{-1}. \)  

**1H NMR (CDCl₃):** δ 1.33 (t, 3H, CH₃); δ 1.65
(d, 2H, β-CH₃); δ 4.30 (q, 2H, OCH₂); δ 4.36 (q, 1H, α-CH). ¹³C NMR (CDCl₃): δ 166.8 (CO); δ 159.0 (t, CN); δ 62.3 (OCH₂); δ 51.5 (t, α-CH); δ 19.1 (β-CH₃); δ 13.6 (2-CH₃).

Characterization of 2-carboxemethoxyethyl isocyanide, CNCH₂CH₂COOCH₃. A viscous oil, bp 66-67 °C @ 13 mmHg. IR (neat, NaCl plates): νCN = 2150, νCO = 1740 cm⁻¹. ¹H NMR (CDCl₃): δ 2.70 (t, 2H, β-CH₂); δ 3.70 (t, 2H, α-CH₂); δ 3.76 (s, 3H, OCH₃). ¹³C NMR (CDCl₃): δ 169.6 (CO); δ 157.1 (t, CN); δ 51.9 (OCH₃); δ 36.8 (t, α-CH₂); δ 33.6 (β-CH₂).

Characterization of 2-carb ethoxyethyl isocyanide, CNCH₂CH₂COOCH₂CH₃. A viscous oil, bp 70-71 °C @ 19 mmHg. IR (neat, NaCl plates): νCN = 2155, νCO = 1738 cm⁻¹. ¹H NMR (CDCl₃): δ 1.22 (t, 3H, 2-CH₃); δ 2.65 (tt, 2H, β-CH₂); δ 3.63 (tt, 2H, α-CH₂); δ 4.13 (q, 2H, OCH₂). ¹³C NMR (CDCl₃): δ 169.1 (CO); δ 157.2 (t, CN); δ 60.8 (OCH₂); δ 36.9 (t, α-CH₂); δ 33.8 (β-CH₂); δ 13.8 (2-CH₃).

Characterization of 1-carboxemethoxypropyl isocyanide, CNCH(CH₂CH₃)COOCH₃. A viscous oil, bp 52-53 °C @ 3 mmHg. IR (neat, NaCl plates): νCN = 2144, νCO = 1757 cm⁻¹. ¹H NMR (CDCl₃): δ 1.09 (t, 3H, γ-CH₃); δ 1.95 (m, 2H, β-CH₂); δ 3.83 (s, 3H, OCH₃); δ 4.20 (t, 1H, α-CH). ¹³C NMR (CDCl₃): δ 166.8 (CO); δ 159.5 (t, CN); δ 57.5 (t, α-CH); δ 53.0 (OCH₃); δ 26.0 (β-CH₂); δ 9.3 (γ-CH₃).

Characterization of 1-carboxethoxypropyl isocyanide, CNCH(CH₂CH₂)COOCH₂CH₃. A viscous oil, bp 56-57 °C @ 3 mmHg. IR (neat, NaCl plates): νCN = 2144, νCO = 1754 cm⁻¹. ¹H NMR (CDCl₃): δ 1.09 (t, 3H, CH₃);
δ 1.32 (t, 3H, 2-CH₃); δ 1.95 (m, 2H, β-CH₂); δ 4.27 (q, 2H, OCH₂);
δ 4.30 (t, 1H, α-CH). ¹³C NMR (CDCl₃): δ 166.3 (CO); δ 159.5 (t, CN); δ 62.1 (OCH₂); δ 57.5 (t, α-CH); δ 26.0 (β-CH₂); δ 13.6 (2-CH₃); δ 9.1 (γ-CH₃).

Characterization of 2-carbomethoxypropyl isocyanide,

CNCH₂CH(CH₃)COOCH₃. A viscous oil, bp 41-42 °C @ 3 mmHg. IR (neat, NaCl plates): νCN = 2147, νCO = 1739 cm⁻¹. ¹H NMR (CDCl₃): δ 1.28 (d, 3H, γ-CH₃); δ 2.80 (hex, 1H, β-CH); δ 3.60 (dq, 2H, α-CH₂); δ 3.75 (s, 3H, OCH₃). ¹³C NMR (CDCl₃): δ 172.5 (CO); δ 157.3 (t, CN); δ 51.9 (OCH₃); δ 43.6 (t, α-CH₂); δ 38.7 (β-CH); δ 14.0 (γ-CH₃).

Characterization of 1-carbomethoxyisopropyl isocyanide,

CNCH(CH₃)CH₂COOCH₃. A viscous oil, bp 80-82 °C @ 10 mmHg. IR (neat, NaCl plates): νCN = 2140, νCO = 1740 cm⁻¹. ¹H NMR (CDCl₃): δ 1.42 (m, 3H, β-CH₃); δ 2.60 (bd, 2H, β-CH₂); δ 3.67 (s, 3H, OCH₃); δ 4.10 (m, 1H, α-CH). ¹³C NMR (CDCl₃): δ 169.4 (CO); δ 155.9 (t, CN); δ 51.8 (OCH₃); δ 46.2 (t, α-CH); δ 41.0 (β-CH₃).

Characterization of 2-carbomethoxyisopropyl isocyanide,

CNCH(CH₃)₂COOCH₃. A viscous oil, bp 70-71 °C @ 26 mmHg. IR (neat, NaCl plates): νCN = 2140, νCO = 1750 cm⁻¹. ¹H NMR (CDCl₃): δ 1.67 (s, 6H, β-CH₃); δ 3.83 (s, 3H, OCH₃). ¹³C NMR (CDCl₃): δ 169.2 (CO); δ 157.5 (t, CN); δ 59.0 (t, J = 6 Hz, α-C); δ 52.9 (OCH₃); δ 27.0 (CH₃).

Characterization of 2-carbobethoxyisopropyl isocyanide,

CNCH(CH₃)₂COOCH₂CH₃. A viscous oil, bp 80-81 °C @ 29 mmHg. IR (neat, NaCl
plates): νCN = 2138, νCO = 1747 cm⁻¹. ¹H NMR (CDCl₃): δ 1.33 (t, 3H, 2-CH₃), δ 1.67 (s, 6H, β-CH₃), δ 4.27 (q, 2H, OCH₂). ¹³C NMR (CDCl₃): δ 169.2 (CO); δ 157.6 (t, CN); δ 62.4 (OCH₂); δ 59.3 (t, α-C); δ 27.1 (β-CH₂); δ 13.7 (2-CH₃).

**Characterization of 2-carbopropoxyisopropyl isocyanide,**

CNC(CH₃)₂COOC₂H₅CH₂CH₃. A viscous oil, bp 41-43 °C @ 1.0 mmHg. IR (neat, NaCl plates): νCN = 2140, νCO = 1748 cm⁻¹. ¹H NMR (CDCl₃): δ 0.98 (t, 3H, 3-CH₃); δ 1.67 (s, 6H, β-CH₃); δ 1.71 (m, 2H, 2-CH₂); δ 4.17 (t, 2H, OCH₂). ¹³C NMR (CDCl₃): δ 169.0 (CO); δ 157.5 (t, CN); δ 67.5 (OCH₂); δ 59.2 (t, α-C); δ 27.0 (β-CH₃); δ 21.4 (2-CH₂); δ 9.7 (3-CH₃).

**Characterization of 2-carboxisoproxyisopropyl isocyanide,**

CNC(CH₃)₂COOC(CH₃)₂. A viscous oil, bp 75-76 °C @ 21 mmHg. IR (neat, NaCl plates): νCN = 2140, νCO = 1745 cm⁻¹. ¹H NMR (CDCl₃): δ 1.30 (d, 6H, 2-CH₃); δ 1.65 (s, α-, β-CH₃); δ 5.07 (t, 1H, OCH). ¹³C NMR (CDCl₃): δ 168.6 (CO); δ 157.5 (t, CN); δ 70.1 (OCH); δ 59.4 (t, α-C); δ 27.0 (β-CH₃); δ 21.1 (2-CH₂).

**Characterization of 2-carbobutoxyisopropyl isocyanide,**

CNC(CH₃)₂COO(CH₃)₃CH₃. A viscous oil, bp 58-60 °C @ 0.7 mmHg. IR (neat, NaCl plates): νCN = 2140, νCO = 1748 cm⁻¹. ¹H NMR (CDCl₃): δ 0.96 (t, 3H, 4-CH₃); δ 1.61 (hex, 2H, 3-CH₂); δ 1.67 (s, 6H, β-CH₃); δ 1.68 (m, 2H, 2-CH₂); δ 4.21 (t, 2H, OCH₂). ¹³C NMR (CDCl₃): δ 169.3 (CO); δ 157.6 (t, CN); δ 66.2 (OCH₂); δ 59.4 (t, α-C); δ 30.1 (2-CH₂); δ 27.2 (β-CH₃); δ 18.7 (3-CH₂); δ 13.4 (4-CH₃).
Characterization of 2-carboisobutoxyisopropyl isocyanide,

\( \text{CNC}(\text{CH}_3)_2\text{COOCH}_2\text{CH}(\text{CH}_3)_2 \). A viscous oil, bp 50-52 °C @ 0.7 mmHg. IR (neat, NaCl plates): \( \nu_{\text{CN}} = 2141, \nu_{\text{CO}} = 1748 \text{ cm}^{-1} \). \( ^{1} \text{H NMR (CDCl}_3 \): \( \delta \)

0.97 (d, 6H, 3-CH\( _3 \)); \( \delta \) 1.68 (s, 6H, \( \beta \)-CH\( _3 \)); \( \delta \) 2.05 (hept, 1H, CH); \( \delta \) 4.00 (d, 2H, OCH\( _2 \)). \( ^{13} \text{C NMR (CDCl}_3 \): \( \delta \) 169.2 (CO); \( \delta \) 157.6 (t, CN); \( \delta \) 72.2 (OCH\( _2 \)); \( \delta \) 59.4 (t, \( \alpha \)-C); \( \delta \) 27.5 (2-CH); \( \delta \) 27.5 (}\( \beta \)-CH\( _3 \)); \( \delta \) 18.6 (3-CH\( _3 \)).

Characterization of 1-carbomethoxyisobutyl isocyanide,

\( \text{CNCH}($\text{CH}(\text{CH}_3)_2$)$\text{COOCH}_3 \). A viscous oil, bp 60-63 °C @ 14 mmHg. IR (neat, NaCl plates): \( \nu_{\text{CN}} = 2142, \nu_{\text{CO}} = 1755 \text{ cm}^{-1} \). \( ^{1} \text{H NMR (CDCl}_3 \): \( \delta \)

1.10 (dd, 6H, \( \gamma \)-CH\( _3 \)); \( \delta \) 2.40 (bs, 1H, \( \beta \)-CH); \( \delta \) 4.19 (s, 3H, OCH\( _3 \)); \( \delta \) 4.21 (d, 1H, \( \alpha \)-CH). \( ^{13} \text{C NMR (CDCl}_3 \): \( \delta \) 166.0 (CO); \( \delta \) 160.0 (t, CN); \( \delta \) 62.4 (t, \( \alpha \)-CH); \( \delta \) 52.7 (OCH\( _3 \)); \( \delta \) 30.7 (\( \beta \)-CH); \( \delta \) 18.8; \( \delta \) 16.1 (\( \gamma \)-CH\( _3 \)).

Characterization of 1,2-bis(carbomethoxyethyl isocyanide,

\( \text{CNCH}($\text{COOCH}_3$)($\text{CH}_2\text{COOCH}_3$) \). A viscous oil, bp 105-107 °C @ 1 mmHg. IR (neat, NaCl plates): \( \nu_{\text{CN}} = 2157, \nu_{\text{CO}} = 1745 \text{ cm}^{-1} \). \( ^{1} \text{H NMR (CDCl}_3 \): \( \delta \)

2.90 (d, 2H, \( \beta \)-CH\( _2 \)); \( \delta \) 3.73; \( \delta \) 3.83 (s, 3H, OCH\( _3 \)); \( \delta \) 4.58 (t, 1H, \( \alpha \)-CH). \( ^{13} \text{C NMR (CDCl}_3 \): \( \delta \) 168.5 (\( \beta \)-CO); \( \delta \) 165.7 (\( \gamma \)-CO); \( \delta \) 160.9 (CN); \( \delta \) 53.4 (OCH\( _3 \)); \( \delta \) 52.1 (OCH\( _3 \)); \( \delta \) 51.9 (t, \( \alpha \)-CH); \( \delta \) 36.9 (\( \beta \)-CH\( _2 \)).

Characterization of 2-aminocarbonylisopropyl isocyanide,

\( \text{CNC}(\text{CH}_3)_2\text{CONH}_2 \). A white crystalline solid, mp 118-120 °C. IR (KBr):

\( \nu_{\text{CN}} = 2140, \nu_{\text{CO}} = 1693, \nu_{\text{NH}} = 1635 \text{ cm}^{-1} \). \( ^{1} \text{H NMR (CDCl}_3 \): \( \delta \)

1.35 (bs, 2H, NH\( _2 \)); \( \delta \) 1.65 (t, 6H, \( \beta \)-CH\( _3 \)). \( ^{13} \text{C NMR (CDCl}_3 \): \( \delta \) 171.7 (CO); \( \delta \) 159.7 (t,CN); \( \delta \) 60.0 (t, \( \alpha \)-C); \( \delta \) 27.4 (\( \beta \)-CH\( _3 \)).
Characterization of 2-N-methylaminocarbonylisopropyl isocyanide,

\( \text{CNC}(\text{CH}_3)\text{CONHCH}_3 \). A white crystalline solid, mp 59-60 °C. IR (CCl₄ solution): \( \nu_{\text{CN}} = 2128, \nu_{\text{CO}} = 1693 \text{ cm}^{-1} \). \( ^1\text{H NMR (CDCl}_3 \): \( \delta 1.65 \) (t, 6H, \( \beta-\text{CH}_3 \)); \( \delta 2.87 \) (d, 3H, N-\text{CH}_3); \( \delta 6.74 \) (bs, 1H, NH). \( ^{13}\text{C NMR (CDCl}_3 \): \( \delta 166.5 \) (CO); \( \delta 159.9 \) (t, CN); \( \delta 60.8 \) (t, \( \alpha-\text{C} \)); \( \delta 27.4 \) (\( \beta-\text{CH}_3 \)); \( \delta 26.4 \) (N-\text{CH}_3).

Characterization of 1-N-methylaminocarbonylisopropyl isocyanide,

\( \text{CNCH}(\text{CH}_3)\text{CH}_2\text{CONHCH}_3 \). A white crystalline solid, mp 49-51 °C. IR (CCl₄ solution): \( \nu_{\text{CN}} = 2142, \nu_{\text{CO}} = 1670 \text{ cm}^{-1} \). \( ^1\text{H NMR (CDCl}_3 \): \( \delta 1.44 \) (m, 3H, \( \beta-\text{CH}_3 \)); \( \delta 2.53 \) (dq, 2H, \( \beta-\text{CH}_2 \)); \( \delta 2.82 \) (d, 3H, NCH₃); \( \delta 4.20 \) (q, 1H, \( \alpha-\text{CH} \)); \( \delta 6.70 \) (bs, 1H, NH). \( ^{13}\text{C NMR (CDCl}_3 \): \( \delta 169.0 \) (CO); \( \delta 154.9 \) (t, CN); \( \delta 47.1 \) (t, \( \alpha-\text{CH} \)); \( \delta 43.3 \) (\( \beta-\text{CH}_2 \)); \( \delta 26.1 \) (NCH₃); \( \delta 21.3 \) (\( \beta-\text{CH}_3 \)).

Characterization of 1-N-methylaminocarboxylpropyl isocyanide,

\( \text{CNCH}(\text{CH}_3\text{CH}_3)\text{CONHCH}_3 \). A white crystalline solid, mp 51-53 °C. IR (CCl₄ solution): \( \nu_{\text{CN}} = 2143, \nu_{\text{CO}} = 1685 \text{ cm}^{-1} \). \( ^1\text{H NMR (CDCl}_3 \): \( \delta 1.08 \) (t, 3H, \( \gamma-\text{CH}_3 \)); \( \delta 2.00 \) (t, 2H, \( \beta-\text{CH}_2 \)); \( \delta 2.88 \) (d, 3H, NCH₃); \( \delta 4.19 \) (t, 1H, \( \alpha-\text{CH} \)); \( \delta 6.82 \) (bs, 1H, NH). \( ^{13}\text{C NMR (CDCl}_3 \): \( \delta 166.0 \) (CO); \( \delta 160.8 \) (t, CN); \( \delta 59.5 \) (t, \( \alpha-\text{CH} \)); \( \delta 26.4 \) (NCH₃); \( \delta 26.1 \) (\( \beta-\text{CH}_2 \)); \( \delta 9.3 \) (\( \gamma-\text{CH}_3 \)).

Characterization of 2-N-methylaminocarboxylethyl isocyanide,

\( \text{CNCH}_2\text{CH}_2\text{CONHCH}_3 \). A white crystalline solid, mp 40-43 °C. IR (CCl₄ solution): \( \nu_{\text{CN}} = 2153, \nu_{\text{CO}} = 1675 \text{ cm}^{-1} \). \( ^1\text{H NMR (CDCl}_3 \): \( \delta 2.60 \) (t, 2H, \( \beta-\text{CH}_2 \)); \( \delta 2.82 \) (d, 3H, NCH₃); \( \delta 3.74 \) (t, 2H, \( \alpha-\text{CH}_2 \)); \( \delta 6.82 \) (bs, 1H, NH). \( ^{13}\text{C NMR (CDCl}_3 \): \( \delta 168.9 \) (CO); \( \delta 156.0 \) (t, CN); \( \delta 37.5 \) (t, \( \alpha-\text{CH}_2 \)); \( \delta 35.3 \) (\( \beta-\text{CH}_2 \)); \( \delta 26.0 \) (NHCH₃).
Characterization of 1-N-methylaminocarbonylethyl isocyanide, CNCH(CH₃)CONHCH₃. A white crystalline solid, mp 30-35 °C. IR (CCl₄ solution): ν CN = 2152, ν CO = 1685 cm⁻¹. ¹H NMR (CDCl₃): δ 1.60 (m, 3H, β-CH₃); δ 2.80 (d, 3H, NCH₃); δ 4.20 (dq, 1H, α-CH); δ 6.50 (bs, 1H, NH). ¹³C NMR (CDCl₃): δ 167.0 (CO); δ 160.1 (t, CN); δ 53.4 (t, α-CH); δ 26.7 (NCH₃); δ 19.8 (β-CH₃).

Characterization of aminocarbonylmethyl isocyanide, CNCH₂CONH₂. A white crystalline solid mp 37-40 °C. IR (CCl₄ solution): ν CN = 2138, ν CO = 1690 cm⁻¹. ¹H NMR (CDCl₃): δ 4.17 (s, 2H, α-CH₂); δ 6.05 (bs, 2H, NH₂). ¹³C-NMR (CDCl₃): δ 166.8 (CO); δ 160.3 (t, CN); δ 45.3 (t, α-CH₂).
Discussion:

The ester isonitriles synthesized here are all foul-smelling oils that react with acids which destroy the isonitrile function or with base which hydrolyzes the ester group. The initial destruction of the isonitrile moiety by aqueous acid can be viewed as the inverse of the dehydration reaction used to form these species.\(^{(11)}\)

\[
\text{H}^+ \quad R-N\equiv C + H_2O \quad \longrightarrow \quad R-NH-CHO
\]

The best accepted Lewis formula for an isonitrile group is the polar triple bond-containing structure. This bond order for the isonitrile is consistent with numerous physical chemical data which aid in the identification and characterization of synthetic products.\(^{(1)}\) In particular the triply-bonded C≡N has a distinct high energy stretching frequency in the IR at 2100 cm\(^{-1}\). Adding various substituents to the isonitrile shifts this absorption only slightly (+ 50 cm\(^{-1}\)), thus providing a characteristic indication of its presence. The well known C=O stretching frequency in the IR at 1600-1800 cm\(^{-1}\) also aids in identifying the presence and integrity of the ester function in these compounds.

The \(^{13}\)C NMR chemical shifts for isonitrile carbons also occur in a narrow and characteristic region of 150-165 ppm relative to TMS.\(^{(6,10)}\) Also nuclear quadrupole coupling in isonitriles is very low,\(^{(9)}\) indicating a near zero electric field gradient about the nitrogen. Thus the observation of \(^{13}\)C - \(^{14}\)N fine structure can be made in a proton-decoupled \(^{13}\)C NMR experiment.\(^{(10)}\) Spin-spin coupling constant measurements are not possible for most other organic nitrogen compounds. Coupling in isonitriles is observed between the \(^{14}\)N and both the terminal carbon and
the carbon α to the C≡N function. Coupling constants are greater for the α-13C–14N bond \( J \approx 8 \text{ Hz} \) than for the terminal 13C≡14N bond \( J \approx 5 \text{ Hz} \). The 13C NMR spectrum also exhibits the characteristic carbonyl peak at 165–175 ppm. Thus the 13C NMR confirms the number of carbon atoms present in these compounds as well as the identity of at least three of the critical components.

The alkyl substituents of these compounds are rather simple and thus produce classical first order splitting patterns in the high resolution 1H NMR.

The amide isonitriles are white crystalline solids at room temperature and dissolve freely in water. Characterization of these compounds over the ester isonitrile starting materials was made on the observation of the classical νCO shift and νCN in the IR spectrum. 13C and 1H NMR also confirmed the loss of the ester function and presence of amide protons.

The large dipolar nature of the isonitrile function is responsible for its good coordinating properties to metals. This structure is also the origin of its foul smell. Interestingly, although these compounds bind tightly to transition metals, they do not appear to be very toxic, with LD50 in mice of \( \leq 10 \text{ mg/kg} \). (11)
Figure 2.1 Reaction sequence for the synthesis of an ester isonitrile ligand.
Synthesis of Esterisonitrile Ligand

\[
\text{HO-}C\text{C-}NH_2 + \text{HCl (9) \rightarrow ROH} \\
\text{acetone/ether} \\
\text{RO-C-C-NH}_{2}\text{Cl} + \text{HCOONa} \\
\text{vac. distillation} \\
\text{RO-C-C-N=C} + \text{NaCl} \\
2\text{HCl} + \text{CO}_2 + \\
\text{-diphosgene} \\
\text{-CH}_2\text{Cl}_2 \\
\text{-vac. distillation}
\]
Figure 2.2 Amidation of an ester isonitrile ligand to yield the aminocarbonylalkyl isonitrile.
Amidation of Esterisonitrile

\[
\begin{align*}
\text{H}_2\text{NCH}_3 & \quad \text{NH}_2\text{CH}_3 \\
\text{C} & \quad \text{MeOH} \\
& \quad \text{C} \\
\text{C} & \quad \text{C} \\
\text{O} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{CH}_3 & \quad \text{CH}_3
\end{align*}
\]
References:


CHAPTER 3

SYNTHESIS AND CHARACTERIZATION OF

HEXAKIS(ISONITRILE)TECHNETIUM(I) COMPLEXES
Introduction:

Hexakisisonitrile complexes of manganese(I) and rhenium(I) have been known for some time and shown to be stable.(5-7) However, the reported synthesis involved reaction of the pre-reduced MnI₂ or ReI₃ as starting materials.(6) The first examples of the related technetium species were made by Davison and Abrams who proved the accessibility of hexakisalkylisonitrile technetium(I) complexes, directly from the Tc(VII) pertechnetate ion, in aqueous aerobic conditions.(2,3) The rapid synthesis of these compounds, in physiologically compatible conditions, is an essential requirement for their production and evaluation as radiopharmaceuticals.(10) The relatively short half-life of the clinically useful isotope (T₁/₂ = 6.02 hours) necessitates a synthesis of the drug that produces a single species, in high yield, without the need for additional purification.

The commercially available source of ⁹⁹Tc is from a generator system based on the decay of the mother isotope ⁹⁹Mo. This generator consists of an alumina oxide absorbent column onto which is loaded ⁹⁹Mo as molybdate. Decay of the oxygenated ⁹⁹Mo(VI) yields the ⁹⁹mTc(VII) species as the mono-anion pertechnetate which is eluted from the column with isotonic saline (0.15 Molar). The pertechnetate is typically present in a concentration of 10⁻⁷ to 10⁻¹⁰ moles/liter. Often the scale down of reaction conditions from the millimolar concentration, used to produce characterizable compounds, to the "radiolabeling" concentrations alters the composition of the products obtained.(7) Consequently it is essential to correlate the identity of the characterized species with the injectable radiopharmaceuticals if structure distribution or activity relationships are to be studied. The confirmation between the products
obtained from these dissimilar reaction conditions was established by dual detection reverse-phase high performance liquid chromatography (RP-HPLC). Fortunately for the mono hapto isonitrile ligands the Tc(I) hexakis species are accessible at both concentration ranges.\(^{(1)}\)

The crystal structure of the hexakis(t-butyl isocyanide) technetium(I) PF\(_6\) compound has demonstrated the six ligands in a slightly distorted octahedral environment with three C-Tc-C angles of 180°.\(^{(9)}\) Such a geometry should produce degenerate Eg and T\(_{2g}\) orbitals with a large ligand field stabilization energy thus yielding the stable diamagnetic d\(^6\), 18-electron species. In the case of the alkyl isonitriles, reaction of the commonly available TcO\(_4^-\) salt with a strong reducing agent in a refluxing alcoholic solution of excess ligand yields almost quantitively the thermodynamically stable hexakis species. However, reaction under similar conditions with an esterisonitrile ligand produces a number of mixed ligand species containing the hydrolyzed and non-hydrolyzed ligands. Consequently an alternative route starting from the prereduced Tc(III) species, Tc(thiourea)\(_6\)(BF\(_4\))\(_3\), was employed to produce characterizable quantites of compounds.\(^{(1)}\)

The isolated hexakis(esterisonitrile)\(^{99}\)technetium(I) compounds were characterized by FAB\(^+\) mass spectrometry, infrared absorption, UV-Vis, \(^{99}\)Tc-NMR, and elemental analysis. The purity was established by reverse phase high performance liquid chromatography (RP-HPLC) and the observed retention times used to identify the desired products of the dilute radiopharmaceutical preparation. Analysis of reaction products at the \(^{99}\)mTc concentration of 10\(^{-9}\) M, by gamma photon detection, permitted the optimization of reaction conditions to yield the desired compounds in high radiochemical yield.
Experimental:

Materials and methods. Technetium-99 in aqueous solution as the NH$_4$ salt of [99TcO$_4$]$^-$ was obtained from NEN Products / DuPont Medical Products, Billerica, MA. All manipulations were carried out in laboratories approved for low-level radioactivity (99Tc is a weak beta emitter with a half-life of 2.15 x 10$^5$ years and a particle energy of 0.292 MeV). Precautions for handling were detailed previously. The metastable radionuclide 99mTc, as sodium pertechnetate, was obtained from a commercial NEN/DuPont (99Mo/99mTc) generator in a 0.15 M saline solution. Technetium-99m decays primarily by isomeric transition from the metastable spin 1/2 state to the spin 9/2 ground state of 99Tc with emission of a single gamma photon of 140 KeV. All manipulations involving the high specific activity 99mTc isotope were performed under guidelines established by the NRC in a laboratory approved for intermediate level radioactivity at the Harvard Medical School.

Infrared spectra, obtained as KBr pellets or as CHCl$_3$ solutions, were recorded in the range of 4000-600 cm$^{-1}$ on a model 1420 Perkin-Elmer spectrophotometer. Elemental analyses were performed by Atlantic Microlab Inc., Atlanta, GA. Fourier transform $^1$H NMR spectra were obtained on a Bruker WM 250 MHz instrument with TMS as the internal standard, $^{13}$C NMR spectra were obtained on a Varian 400 MHz instrument at 100 MHz, with CDCl$_3$ as the lock solvent/standard. Fast-Atom-Bombardment mass spectra were run on a Varian MAT 731 mass spectrometer from a glycerol matrix. Doubly distilled, deionized water was used; all other solvents and chemicals were of reagent grade and used as received.

Reverse-Phase thin-layer chromatography (RPTLC) was carried out on Whatman MK Cl8 plates developed in (2:3:3:2) tetrahydrofuran, methanol,
acetonitrile, aqueous buffer (0.5 M, (NH₄)₂Ac). Measurement of
retention time (R_t) and peak quantitation were obtained by a TLC plot
reader using a collimated NaI crystal interfaced through a Canberra 2007P
photo multiplier tube to a 2015A single channel amplifier and model 14812
rate meter (Canberra Industries Inc., Meriden, CT). The printout and
integration of this signal was obtained on a Hewlett Packard 3390A
Integrator.

The high performance liquid chromatography (HPLC) instrumentation
used included the following modules (Waters Associates, Milford, MA): two
M6000A Solvent Delivery Systems, U6K Universal Injector, M710B WISP
(Waters Intelligent Sample Processor), M720 System Controller, M730 Data
Module—dual pen recorder, Model 440 UV Absorbance Detector, NaI crystal—
2007P PMT (Canberra Industries Inc. Meriden, CT), Canberra model 3102 HV
Power Supply, model 2015A Spectroscopy Amplifier/Timing SCA, and a model
1481L Ratemeter.¹¹ Reverse-phase chromatography techniques were used
in sample analysis. Analytical separations were performed on a Brownlee
Labs Cartridge System employing a 100mm x 4.6mm column containing a RP-8
5 micron spherical partical stationary phase and an Aquapore RP-300 guard
cartridge (Brownlee #OS-8MPand #C03-GU, Rainin Instrument Co. Woburn, MA).

Technetium-99 NMR spectrometry. Spectra were obtained on a XL-400
NMR spectrometer, Varian Assoc., Palo Alto, CA employing a 94 kilogauss
Nb-Ti superconducting magnet with a 5.4 mm bore, broad band probe.
Technetium-99, as TcO₄⁻, resonates with a frequency of 90.03 MHz at this
field strength. Spectra were obtained with CDCl₃ as the lock solvent and
Tc(TBI)₆⁺ in CDCl₃ as an external reference. Chemical shifts are
reported in ppm relative to ⁹⁹Tc(t-butyl isocyanide)₆⁺ which resonates at
-1908 ppm upfield, "shielded", from TcO₄⁻.²⁰ A typical experiment
consisted of 512-1024 transients obtained with an acquisition time of 0.15 sec with no recycle delay and a sweep width of 20 KHz.

**Preparation of hexakis(carbomethoxymethyl isocyanide)technetium(I)-hexafluorophosphate;** \(\text{Tc(CNCH}_2\text{COOCH}_3\text{)}_6\text{PF}_6\). To a 100-mL three-neck flask were added \(\text{Tc(SC(NH}_2\text{)}_2\text{(BF}_4\text{)}_6\) (0.42 g, 0.47 mol), magnetic stir bar, and methanol (50 mL). A condenser was attached and the system purged with argon for 15 min prior to addition of carbomethoxymethyl isocyanide (1.5 mL, 15 mmol). The bright red solution was refluxed for 30 min during which the color faded to pale orange. Addition of \(\text{NH}_4\text{PF}_6\) (0.5 g) in water followed by cooling overnight yielded a brown precipitate. The solid was filtered free of the supernate and recrystallized from acetone/diethyl ether six times to yield the slightly off-white product (0.17 g, 0.21 mmol, 45% yield based on Tc). Anal. Calcd. for \(\text{C}_{24}\text{H}_{30}\text{N}_6\text{O}_{12}\text{PF}_6\text{Tc}\): C, 34.36; H, 3.58; N, 10.02. found: C, 34.77; H, 3.08; N, 10.16. FAB (positive ion mode): \(m/z = 693\). IR (CHCl\(_3\) solution): \(\nu_{\text{CN}} = 2116\), \(\nu_{\text{CO}} = 1745\) cm\(^{-1}\). \(^1H\) NMR (CDCl\(_3\)): \(\delta = 3.82\) (s, 3H, OCH\(_3\)); \(\delta = 4.53\) (bs, 2H, \(\alpha\)-CH\(_2\)). \(^{13}C\) NMR (CDCl\(_3\)): \(\delta = 174.8\) (CO); \(\delta = 53.0\) (OCH\(_3\)); \(\delta = 45.4\) (\(\alpha\)-CH\(_2\)). \(^{99}\text{Tc}\) NMR (CDCl\(_3\)): \(\delta = -32.4\) versus \(\text{Tc(TBI)}_6\)^{+}.

**Preparation of hexakis(carboethoxymethyl isocyanide)technetium(I)-tetrafluoroborate;** \(\text{Tc(CNCH}_2\text{COOCH}_2\text{CH}_3\text{)}_6\text{BF}_4\). To a 100-mL round-bottom flask was transferred \(\text{Tc(SC(NH}_2\text{)}_2\text{(BF}_4\text{)}_3\) (0.58 g, 0.71 mmol),\(^{(1)}\) a magnetic stir bar, and dry ethanol (35 mL). The system was purged with argon for 20 min prior to the addition, via cannula, of the neat carboethoxymethyl isocyanide (1.75 mL, 15.4 mmol). The clear bright orange solution was refluxed for 30 min, resulting in a pale orange color, and the volume reduced to 15 mL under reduced pressure.
Diethyl ether (10 mL) was added to precipitate the crude yellowish green solid. The product was filtered through a medium porosity glass frit and recrystallized from an acetone/ether mixture (2 times) to yield the white fluffy microcrystalline product (0.384 g, 0.44 mmol, 62% yield based on Tc). Anal. Calcd. for C$_{30}$H$_{42}$N$_6$O$_{12}$F$_4$BTc: C, 41.67; H, 4.86; N, 9.72. found: C, 42.12; H, 4.70; N, 10.31. FAB (positive ion mode): m/z = 777. IR (CHCl$_3$ solution): νCN = 2120, νCO = 1747 cm$^{-1}$. $^1$H NMR (CDCl$_3$): δ 1.30 (t, 3H, CH$_3$); δ 4.25 (q, 2H, OCH$_2$); δ 4.50 (bs, 2H, α-CH$_2$). $^{13}$C NMR (CDCl$_3$): δ 165.6 (CO); δ 62.4 (OCH$_2$); δ 45.6 (α-CH$_2$); δ 14.1 (CH$_3$). $^{99}$Tc NMR (CDCl$_3$): δ -31.77 ppm.

Preparation of hexakis(carbo-t-butoxymethyl isocyanide)technetium(I) hexafluorophosphate, (Tc(CNCH$_2$COOC(CH$_3$)$_3$)$_6$PF$_6$). To a 100-mL three-neck flask was transferred Tc(SC(NH$_2$)$_2$)$_6$(BF$_4$)$_3$ (0.164 g, 0.20 mmol), t-butanol (30 mL), acetone (50 mL), and a magnetic stir bar. The system was purged with argon, via cannula, for 15 min prior to addition of the neat carbo-t-butoxymethyl isocyanide (1.0 mL, 7.1 mmol). This solution was heated at reflux for one hour during which the color changed from bright orange to brown. The solvents were removed under reduced pressure and the brown oil dissolved in CH$_2$Cl$_2$ and loaded on to a neutral alumina column (Woelm) (8 x 10cm). The first light yellow band to elute with excess CH$_2$Cl$_2$ was collected and the volume reduced to 5 mL in vacuo. The addition of hexane (30 mL) and diethyl ether (10 mL) followed by slow cooling overnight yielded long white needles of the product which were filtered, washed with ether, and dried in vacuo. Anal. Calcd. for C$_{42}$H$_{66}$N$_6$O$_{12}$P$_6$Tc: C, 46.24; H, 6.06; N, 7.71. found: C, 45.76; H, 6.26; N, 8.09. FAB (positive ion mode): m/z = 945. IR (CHCl$_3$ solution):
\[ \nu_{CN} = 2128, \nu_{CO} = 1752 \text{ cm}^{-1}. \]

\[ ^1H \text{NMR (CDCl}_3\): \delta 1.47 (s, 9H, CH}_3\); \delta 4.37 (bs, 2H, \alpha-CH}_2\). \]

\[ ^{13}C \text{NMR (CDCl}_3\): \delta 163.7 (CO); \delta 83.1 (OC); \delta 45.9 (\alpha-CH}_2\); \delta 27.6 (CH}_3\). \]

\[ ^{99}Tc \text{NMR (CDCl}_3\): \delta -31.95. \]

**Preparation of hexakis(2-carbomethoxyisopropyl isocyanide)technetium (I)chloride, (Tc(GNC(CH}_3\)_2COOCH}_3\)_6Cl.** To a 20-mL scintillation vial were added sodium dithionite (0.15 g, 0.86 mmol), methanol (0.5 mL), 2-carbomethoxyisopropyl isocyanide (150 \( \mu \)L, 1.18 mmol), and NH\(_4\)TcO\(_4\) (0.04 mmol) in water (2 mL). The vial was sealed and incubated for 40 min at 60 °C. One half of this solution was then loaded on to a Sep-Pak™ Cartridge (Waters Associates, Milford MA) and washed with water (10 mL). The pure product was eluted with methanol (4 mL) and the solvent removed under reduced pressure. FAB (positive ion mode): m/z = 861. IR (KBr):

\[ \nu_{CN} 2093, \nu_{CO} 1748 \text{ cm}^{-1}. \]

\[ ^1H \text{NMR (CDCl}_3\): \delta 1.65 (s, 6H, \beta-CH}_3\); \delta 3.86 (s, 3H, OCH}_3\). \]

\[ ^{13}C \text{NMR (CDCl}_3\): \delta 169.1 (CO); \delta 60.8 (s, \alpha-C); \delta 52.8 (OCH}_3\). \]

\[ ^{99}Tc \text{NMR (CH}_3\text{CH}_2\text{OH): } \delta -22.6. \]

**Preparation of "no carrier added" \(^{99m}\text{Tc}(2\text{-carbomethoxyisopropyl isocyanide})\)_6^+, \(^{99m}\text{Tc}(\text{CPI})\)_6^+.** Into a sterile 5 mL glass serum vial was weighed out ~5.0 mg sodium dithionite (Na\(_2\)S\(_2\)O\(_4\)). (Note: sodium dithionite was kept under a dry inert atmosphere after opening otherwise it would deteriorate rapidly.) To the serum vial was added 0.4 mL 95% ethanol and 10 \( \mu \)L of the neat CPI ligand. The vial was then sealed with a rubber stopper and 0.6 mL of \(^{99m}\text{TcO}_4^-\), generator eluate (NCA), was added. The reaction mixture was then shaken and heated for 35 min in a 60 °C water bath. The radiochemical yield and purity were both greater than 97%. Purity was tested using Whatman C-18 reverse phase TLC plates with a 3:3:2:2 ratio of methanol, acetonitrile, tetrahydrofuran and
aqueous buffer (0.5 M, \( \text{NH}_4\text{OAc} \)). The Tc-CPI cationic complex has a \( R_f \) of
0.7 in this system, with TcO_2 at \( R_f = 0 \) and TcO_4^- at \( R_f = 1 \).

**Purification of \( ^{99m} \text{Tc-CPI} \) from the reaction mixture.** The
\( ^{99m} \text{Tc(CPI)}_6^+ \) complex was separated from excess dithionite and free
isonitrile in the reaction mixture using a disposable SEP-PAK C-18
cartridge (Waters Associates, Milford, MA). The eluate from these
cartridges was found to be pyrogen-free and could be sterilized by
passing it through a Minisart NML 0.2 \( \mu \)M filter (Sartorius GmbH, W.
Germany). The contents from the \( ^{99m} \text{Tc(CPI)}_6^+ \) reaction vial were loaded
onto a pre-wet (EtOH) SEP-PAK cartridge via syringe. The cartridge was
then washed with 2 x 5 mL portions of 0.15 M saline followed by 10 mL of
35% EtOH/H_2O. The pure \( ^{99m} \text{Tc(CPI)}_6^+ \) was then eluted from the cartridge
with 2 mL 95% EtOH/saline. This 2 mL of eluate was then buffered with
0.5 mL ammonium acetate (0.5 M). The buffered solution was diluted with 6
mL saline (0.15 M) and filtered through the 0.2 \( \mu \)M filter. The eluate and
wash solutions from the SEP-PAK were tested for free CPI ligand using a
procedure adapted from the article by Crabtree. (18)
Results and Discussion:

The reaction of the pertechnetate ion with sodium hydrosulfite, Na$_2$S$_2$O$_4$, in refluxing ethanolic aqueous base in the presence of excess isocyanide ligand produces the hexakis(alkyl isonitrile)technetium(I) cation in fair to excellent yields. However, with the ester derivatives, the aqueous base necessary to solubilize the sodium hydrosulfite produces hydrolysis of the ester linkages and the subsequent composite of mixed-ligand complexes. Synthesis of the desired hexakis(ester isonitrile)technetium(I) complexes has been achieved employing the more labile technetium(III)(thiourea)$_6$(tetrafluoroborate)$_3$ complex as starting material. These complexes are most easily isolated and recrystallized as the hexafluorophosphate salt. The detailed synthesis for several of these compounds have been presented, vida supra, as well as their complete characterizations. Table I lists the 27 isonitrile technetium complexes that were synthesized for biological evaluation and their characterization data including IR, FAB-MS, $^{99}$Tc NMR, and RP-HPLC retention times for comparison.

The hexakis(ester isonitrile)technetium(I) species are soluble in polar organic solvents and air and water stable at pH 4-8. The crystalline solids of these salts are white to pale brown in color. This color difference has been observed for other technetium and rhenium complexes of isonitriles and is due to the tailing of the 300 nm charge-transfer absorption band in the UV region.

The lipophilicity of these technetium complexes increases with increased alkyl substitution and decreased branching as indicated by comparing the molecular weights with the HPLC-retention times. Reverse phase HPLC retention times have been used to obtain a relative measure
of "lipophilicity" for a series of derivatized similar complexes.\cite{15,16}
It should be noted that in these studies a C-8 alkyl bonded silica was
used for the stationary phase. Studies comparing RP-HPLC retention times
with octanol/water partition coefficients are at best erratic. Much of
the mismatch is due to the different natures of the "organic phase"\cite{16}
however, minor amounts of impurities can cause large errors when
measuring partition coefficients of highly lipophilic compounds. For
these reasons RP-HPLC retention times were used as a relative ordering of
lipophilicity for the tested compounds. Most of the complexes
synthesized were designed to have retention times less than Tc(t-butyl
isocyanide)$_6^+$.

The infrared spectra of all these Tc(ester isonitrile)$_6^+$ complexes
exhibit intense absorptions between 2100 and 2130 cm$^{-1}$ due to the C\equiv N
stretching modes. For all hexakis(isonitrile)technetium(I) complexes the
$\nu_{\text{CN}}$ is 50-80 cm$^{-1}$ lower in energy than the band for the uncomplexed
ligand. This corresponds to a decrease in the C\equiv N bond order and is
consistent with extensive $\pi$ back bonding from the Tc(I) core to the
isocyanide ligands. The CCl$_4$ solution spectra of these complexes
show a single broad peak near 2100 cm$^{-1}$ for all the tested ester
isonitriles. In contrast, some of the more symmetrical alkyl isonitrile
complexes exhibit splitting of the C\equiv N band \cite{1}. This splitting is
consistent with the superposition of the C$_3$ and C$_2$ alkyl symmetry on the
0$_h$ symmetry of the metal core to give an overall S$_6$ symmetry with the
expected A$_u$ and E$_u$ bands. As the crystal structure shows, the Tc(t-butyl
isocyanide)$_6$PF$_6$ complex has a slightly distorted O$_h$ geometry in the solid
state.\cite{9} Molecular modeling of the ester-isonitrile complexes indicate
more than 16 Van der Waals interactions which would indicate a lower
symmetry for these complexes. The observed effect is the broadening of
the C=N stretching frequencies in the IR. The variation of ester and amide groups had only a small effect on the C=N stretching frequency but the larger, anticipated effect on the closer carbonyl stretch, Figure 3.1.

Previous work has shown field desorption mass spectrometry (FDMS), (17) and fast atom bombardment mass spectrometry (FAB-MS), (18) can be used to characterize Werner-type coordination compounds that are involatile salts. Single spectra resulted from anionic technetium complexes in both the negative (-FDMS) and positive (+FDMS) ion modes. (19) For the hexakis(ester isonitrile)technetium(I) compounds reported here the strong signals associated with the monocation, Tc(CNCR₂COOR)⁺, are the only features found in the (+FD) mass spectrum. The FAB-MS positive ion mode provided greater fragmentation and thus more insight to the identity and stability of various technetium isonitrile complexes. (8)

Figure 3.2 is a representative spectrum for the hexakis(carb oxy methyl isocyanide)technetium(I) cation with a m/z = 945 mass units. The three subsequent peaks correspond to the three most prevalent forms of fragmentation observed. The 889 peak represents loss of a terminal alcohol group corresponding to mono-hydrolysis. The major fragmentation species is due to dealkylation of a single isonitrile ligand with retention of the six coordinate geometry yielding a structure of Tc(CNCH₂COOC(CH₃)₃)₅(CNH)⁺. The other commonly observed fragment corresponds to the loss of an entire isonitrile ligand to produce the penta-coordinate Tc[CNCH₂COOC(CH₃)₃]₅⁺ of unknown geometry. Figure 3.3 shows a (+)FAB spectra for Tc(2-carboxyisopropyl isocyanide)₆⁺ which demonstrates continued dealkylation producing large concentrations of fragments for the mono-, di-, and tri-isocyanic acid substituted species. These species correlate with reports of dealkylation of metal
isocyanide complexes by chemical means. (8)

The isotope technetium-99, with a spin of 9/2 and a sensitivity at constant field relative to hydrogen of 0.38, gives easily observable NMR signals from micromolar solutions of diamagnetic complexes. (20) Although $^{99}$Tc possess an appreciable quadrupole moment, $Q = -0.19 \times 10^{-28} \text{ m}^2$, the effect of quadrupole line broadening is attenuated by the large size of the spin. Thus the symmetrical hexakis(isonitrile)technetium(I) species with octahedral geometry produce single resonances with line widths of 1-3 ppm. Figure 3.4 is a $^{99}$Tc NMR spectrum obtained on a 2 mL sample of hexakis(2-carbomethoxyisopropyl isocyanide)technetium(I) at a concentration of 25 $\mu$M after 1024 transients. The chemical shift is $-22.8$ ppm from the external reference Tc(t-butyl isocyanide)$_6^+$ which is $-1908$ ppm shifted upfield from the d⁶, Tc(VII) in TcO₄⁻. The small peak downfield from the main peak is due to an impurity of the mono-hydrolyzed species. From the various isonitrile complexes synthesized it appeared that varying the ester functionality had a marginal effect on chemical shift. However, alkyl substitution on the carbon alpha to the isonitrile group produced larger effects. The sterically hindered ligands CNCH(CH(CH₃)₂)COOCH₃, CNCH(CH₂CH₃)COOCH₃, and CNCH(COOCH₃)(CH₂COOCH₃) produced the largest upfield shift of the $^{99}$Tc resonance. These bulky ligands may result in less symmetrical geometries for the hexakis complexes thus decreasing 10Dq and increasing the paramagnetic component of the local magnetic field.

For a technetium complex to be successful as a radiopharmaceutical its synthesis from the commercially available $^{99}$Tc generator eluate must be rapid and proceed in good radiochemical yield and purity. Figure 3.5 depicts the scheme used for the "kit" preparation of $^{99}$MTc(CPI)$_6^+$ at the
"no carrier added" (NCA) concentration. This protocol produced complete reduction of the pertechnetate ion to the technetium(I) species in greater than 98% yield. Identity and radiochemical purity were established by radiometric detection of the RP-TLC and RP-HPLC chromatographs of this "kit" preparation and their comparison with a sample of the characterized $^{99}$Tc(CPI)$_6^+$. Figure 3.6 shows a dual pen trace of the RP-HPLC with a NaI crystal radiometric detector for the gamma-emitting isotope and the UV trace for the carrier $^{99}$Tc(CPI)$_6$PF$_6$ complex. Characterization by HPLC was used to maximize the yield of each "kit" reaction for different isonitrile ligands and served as the purity check performed before each biological study.
Table 3.1 List of functionalized hexakis(isonitrile)technetium(I) complexes made from $^{99}\text{TcO}_4^-$, IR spectra were obtained of CCl$_4$ solutions and $^{99}\text{Tc}$ NMR performed in CH$_3$OH with CDCl$_3$ as the external lock/standard.
<table>
<thead>
<tr>
<th>$^{99}\text{Tc}$-Complexes</th>
<th>FAB-MS (M/Z+)</th>
<th>HPLC (min.)</th>
<th>$\nu_{\text{CN}}$ (cm$^{-1}$)</th>
<th>$\nu_{\text{CO}}$ (cm$^{-1}$)</th>
<th>$^{99}\text{Tc}$-NMR (ppm)</th>
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<td>$\text{Tc(CNCH}_2\text{COOCH}_3\text{)}_6^+$</td>
<td>693</td>
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<td>1747</td>
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<td>$\text{Tc(CNCH}_2\text{COOCH}_2\text{CH}_2\text{CH}_3\text{)}_6^+$</td>
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<td>9.5</td>
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<td>1745</td>
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Figure 3.1 Infrared absorption spectra of hexakis(2-carbomethoxyisopropyl-isocyanide)technetium(I)chloride in a KBr pellet. The sharp bands at 2100 and 1750 cm\(^{-1}\) are characteristic for the C≡N and C=O stretching modes.
Figure 3.2  Fast Atom Bombardment Mass Spectrum of

\[\text{hexakis(carbo-}t\text{-butoxymethyl isocyanide)technetium(I)},\]

obtained in the positive ion mode.
Figure 3.3 Fast Atom Bombardment Mass Spectrum of

hexakis(2-carboxethoxyisopropyl isocyanide)technetium(I),

obtained in the positive ion mode.
Figure 3.4 $^{99}$Tc NMR spectrum in CDCl$_3$ of hexakis(2-carboxymethoxyisopropylisocyanide)technetium(I). Obtained after 1024 transients with a sweep width of 20KHz and an acquisition time of 0.15 seconds.
$^{99}\text{Tc(CNC(CH}_3\text{)}_2\text{COOCH}_3\text{)}_6^+$

at pH 4.35 , 4 mM

in etOH/H\text{}_2\text{O}
Figure 3.5 Schematic for the no carrier added "kit" preparation of 
$^{99m}$Tc(2-carbomethoxyisopropyl isocyanide)$_6^{4+}$Cl$^-$ from 
$^{99m}$TcO$_4^{-}$Na$^+$ in eluate from a $^{99}$Mo/$^{99m}$Tc generator.
Preparation of Carrier Free $^{99m}$Technetiumhexakis(2-carbomethoxyisopropylisonitrile)

$^{99m}$TcO$_4^-$ / Saline + 5 μl CNC(CH$_3$)$_2$COOCH$_3$ $\xrightarrow{60^\circ C/40\text{ min}}$ $\left[\text{Tc(CNC(CH$_3$)$_2$COOCH$_3$)$_6}\right]^{+1}$

-5 mg Na$_2$S$_2$O$_4$

-0.5 ml MeOH

>98% by RPHPLC and TLC
Figure 3.6 Reverse-phase high performance liquid chromatograph of a mixture of characterized $^{99}\text{Tc(CPI)}_6^{+}PF_6$, UV absorbance trace, and $^{99m}\text{Tc(CPI)}_6^{+}Cl^-$ from a "kit" preparation, radiometric detection (bottom trace). Note 1 cm offset is due to an overlap of pens on the recorder. The dead time between detectors is <0.05 min at 2 mL/min.
Detector
UV Absorbance
254 nm
1.0 AUFS
1.0 cm path length

MIX OF $^{99}\text{Tc}(\text{CNC}(\text{CH}_3)_2\text{COOCH}_3)_6\text{PF}_6$

AND

$^{99m}\text{Tc}(\text{CNC}(\text{CH}_3)_2\text{COOCH}_3)_6^@$ KIT

Detector
NaI gamma detector
500 cps Full scale
normal loop
1.0 uCi injected

HPLC System
Column: Brownlee RP-8
10 cm x 4.6 mm
5 um spherical part.
Moble phase: Gradient
100% .05 M (NH$_4$)$_2$SO$_4$ to
95% Methanol, 5 min linear.
References:


CHAPTER 4

BIOLOGICAL EVALUATION OF $^{99m}$Tc–ISONITRILE COMPLEXES
**Introduction:**

To function as a successful diagnostic myocardial perfusion agent a compound must be handled favorably by numerous physiological systems. Not only must its accumulation in the target agent, the heart, be high but also clearance from nontarget organs, the background, must be rapid. This means there should be minimal activity in the lungs, liver, and blood. The previously successful compound hexakis(t-butylisocyanide) technetium\(^{(1)}\) showed high myocardial extraction which was proportional to blood flow.\(^{(2)}\) However, the high lipophilic character of this compound produced nonspecific accumulation into the lungs, which is the first capillary system an intravenously injected agent encounters. Since only ~6 percent of the output from the heart enters the coronary arteries, the remaining 94% of the bolus is exposed to the other organs including both excretory systems before returning to the heart for recirculation. Thus the majority of the injected dose is localized in the liver, which is the primary route of elimination for lipophilic substances. In addition, although the initial rate of clearance from the blood is rapid, the slow washout of activity from the lungs produces rather high blood activity proximal to the heart. The functionalized isonitrile complexes provided a systematic method to alter the rates of non-target organ clearance while attempting to maintain myocardial uptake.

The most efficient method to determine if a compound possesses the multiple desired properties in vivo is to observe the biodistribution over time in a suitable animal model. The evaluation of technetium complexes as potential myocardial perfusion agents is complicated by the absence of a single acceptable animal model for
humans. Indeed, even primates do not appear to approximate humans sufficiently to confirm a probable candidate. The publication of "Noah's ark experiments" demonstrates the desperation of researchers approaching this problem. The current consensus is that swine is the most critical model for the human cardiovascular system. This conclusion is based on the observation that compounds that show no heart uptake in humans also show no uptake in pigs. Some preliminary human studies however, indicate that it is not only similarities in myocardial physiology but also differences in blood chemistry that affect the availability of agents for heart localization. Specifically, either binding to blood components or alteration of the agents themselves can complicate the localization process. Obviously any evaluation of compounds must address their integrity in vivo to make conclusions on their biological distributions.

Rabbits were chosen as the initial screening model for these compounds for several reasons. Most significantly, with all the technetium compounds tested, rabbits appear to be an optimistic model for myocardial uptake. In particular since hexakis(t-butyliocyanide) technetium(I) distribution in rabbits mimics the prolonged lung and liver activity observed in humans it was felt that optimizing imaging properties in this species should be an acceptable first approach. Also, unlike rats, rabbits have a gall bladder which helps in the quantitation of hepatobiliary clearance rates. Rabbits are also attractive to work with because their size enables simultaneous whole body observation, with good spatial resolution on an Anger camera. Whereas these agents were being surveyed for several in vivo characteristics, data could be accumulated sequentially over time and
analyzed in a dynamic mode. Another advantage of the imaging studies was that the animals were not sacrificed and were used repeatedly to limit intraspecies variation between tests of different compounds. The screen consisted of injecting the compound into an anesthetized rabbit and obtaining sequential images at 60 second intervals for one hour. Regions of interest were then drawn over specific organs and the data plotted as normalized activity density per organ versus time.

For the control a "gold standard" to compare these complexes with, Tc(t-butyl isocyanide)$_6^+$, was studied in this model system. From this data analysis several criteria were established to designate an improved imaging agent. The ratios of activity for heart/lung and heart/liver should be maximized. The half time for clearance of activity from the liver ($T_{1/2}$) should be minimized and the rate of activity washout from the heart should be slow. In general it was observed that minor modifications in ligand structure produced major changes in distribution and clearance kinetics. Uptake of these compounds into myocardial tissue could not be explained simply on the basis of lipophilicity. Of all the compounds tested, Tc(2-carbomethoxyisopropyl isocyanide)$_6^+$, Tc(CPI)$_6^+$, best exhibited these criteria. Further quantitative biodistribution in guinea pigs confirmed the distribution and kinetics screen in rabbits. However, preliminary toxicity testing in mice and rats showed drastically different biodistributions. Imaging studies performed by NEN/DuPont, Billerica, MA on swine (7) and subsequent human (8) clinical studies have confirmed the rabbit model results. The absence of myocardial uptake in rodents has been explained by enzyme hydrolysis experiments as detailed in Chapter 5.
Experimental section:

Preparation of $^{99m}$Tc(ester isonitrile)$_6^{+}$ complexes. All imaging studies were performed with fresh (<3 hours) preparations of the technetium-$99m$ complex synthesized from a "kit type" reaction as outlined in Chapter 3. Agents were tested for radiochemical purity by scintillation detection of reverse phase HPLC and TLC analysis. Purity exceeded 90 percent in all cases. To maintain consistency all preparations were diluted with (25%, v/v) ethanol/saline (0.15 M) to a specific activity of 2.0 mCi/mL.

Imaging studies on the Anger camera. Dynamic imaging studies were performed on a GE 400 AC Star camera, General Electric Co.; Milwaukee, WI. Images were obtained with a Low Energy All Purpose (LEAP) collimator which obtained a distal resolution of 6.3 mm (full width half max) when using a 20% energy window centered at 140 KeV. Data was acquired in a $64 \times 64$ word matrix sequentially for 60 seconds per frame for 60 minutes. Regions of interest used for calculating curves were a $4 \times 4$ pixel square and the data analyzed on a Nova 4 computer.

Rabbits used in these studies were New Zealand Albinos obtained from Pine Acre Rabbitry, Assonet, MA., were between 2 and 3 years old and weighed between 3.5 to 4.0 Kg. All animals were given food and water ad libitum up to one hour before the studies began. Rabbits were pre-anesthetized with Ketamine® (25 mg per Kg) and Acepromazine® (0.25 mg per Kg) intra-muscularly, followed by intravenously administered Nembutal® (25 mg per Kg). Technetium compounds were injected intravenously through an ear vein and acquisition begun immediately in the anterior position.
**Biodistribution:** Mice (male CD-1) and rats (male CD) were obtained from Charles River, Wilmington, MA. Mice weighed 25-35 g and rats weighed 400-650 g. Male and female Hartley guinea pigs were obtained from Elm Hill, Chelmsford, MA and weighed 300-600 g. Five-day-old 2 Leghorn chicks (SOF- utility chicks) were obtained from Spafa, Norwich, CT and weighed 40-60 g. Animals were injected with ~100 μCi of $^{99m}$Tc(CPI)$_6^+$ in 25% ethanol/saline (0.15M) at a specific activity of 1 mCi/mL (mice, rats, guinea pigs) or 2 mCi/mL (chicks). Mice were injected unanesthetized via a tail vein and sacrificed by cervical dislocation at 0.5, 5, 15, 30 and 60 minutes post injection. Rats were injected intravenously via femoral cutdown while under sodium pentobarbital anesthetic (50 mg per kg body weight) and sacrificed by cardiac puncture at 5 and 60 minutes. Guinea pigs were injected via femoral cutdown while under Nembutal® anesthesia and sacrificed by cardiac puncture at 5, 15, 30, 60, 150, and 240 min. Chicks were injected intravenously (unanesthetized) via the central wing vein and sacrificed using ether at 5, 30, and 60 min.

Organs were excised, weighed, placed into glass tubes and counted along with background and four standards (4 x 50 μL of the solution used for each experiment) on a Packard Auto-Gamma 500 NaI(Tl) automated γ counter.
Results and Discussion:

Screening of compounds in the rabbit model. Anterior views of anesthetized rabbits were obtained beginning immediately following intravenous injection, via the ear vein, of the $^{99m}$technetium compounds. Images were acquired for 60 seconds per frame sequentially for one hour. Figure 4.1 shows a schematic for the anatomy of a rabbit. The 15, 30, and 60 minute post injection 60 sec images for Tc(t-butyl isocyanide)$_6^{+}$ complex are shown in Figure 4.2 as well as the clearance curves for activity in the heart, lungs, kidney, and liver. All curves presented are normalized to count per minute per pixel, for each organ, versus time. Noticeably for this compound, the slow clearance of activity in the lungs results in a low heart/lung ratio until >20 minutes post injection. Also the gradual uptake and retention in the liver produces a consistent heart/liver ratio <0.5. The plateau for the activity in the kidney indicates no renal clearance and parallels the fixed localization observed in the liver and heart.

In contrast, as the next 22 figures indicate, all the ester isonitrile complexes demonstrate hepato-biliary clearance although at very different rates. Table 6.1 lists the heart/lung and heart/liver ratios at 5 and 30 minutes post injection as well as the half times for clearance from the liver.

The analysis reflects several trends in the in vivo behavior within a class of compounds. For a particular series of alkyl isonitrile derivatives, increasing the size and decreasing the branching of the ester fuction increases the lipophilicity of the complex, as measured by RP-HPLC retention times. The greater lipophilic nature of the complex then favors hepato-biliary accumulation over renal clearance from the
bloodstream. For the derivatized methyl and isopropyl isonitrile ligands prepared, Figures 4.3-4.9 and 4.17-4.22, the ratio of heart to liver activity at 5 minutes post injection decreases down a series because of higher liver uptake. Nonspecific binding or sticking to lung fibroblast cells also increased for the more lipophilic compounds. Thus at 5 minutes post injection the heart to lung ratio decreased to 1.0 with increasing lipophilicity.

As the size of the ester group is increased within a series it appears that the rate of hepatobiliary clearance decreases. However, this trend is augmented by the observation that methylester isonitriles are usually cleared from the liver more slowly then the ethylester derivatives. This data correlates well with studies on in vitro enzymatic hydrolysis in rabbit plasma as detailed in Chapter 5. As was mentioned, all these esterisonitrile complexes exhibited hepatobiliary clearance, even the carbo-t-butoxymethyl isocyanide complex. Since t-butyl esters are not commonly found in biological systems and t-butyl esterases have not been reported, these results seemed not to follow the excretion via hydrolysis theory. Indeed, plasma enzymatic hydrolysis studies show an absence of hydrolysis of the larger alkyl esters' substituents. These data indicate that hepatobiliary clearance occurs, as in most biological systems, by more than one mechanism.

Ratios of heart to lung activities seemed to pass through a maximum at a relative lipophilicity of 9.0 minutes as measured by HPLC retention times. Compounds with lower molecular weights were too hydrophilic and showed reduced heart uptake where more lipophilic species exhibited the higher nonspecific background retention. However, the analysis is not that simple because for the 6 structural isomers of
molecular weight 861, only one possessed all the characteristics desirable for a perfusion agent. Technetium hexakis(2-carbomethoxy-isopropyl isocyanide) distribution, Figure 4.17, in the rabbit model exhibited rapid initial uptake into the myocardium. There was minimal uptake into the lung tissue as demonstrated by the rapid washout which was due mostly to blood clearance. In addition the rapid clearance of blood activity into the liver and kidneys was followed by rapid hepatobiliary excretion into the gut with a $T_{1/2} = 16$ minutes for liver clearance. The kidneys also demonstrated renal clearance into the urinary bladder with a $T_{1/2} = 8$ minutes. The rapid decrease in liver activity produced a heart to liver ratio of greater than 1.0 by 10 minutes post injection. The heart to liver ratio did reach a maximum at 10 minutes but decreased slowly as a result of slow myocardial washout.

**Imaging studies of amide isonitrile complexes of technetium.**

Screening of aminocarbonylalkyl isonitrile complexes for myocardial proved disappointing as indicated in Table 4.2. Although these compounds did exhibit low lung uptake and rapid liver clearance, they showed no significant accumulation in myocardial tissue. The apparent heart activity is due to slow blood clearance. The compounds tested were much more hydrophilic than complexes of the related ester ligands, which may account for the low heart uptake. This more hydrophilic character however produced the anticipated results of high kidney uptake followed by rapid renal clearance. These complexes show an exciting potential for a cationic technetium-based kidney agent.

**Imaging studies with carboxyalkyl isonitrile complexes.** Base hydrolysis of the preformed hexakis(ester isonitrile)technetium(I)
complex yielded the hexakis (carboxyalkyl isonitrile)technetium(I) which possessed a ~5 charge at physiological pH (Chapter 5). Rabbit imaging studies of these hydrophilic species demonstrated the expected behavior of high kidney uptake and rapid renal clearance to the urinary bladder (Figures 4.29-4.33). The rate of kidney clearance appeared to decrease as the size of the alkyl group on the isonitrile was increased, Table 4.3. The observed rapid liver clearance was partially due to blood pool activity, however, some hepatobiliary clearance into the gut was observed as evidenced by the peaks in the kidney clearance curves due to the intestines overlaying the kidneys. The heart to lung and liver ratios of ~1.0 for all these compounds are a result of blood pool retention. Only the complex of 1-carboxyisobutyl isocyanide was lipophilic enough to have significant liver uptake even with its high anionic charge.

**Biodistribution studies:** To further quantitate the in vivo fate of this potential agent, hexakis(2-carboxymethoxyisopropyl isocyanide) Technetium(I), biodistribution as a function of time was performed in guinea pigs. Table 4.4 shows the percent injected dose per organ and percent injected dose per gram of tissue in at 5, 15, 30, 60 and 120 minutes post injection. As was observed in the rabbit imaging studies, this compound is rapidly taken up into myocardial tissue from which it washes out slowly with a $t_{1/2} = 75$ minutes. The remainder of the activity is rapidly cleared from the blood primarily to the liver, kidneys and skeletal muscle. The kinetics for clearance correlated with the rabbit imaging data.

Further biodistribution studies in rats and mice (Table 4.5), performed in order to establish toxicity and doseometry for human
clinical trials, however showed no myocardial localization, even at 30 seconds post injection. These species did demonstrate rapid blood clearance with localization of activity in the liver and kidneys followed by excretion into the gut and bladder respectively. The nature of this interspecies variation is addressed in Chapter 5.
Discussion:

Initial experiments in human volunteers and phase II clinical trials have been performed by NEN/DuPont and others with the hexakis(2-carbomethoxyisopropyl isocyanide)technetium(I), Tc(CPI)$_6^+$ complex.\(^8\) The results are very encouraging since the observed clinical behavior mimics the rabbit model imaging data. Studies in isolated perfused rabbit hearts with artificially induced infarcts have shown that Tc(CPI)$_6^+$ distributes linearly with blood flow to heart muscle. As observed in the rabbit imaging studies this compound does slowly washout of normal myocardial tissue, however unlike Tl-201, no redistribution of activity is observed in transient ischemic patients.

The property of slow washout is ideal for the clinical protocol being used. In these studies the suspected CAD patient is physically stressed on a treadmill and the agent is injected at the point of full exertion or onset of angina. The images can be obtained immediately or anytime within 2 hours, allowing sufficient time for accumulation of a series of images for single photon emission tomography. The patient is then reinjected in a resting condition at ~3 hours after the initial injection and images are obtained to assess the presence of a transient defect. Thus the slow washout of activity from the heart facilitates the completion of the study in one day and also allows sufficient time to perform extensive imaging.
Table 4.1  Anger camera biodistribution dynamics in rabbits for some $^{99m}\text{Tc}$(carboalkoxyalkyl isonitrile)$_6^+$ complexes.
<table>
<thead>
<tr>
<th>$^{99m}$Tc-Complexes</th>
<th>Heart/Lung 5'</th>
<th>Heart/Lung 30'</th>
<th>Heart/Liver 5'</th>
<th>Heart/Liver 30'</th>
<th>Liver $T_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tc(CNCH$_2$COOCH$_3$)$_6^{+}$</td>
<td>1.52</td>
<td>1.33</td>
<td>0.63</td>
<td>0.74</td>
<td>25.</td>
</tr>
<tr>
<td>Tc(CNCH$_2$COOCH$_2$CH$_3$)$_6^{+}$</td>
<td>2.14</td>
<td>1.10</td>
<td>0.75</td>
<td>0.57</td>
<td>15.</td>
</tr>
<tr>
<td>Tc(CNCH$_2$COOCH$_2$CH$_2$CH$_3$)$_6^{+}$</td>
<td>1.41</td>
<td>1.54</td>
<td>0.38</td>
<td>0.37</td>
<td>28.</td>
</tr>
<tr>
<td>Tc(CNCH$_2$COOCH(CH$_3$)$_2$)$_6^{+}$</td>
<td>1.25</td>
<td>1.66</td>
<td>0.37</td>
<td>1.00</td>
<td>8.</td>
</tr>
<tr>
<td>Tc(CNCH$_2$COO(CH$_2$)$_3$CH$_3$)$_6^{+}$</td>
<td>1.00</td>
<td>1.00</td>
<td>0.25</td>
<td>0.33</td>
<td>14.</td>
</tr>
<tr>
<td>Tc(CNCH$_2$COOCH$_2$CH(CH$_3$)$_2$)$_6^{+}$</td>
<td>1.00</td>
<td>1.00</td>
<td>0.16</td>
<td>0.13</td>
<td>23.</td>
</tr>
<tr>
<td>Tc(CNCH$_2$COOC(CH$_3$)$_3$)$_6^{+}$</td>
<td>1.00</td>
<td>1.57</td>
<td>0.29</td>
<td>0.48</td>
<td>21.</td>
</tr>
<tr>
<td>Tc(CNCH(CH$_3$)COOCH$_3$)$_6^{+}$</td>
<td>1.87</td>
<td>1.50</td>
<td>0.85</td>
<td>0.75</td>
<td>55.</td>
</tr>
<tr>
<td>Tc(CNCH(CH$_3$)COOCH$_2$CH$_3$)$_6^{+}$</td>
<td>1.18</td>
<td>1.25</td>
<td>0.47</td>
<td>0.45</td>
<td>16.</td>
</tr>
<tr>
<td>Tc(CNCH$_2$CH$_2$COOCH$_3$)$_6^{+}$</td>
<td>1.29</td>
<td>1.36</td>
<td>0.69</td>
<td>0.66</td>
<td>90.</td>
</tr>
<tr>
<td>Tc(CNCH$_2$CH$_2$COOCH$_2$CH$_3$)$_6^{+}$</td>
<td>1.80</td>
<td>1.40</td>
<td>0.84</td>
<td>0.74</td>
<td>12.</td>
</tr>
<tr>
<td>Tc(CNC(CH$_3$)$_2$COOCH$_3$)$_6^{+}$</td>
<td>2.40</td>
<td>3.40</td>
<td>0.90</td>
<td>1.70</td>
<td>16.</td>
</tr>
<tr>
<td>Tc(CNC(CH$_3$)$_2$COOCH$_2$CH$_3$)$_6^{+}$</td>
<td>5.25</td>
<td>5.00</td>
<td>0.35</td>
<td>0.43</td>
<td>22.</td>
</tr>
<tr>
<td>Tc(CNC(CH$_3$)$_2$COOCH$_2$CH$_2$CH$_3$)$_6^{+}$</td>
<td>1.88</td>
<td>2.00</td>
<td>0.51</td>
<td>0.50</td>
<td>40.</td>
</tr>
<tr>
<td>Tc(CNC(CH$_3$)$_2$COOCH$_2$CH(CH$_3$)$_2$)$_6^{+}$</td>
<td>1.00</td>
<td>1.00</td>
<td>0.41</td>
<td>0.37</td>
<td>42.</td>
</tr>
<tr>
<td>Tc(CNC(CH$_3$)$_2$COOCH$_2$CH(CH$_3$)$_3$)$_6^{+}$</td>
<td>1.00</td>
<td>1.00</td>
<td>0.20</td>
<td>0.09</td>
<td>120.</td>
</tr>
<tr>
<td>Tc(CNC(CH$_3$)$_2$COOCH$_2$CH(CH$_3$)$_2$)$_6^{+}$</td>
<td>2.50</td>
<td>2.00</td>
<td>0.36</td>
<td>0.14</td>
<td>70.</td>
</tr>
<tr>
<td>Tc(CNCH(CH$_2$CH$_3$)COOCH$_3$)$_6^{+}$</td>
<td>1.54</td>
<td>1.00</td>
<td>0.50</td>
<td>0.35</td>
<td>18.</td>
</tr>
<tr>
<td>Tc(CNCH(CH$_2$CH$_3$)COOCH$_2$CH$_3$)$_6^{+}$</td>
<td>1.00</td>
<td>1.10</td>
<td>0.52</td>
<td>0.47</td>
<td>18.</td>
</tr>
<tr>
<td>Tc(CNCH(CH$_3$)CH$_2$COOCH$_3$)$_6^{+}$</td>
<td>2.40</td>
<td>1.75</td>
<td>0.77</td>
<td>1.00</td>
<td>15.</td>
</tr>
<tr>
<td>Tc(CNCH$_2$CH(CH$_3$)COOCH$_3$)$_6^{+}$</td>
<td>2.38</td>
<td>2.16</td>
<td>0.62</td>
<td>0.76</td>
<td>16.</td>
</tr>
<tr>
<td>Tc(CNCH(CH(CH$_3$)$_2$)COOCH$_3$)$_6^{+}$</td>
<td>1.20</td>
<td>1.25</td>
<td>0.77</td>
<td>0.53</td>
<td>75.</td>
</tr>
<tr>
<td>Tc(CNCH(COOCH$_3$)CH$_2$COOCH$_3$)$_6^{+}$</td>
<td>1.33</td>
<td>1.00</td>
<td>0.47</td>
<td>0.46</td>
<td>22.</td>
</tr>
</tbody>
</table>
Table 4.2  Anger camera biodistribution dynamics in rabbits for some $^{99m}Tc(N\text{-methylaminocarbonylalkyl isonitrile})_6^+$ complexes.

Table 4.3  Anger camera biodistribution dynamics in rabbits for some $^{99m}Tc(\text{carboxylalkyl isonitrile})_6^{-5}$ complexes.
Table 4.2 Anger camera biodistribution dynamics in rabbits of some hexakis(N-methylaminocarbonylalkyl isonitrile)technetium(I) complexes.

<table>
<thead>
<tr>
<th>$^{99m}$Tc-complexes</th>
<th>Ht/Lun 15'</th>
<th>Ht/Liv 15'</th>
<th>Liver T1/2</th>
<th>Kidneys T1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tc(CNC(CH3)2CONH2)$_6^+$</td>
<td>1.20</td>
<td>1.20</td>
<td>10 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Tc(CNC(CH3)2CONHCH3)$_6^+$</td>
<td>1.00</td>
<td>0.25</td>
<td>30 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Tc(CH2CH2CONHCH3)$_6^+$</td>
<td>1.00</td>
<td>1.20</td>
<td>---</td>
<td>9 min</td>
</tr>
</tbody>
</table>

Table 4.3 Anger camera biodistribution dynamics in rabbits of various hexakis(carboxylate isonitrile)technetium(I) complexes.

<table>
<thead>
<tr>
<th>$^{99m}$Tc-complexes</th>
<th>Ht/Lun 15'</th>
<th>Ht/Liv 15'</th>
<th>Liver T1/2</th>
<th>Kidneys T1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tc(CNCH$_2$COO$^-$)$_6^{5-}$</td>
<td>1.10</td>
<td>1.25</td>
<td>10 min</td>
<td>6 min</td>
</tr>
<tr>
<td>Tc(CNCH$_2$CH$_2$COO$^-$)$_6^{5-}$</td>
<td>1.10</td>
<td>1.10</td>
<td>9 min</td>
<td>8 min</td>
</tr>
<tr>
<td>Tc(CNCH(CH$_2$CH$_3$)COO$^-$)$_6^{5-}$</td>
<td>1.00</td>
<td>1.00</td>
<td>10 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Tc(CNCH(CH$_3$)$_2$COO$^-$)$_6^{5-}$</td>
<td>1.00</td>
<td>1.00</td>
<td>10 min</td>
<td>11 min</td>
</tr>
<tr>
<td>Tc(CNCH(CH(CH$_3$)$_2$)COO$^-$)$_6^{5-}$</td>
<td>1.00</td>
<td>0.50</td>
<td>20 min</td>
<td>14 min</td>
</tr>
</tbody>
</table>
Table 4.4  Biodistribution data in guinea pigs at 5, 15, 30, 60, and 120 min post injection of $^{99m}$Tc(CPI)$_6^+$. 
% ID per gram of Tc(cpl)$_6^+$ in Guinea Pigs
Table 4.5  Biodistribution studies for $^{99m}$Tc(CPI)$_6^+$ in rats, mice, guinea pigs, and chicks at 5 min post injection.
<table>
<thead>
<tr>
<th></th>
<th>Guinea Pig</th>
<th>Rat</th>
<th>Mouse</th>
<th>Chick</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%ID/Org</td>
<td>%ID/Gram</td>
<td>%ID/Org</td>
<td>%ID/Gram</td>
</tr>
<tr>
<td>Heart</td>
<td>1.25±0.32</td>
<td>0.90±0.20</td>
<td>0.066±0.003</td>
<td>0.057±0.002</td>
</tr>
<tr>
<td>Blood</td>
<td>1.40±0.27</td>
<td>0.053±0.008</td>
<td>3.82±0.33</td>
<td>0.16±0.018</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.91±0.09</td>
<td>0.38±0.11</td>
<td>0.25±0.064</td>
<td>0.12±0.015</td>
</tr>
<tr>
<td>Liver</td>
<td>13.3±2.20</td>
<td>0.87±0.29</td>
<td>20.0±2.15</td>
<td>1.27±0.11</td>
</tr>
<tr>
<td>Gut</td>
<td>34.4±4.29</td>
<td>-----</td>
<td>37.5±5.72</td>
<td>-----</td>
</tr>
<tr>
<td>Kidneys</td>
<td>10.4±2.55</td>
<td>3.04±0.78</td>
<td>2.71±0.88</td>
<td>0.91±0.27</td>
</tr>
<tr>
<td>Bladder</td>
<td>0.51±0.35</td>
<td>-----</td>
<td>9.30±3.84</td>
<td>-----</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.58±0.10</td>
<td>1.01±0.23</td>
<td>0.048±0.011</td>
<td>0.06±0.007</td>
</tr>
<tr>
<td>Brain</td>
<td>0.029±0.004</td>
<td>0.008±0.001</td>
<td>0.015±0.003</td>
<td>0.008±0.001</td>
</tr>
<tr>
<td>Muscle</td>
<td>14.2±4.50</td>
<td>0.079±0.020</td>
<td>7.78±1.63</td>
<td>0.049±0.11</td>
</tr>
</tbody>
</table>

± = Standard deviation (n=5), where n=5 for all species
Figure 4.1 Schematic representation of the soft tissue anatomy of a rabbit as viewed from the anterior position.
FIGURE 4.2 Region of interest clearance curves for Heart, Liver, Lung, Kidneys, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(t-butyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.3 Region of interest clearance curves for Heart, Liver, Lung, Kidneys, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(carbomethoxymethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.4 Region of interest clearance curves for Heart, Liver, Lung, Kidneys, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(carboethoxymethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.5 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(carboisoproxymethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.6 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 5, 30, and 60 minutes post injection of $^{99m}$Tc(carbopropoxymethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.7 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 5, 30, and 60 minutes post injection of $^{99m}$Tc(carboisobutoxymethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.8 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 5, 30, and 60 minutes post injection of $^{99m}$Tc(carbobutoxymethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.9 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(carbotert-butoxymethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.10 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(1-carbomethoxyethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.11 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(1-carboethoxyethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.12 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(2-carbomethoxyethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.13 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 5, 30, and 60 minutes post injection of $^{99m}$Tc(2-carboethoxyethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.14 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 5, 30, and 60 minutes post injection of $^{99m}\text{Tc}(1\text{-carbomethoxypropyl isocyanide})_6^+$ in an anesthetized rabbit.
FIGURE 4.15 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(1-carboethoxypropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.16 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 5, 30, and 60 minutes post injection of $^{99m}$Tc(2-carbomethoxypropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.17 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 5, 30, and 60 minutes post injection of $^{99m}$Tc(l-carbomethoxyisopropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.18 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(2-carboxyethyl)isocyanide in an anesthetized rabbit.
FIGURE 4.19 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(2-carboethoxyisopropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 420. Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(2-carboisopropoxyisopropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.21 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(2-carbopropoxyisopropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.22 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 5, 30, and 60 minutes post injection of $^{99m}$Tc(2-carboisobutoxyisopropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.23 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(2-carbobutoxyisopropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.24 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(l-carbomethoxyisobutyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.25 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(1,2-biscarbomethoxyethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.26 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(2-N-methylaminocarbonylethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.27 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(1-N-methylaminocarbonylethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.28 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(2-N-methylaminocarbonylisopropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.29 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(carboxymethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.30 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(2-carboxylethyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.31 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(1-carboxylpropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.32 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(2-carboxylisopropyl isocyanide)$_6^+$ in an anesthetized rabbit.
FIGURE 4.33 Region of interest clearance curves for Heart, Liver, Lung, and Anger camera images at 15, 30, and 60 minutes post injection of $^{99m}$Tc(1-carboxylisobutyl isocyanide)$_6^+$ in an anesthetized rabbit.
References:


CHAPTER 5

ANALYSIS OF HYDROLYSIS PRODUCTS AND METABOLITES OF $^{99m}$Tc(CPI)$_6^+$
Introduction:

Establishing the structural integrity of a compound in vivo is essential to understanding its biological distribution.\(^{(1)}\) Analysis of radiopharmaceuticals in particular is complicated by the very low concentrations of the agents that are injected.\(^{(2)}\) Combining this small initial quantity with the dilution of the biological system makes characterization of possible metabolites impossible by conventional techniques. Increasing the amount of a drug that is given may distort the data if a metabolic process is a saturable one. Analysis of the kinetics for an enzymatic system in particular is very sensitive to substrate concentrations\(^{(3)}\) and results will be different at the radiopharmaceutical concentrations of 10\(^{-9}\) molar versus the 10\(^{-6}\) molar concentrations needed for mass spectrometry or spectroscopic techniques.\(^{(4)}\) However, a meaningful observation and quantification of an agent's integrity can be accomplished by radiometric detection of a chromatographic system that separates the various species present, provided the nature of the peaks can be established.\(^{(2)}\)

The rationale behind functionalizing these isonitrile complexes with ester and amide groups was to have the agents serve as substrates for esterases or proteinases. In theory, hydrolysis of these groups would yield a more hydrophilic compound of anionic charge that could be metabolically excreted from the non-target organs, i.e. the lungs and liver.

\[
\text{Liver / Lungs / Plasma} \\
\begin{align*}
\text{Enzymatic} & \\
Tc(CNCH}_2\text{COOR})_6^+ & \quad \overset{\text{------------}}{\longrightarrow} & \quad Tc(CNCH}_2\text{COO}^-)_6^{-5} + R-OH \\
6 \text{OH}^- &
\end{align*}
\]
Before analysis of possible metabolic products could be performed it had to be established that the hydrolyzed species could be formed, that they were stable, and possessed the anticipated reduced lipophilicity and high anionic charge under physiological conditions. Base-catalyzed hydrolysis of Tc(2-carbomethoxyisopropyl isocyanide)$_6^+$ in millimolar concentrations produced quantities of compounds that could be studied by FAB-MS, $^{99}$Tc NMR, and IR.

The characterized hydrolyzed species were then associated with specific retention times of a reverse-phase high-pressure liquid chromatography (RP-HPLC) system. With the identity of the radiometric peaks in hand a systematic study of the in vitro enzymatic kinetics could be performed using physiologically relative concentrations of substrate and enzyme. The molecularity of the hydrolysis reaction was found to be first order in both substrate and enzyme for several animal species. This study also allowed the selection of pseudo first order conditions for evaluating other kinetic parameters.\(^{(5)}\) It was observed that a substantial amount of ester hydrolysis occurred in blood plasma and that the variation in rates between humans and rodents was greater than a factor of 200. Biodistribution and imaging studies\(^{(6)}\) of the hydrolyzed products confirmed that rapid hydrolysis of the initial agent could account for the interspecies variation in myocardial uptake.

An important question for predicting possible differences in biodistribution of compounds between humans and the animal model was whether the enzymes involved had drastically different structures and substrate preferences, or whether they were simply present in much higher concentrations in rodents. Measurements of the observed rates of hydrolysis versus substrate concentrations at various temperatures
allowed the comparison of $K_m$, $V_{max}$, $\Delta H$, and $\Delta S$ for different species.\(^{(7)}\)

These studies indicate an active site in rat and mouse esterases that is significantly different from other mammalian esterases.

If, indeed, two completely different enzyme systems are at work, then it is possible that the rabbit screening model may miss a compound with potential for human use. To confirm the rabbit model, rates of hydrolysis for all the compounds tested were measured in rat, rabbit, and human plasma in vitro at 37 °C. In all cases the rate of hydrolysis was much more rapid in rodent plasma than in human or rabbit plasma. These results indicate that the rabbit is indeed an optimistic model and thus a good initial screening system.
Experimental:

Materials. Rabbit plasma was obtained from New Zealand Albino rabbits obtained from Pine Acre Rabbitry, Assonet, MA. Approximately 5 mL of blood was withdrawn from the ear vein and transferred to a heparinized "green top" test tube Vacutainer® (Becton-Dickinson, Rutherford, NJ). Albino mice (male CD-1) and rats (CD) were obtained from Charles River, Wilmington, MA. Blood was withdrawn by cardiac puncture after ether anesthesia and immediately transferred to a heparinized test tube. Human blood was obtained from male and female "healthy" graduate student "volunteers" of the M.I.T. variety. Blood samples were spun in a centrifuge at 3000 x g for 30 minutes at 4 °C, the plasma was removed and kept at 4 °C until used. No samples were used more than 72 hours after being withdrawn from the animal.

Technetium-99 NMR spectrometry. Spectra were obtained on a XL-400 NMR spectrometer, Varian Assoc., Palo Alto, CA employing a 94 kilogauss Nb-Ti superconducting magnet with a 5.4 mm bore broad band probe. Technetium-99 as TcO₄⁻ resonates with a frequency of 90.03 MHz at this field strength.(9) Spectra were obtained with methanol or water as the solvent, methanol-d₄ as the lock solvent, and Tc(TBI)₆⁺ in methanol as an external reference.(10)

Reversed-phase high-performance liquid chromatography, RP-HPLC. Analysis of "carrier" and "carrier free" preparations and hydrolysis products of Tc(2-carbomethoxyisopropyl isocyanide)₆⁺ were carried out on a Waters Chromatography System as described in Chapter 3. The stationary phase consisted of C-8 bonded spherical silica particles (5 μM) in a 10 X 0.46 cm cartridge column (Brownlee Assoc.). The gradient mobile phase was
100% aqueous buffer (0.05 M (NH₄)₂SO₄, pH 6.0) to 95% methanol in 10 min in a linear mode.

**Preparation of hydrolysis products of Tc(CPI)₆⁺.** The starting material ⁹⁹Tc(2-carbomethoxyisopropyl isocyanide)₆Cl was prepared at the "spiked carrier added" concentration as detailed in Chapter 3 and separated from the excess ligands and reactants by the Sep-Pak® protocol also outlined in Chapter 3. This compound was dissolved in 25% ethanol/water in an NMR tube, and the ⁹⁹Tc NMR observed after sequential addition of NaOH/H₂O in molar fraction increments of 0.15 : 1.0 (OH⁻/Tc). Addition of base was continued until a molar ratio of 3:1 had been reached. At this point the pH was lowered with HCl/H₂O in 0.05 pH unit increments and the chemical shifts observed. A similar set of experiments were performed and analyzed by HPLC.

Addition of seven equivalents of NaOH to one technetium and incubating at 40 °C for 20 minutes yielded a single peak by both ⁹⁹Tc NMR and RP-HPLC. This solution was purified by loading onto a C-18 Sep-Pak® followed by elution with methanol, evaporation, and loading onto a SiO column. The activity was eluted with 0.1 M acetic acid/methanol, the volume reduced in vacuo, and the residue analyzed by FAB-MS. The pure identified compound was dissolved in D₂O and its ⁹⁹Tc NMR chemical shift measured at various intervals from pH 11.0 to 0.5.

The mono-hydrolyzed, Tc(CNC(CH₃)₂COOCH₃)₅(CNC(CH₃)₂COO⁻)₁⁰, neutral material was made by partial hydrolysis of the parent Tc(CPI)₆⁺ and separated from its precursor by ion pairing reverse-phase chromatography on a C-18 Sep-Pak®. This compound was also analyzed by FAB-MS.
**Kinetics protocol.** For each hydrolysis experiment 200 µL of plasma, or the appropriate dilution with saline, was pipetted to a borosilicate culture tube (Fisher Scientific, Pittsburgh, PA) and equilibrated to the specific temperature in a water bath. The $^{99m}Tc$(ester isonitrile)$_6^+$ complex in 20 µL of 25% ethanol/saline (0.15 M) was added, the contents shaken and returned to the water bath for the indicated incubation time. The hydrolysis was halted by precipitating the plasma proteins by adding 1.0 ml cold (4 °C) absolute ethanol and chilling in ice followed by centrifugation at 4 °C. A quantitative analysis indicated that the $^{99m}Tc$-complex did not precipitate with the proteins. The ethanolic supernatant was then analyzed by radiometric detection of a reverse-phase high-pressure liquid chromatographic system. Retention times of peaks were identified by comparison with chromatographs of characterized base-hydrolyzed ester complexes.
Results and Discussion:

Characterization of hexakis(2-carbomethoxyisopropyl isocyanide)-technetium(I) hydrolysis products. The $^{99}$Tc NMR of these symmetrical $d^6$ diamagnetic octahedral isonitrile complexes in micromolar solution gives sharp single peaks, 1-3 ppm FWHM, (10) The complex hexakis(carbomethoxy-isopropyl isocyanide)technetium(I), Tc(CPI)$_6^{+}$, has a characteristic resonance at 1931 ppm upfield from TcO$_4^{-}$. The fact that the hydrolysis products of Tc(CPI)$_6^{+}$ have different chemical shifts provides a convenient way to study this reaction. Hydrolysis of the first ester linkage produces a 5 ppm downfield shift from the parent compound. This effect proved to be additive for hydrolysis of each of the remaining five ligands. By observing the $^{99}$Tc NMR chemical shift of the ester isonitrile complex with incremental addition of aqueous sodium hydroxide the production of the sequentially hydrolyzed mixed ligand compounds could be quantitatively followed.

A comparison of radiometric detection HPLC analysis of these species with $^{99}$Tc magnetic resonance peak integration confirmed this quantitation. Figure 5.1 shows the $^{99}$Tc NMR spectrum for a 3:1 stoichiometric addition of NaOH to $^{99}$Tc(CPI)$_6^{+}$. As predicted for random hydrolysis, seven peaks were observed corresponding to starting material and the mono-, di-, tri-, tetra-, penta-, and hexa-hydrolyzed species. Continued addition of sodium hydroxide resulted in a single peak in the $^{99}$Tc NMR spectrum, 30 ppm downfield of Tc(CPI)$_6^{+}$. This sample was evaporated, excess salt removed, and analyzed by FAB-MS and IR. Figure 5.2 shows the mass spectrum with the anticipated $M^+$ peak at 777 mass units corresponding to the protonated peracid species Tc(CNC(CH$_3$)$_2$COOH)$_6^{+}$ and the six sequential ($M^+$, + 22 mass units) substitutions of Na$^+$ for H$^+$. 
Since this compound was purified by column chromatography and eluted with methanol, a mixture of protonated acid and the sodium salt was obtained. The peaks of mass units less than 777 corresponded to degradation fragments that occurred on the probe as predicted from spectra of the parent ester isonitrile species in Chapter 3. An infrared spectrum of the totally hydrolyzed species exhibited a $\nu_{\text{CN}}$ stretch at 2197 cm$^{-1}$, or 4 cm$^{-1}$ higher than the band for the ester isonitrile complex, Figure 5.3. Also noticeable was the split $\nu_{\text{CO}}$ stretch at 1708 and 1643 cm$^{-1}$ corresponding to the sodium salt and protonated free acid isonitrile ligands.

To further characterize the intermediate compounds and confirm that hydrolysis was not simply occurring on the mass spectrometer probe, a sample of the first three hydrolysis products was analyzed by FAB-MS. Figure 5.4 shows the spectra obtained with the m/z peaks at 847, 833, and 189 corresponding to the mono-, di-, and tri-hydrolyzed species, as well as their respective sodium salts. Figure 5.5 is the reverse-phase HPLC of this mixture with the UV absorption detected concentrations. The peaks are split in this chromatograph because of the existence of isomers for the di- and tri-hydrolyzed products. A rough comparison of FAB-MS peak heights with the HPLC integration reveals that significant hydrolysis does not occur on the probe.

The $^{99m}$Tc chemical shift of the peracid species, Tc(CN(C$_2$H$_5$)$_2$COOH)$_6^{2+}$, was remarkably pH dependent. Measurement of the resonance frequency with decreasing pH, Figure 5.6, demonstrated a gradual upfield shift to the frequency of the parent ester compound. A plot of chemical shift versus pH produced a sigmoidal curve from which a single $pK_a = 2.95 \pm 0.05$ was calculated for this complex, Figure 5.7. At this pH the compound exists
in a dynamic equilibrium where the average formula was considered to be 
\[ \text{Tc(CNC(CH_3)_2COOCH_3)_3(CNC(CH_3)_2COO^-)_3}^-2 \]. No breaks in this curve were 
oberved indicating that, within experimental error, all the acid 
isonitrile ligands have the same pK_a.

The mixed ligand complexes (acid and ester isonitriles) also 
exhibited a pH dependent chemical shift, Figure 5.8. Spectra for a 
mixture of all seven compounds were obtained at various pH's. The 
resonance frequency for all six of the hydrolyzed species gradually 
shifted back to overlap with the parent cationic hexakis(ester 
isonitrile)technetium(I) complex. A plot of the difference in chemical 
shift between each hydrolysis species and the parent compound indicated 
that all these species have nearly the same pK_a, Figure 5.9. Notice 
that this pK_a was different from the one calculated for the totally 
hydrolyzed complex due to the presence of methanol, which was required 
to solubilize the lipophilic ester compound.

The additive deshielding shift in resonance frequency with 
subsequent hydrolysis is not completely understood. The major 
determinate of chemical shift for technetium compounds appears to be due 
to varying percentages of paramagnetism. The infrared spectrum for 
Tc(CNC(CH_3)_2COOH)_6^+ exhibits a 4 cm⁻¹ blue shift, indicating a slightly 
stronger C=\text{N} bond or less Tc-to-ligand back-bonding. This would 
indicate that the hydrolyzed ligand is a poorer \sigma\text{-donor ligand than} 
the ester isonitrile. A weaker ligand field may allow an increased, 
albeit small, percentage of paramagnetic character.\(^8\)

**Assignment of hydrolysis species.** A RP-HPLC analysis for a no 
carrier added \(^{99}\text{mTc(CPI)}_6^+ \) "kit" preparation, buffered at pH 10.0, is 
shown in Figure 5.10. As can be seen with time the hexakis(ester
isonitrile) Technetium complex decreased and subsequent peaks with shorter retention times, i.e., more hydrophilic species, grow in and subsequently disappear. If hydrolysis is allowed to continue a total of nine new peaks develop and eventually progress to the single most hydrophilic hexa-hydrolyzed species. The extra three peaks are due to the cis, trans, fac, and mer isomers of the di-, tetra-, and tri-hydrolyzed complexes.

Comparison of the HPLC peaks integration ratios with ratios predicted for random sequential hydrolysis allows the assignment of the various isomers. As indicated in Figure 5.11, hydrolysis of the mono-hydrolyzed species can occur at any of the four ligands adjacent to the hydrolyzed ligand or at the single dissimilar site opposite to it. Thus the theoretically predicted ratio for cis-to-trans isomer of the di-hydrolyzed species should be 4:1. A similar argument predicts ratios of 1.0:1.5 fac-to-mer for the tri-hydrolyzed species and 4:1 cis-to-trans for the tetra-hydrolyzed species. Figure 5.12 shows a RP-HPLC chromatograph with a comparison of calculated and theoretical peak ratios for the Tc(CPI)₆⁺ hydrolysis products. The near perfect comparison for these values is strong evidence for assigning the order of peak elution for this RP-HPLC system. With confirmation of the identity of these peaks a study of the enzyme kinetics could be performed.

Analysis of enzymatic hydrolysis of Tc(CPI)₆⁺. Incubation of carrier-free levels of ⁹⁹ᵐTc(CPI)₆⁺ with plasma from several species in vitro demonstrated significant amounts of hydrolysis. Measurements of initial rates of hydrolysis in human and rat plasma as a function of Tc(CPI)₆⁺ concentration and plasma dilutions were made and plotted as log(rate) versus log(plasma), Figures 5.13 and 5.14. These graphs, with
a slope equal to one, indicate that the hydrolysis is first order in both enzyme and substrate. Also noticed was that for rat plasma the reaction did not approach first order in enzyme concentration until diluted by a factor of 200. The limited solubility of the ester complex, Tc(CPI)₆⁺, prohibited sufficient addition to saturate the rat esterase system at normal plasma concentrations.

For a steady state approximation, the enzymatic hydrolysis of Tc(2-carboxethoxyisopropyl isocyanide)₆⁺ can be considered to follow the equation below.(7)

\[
\begin{align*}
\text{H}_2\text{O} + \text{E} + \text{S} & \xrightarrow{k_1} \text{ES} \xrightarrow{k_2} \text{E} + \text{P} + \text{MeOH} \\
& \xleftarrow{k_3} \\
\end{align*}
\]

Since the water molecule needed to balance the equation is present in such large excess, the kinetics can be simplified and assumed to follow second order.(5)

\[
\text{Rate} = k_2[E] = \frac{k_1 k_2 [E][S]}{k_1 + k_2 + k_1[S]} = \frac{k_2 [E] [S]}{k_1 + k_2 + [S]}
\]

This equation is typically consolidated to give

\[
\text{Rate} = \frac{v_{\text{max}} [S]}{K_M + [S]} \quad \text{where} \quad K_M = \frac{k_1 + k_2}{k_1} \quad \text{and} \quad v_{\text{max}} = [E]k_2
\]

This is considered the standard Michaelis-Menten equation. By rearranging and multiplying by \([S]\) to yield

\[
\frac{[S]}{\text{Rate}} = \frac{K_M}{v_{\text{max}}} + \frac{[S]}{v_{\text{max}}}
\]

the data can be plotted in a straight line where the slope = \(1/v_{\text{max}}\) and
the y-intercept is $K_M/V_{\text{max}}$. This Hanes plot minimizes the effect of random errors at lower rates.\(^{(5)}\) Figure 5.15 shows a Hanes plot for the effect on hydrolysis rate by Tc(CPI)$_6^+$ concentration. These values have been normalized to the enzyme concentration of normal plasma; note the factor of 1000 difference in Tc(CPI)$_6^+$ concentration.

For an enzymatic system that follows the mechanism above, $V_{\text{max}} = k_2[E]$ and is thus a relative term whose value is enzyme concentration dependent. Since it was unknown whether the enzyme concentration in rodents is different or if the rapid rate of hydrolysis is due to different enzymes, a comparison of $V_{\text{max}}$ does not give meaningful information.

However, the Michaelis constant, $K_M$, is a fundamental constant of an enzyme, independent of its concentration.\(^{(7)}\) Comparing $K_M$'s for hydrolysis of Tc(CPI)$_6^+$ by the two enzyme systems shows that the rat enzyme is 700 times faster than the human enzyme system. For simple steady-state conditions $K_M = (k_{-1} + k_2) / k_1$ so this increase could be due to a better "fit" of substrate to the rodent enzyme, i.e., a higher equilibrium constant ($k_1/k_{-1}$) or a faster rate of hydrolysis after the substrate binds to the enzyme ($k_2$). However, there is danger in comparing these data for two different enzyme systems if both do not follow the same steady-state mechanism.

Enzyme systems can be considered to obey the Arrhenius equation for reaction rates as a function of temperature to yield information on the activation energy barrier, $E_a$, for a reaction.\(^{(5)}\) Assuming that the rate-limiting step is the conversion of the enzyme-substrate complex, ES, to products for either a rapid equilibrium or steady state mechanism, then the overall rate of hydrolysis should be dependent on the $E_a$ for this step as shown in the Arrhenius equation below.
\[
\log k = \frac{-E_a}{2.3 R} \frac{1}{T} + \log A
\]

A plot of log(rate) versus inverse temperature (Figure 5.16) for hydrolysis in rat and human plasma shows a different behavior for these two systems. At low temperature the hydrolysis rate in rat plasma falls off faster than that for human plasma. Temperature-dependent behaviour such as this is common for enzyme systems where a different step becomes rate-limiting. However, it is noteworthy that this effect is more pronounced in the rodent enzymatic system. These plots do indicate that at physiological temperature the \(E_a\) for hydrolysis in rat plasma is 12,200 cal/mole, more than 10% lower than in human plasma (13,400 cal/mole).

In transition-state theory, the constant \(A\) in the Arrhenius equation ought to be equal to the frequency of collisions, \(Z\), for a bimolecular reaction, times a factor \(P\), which is the probability factor of the proportion of molecules that collide with the correct orientation for them to react. The \(E_a\) and \(A\) values are related to the enthalpy and entropy of activation by the following equations

\[
\Delta S^* = R \ln (ANh/RT) - R
\]

\[
\Delta H^* = E_a - RT
\]

where \(h\) = Planck's constant, \(N\) = Avogadro's number, and \(R\) is the gas constant. Inserting the data obtained from the Arrhenius plot produced values of \(\Delta H^* = 12,200\) cal/mole and \(\Delta S^* = -13.8\) eu for \(Tc(CPI)_6^+\) hydrolysis in rat plasma and \(\Delta H^* = 13,400\) cal/mole and \(\Delta S^* = -10.9\) eu for hydrolysis in human plasma, at near physiological temperatures.

The effect on the rate constant of by \(\Delta S^*\) and \(\Delta H^*\) is given by

\[
k = \frac{K_B}{h} T \exp(\Delta S^*/R) \exp(-\Delta H^*/RT)
\]
Again inserting the values obtained above for these two enzyme systems accounts for a difference of a factor of 10 due to the lowering of the activation energy in the rat enzyme system.

By measuring hydrolysis over time another significant difference was observed between these two enzyme systems. Figure 5.17 shows that for a 1:50 dilution of both mouse and rat plasma the reaction of all available Tc(CPI)$_6$$^\text{+}$ ($\sim$2 X 10$^{-12}$ moles) has proceeded to the di-hydrolyzed species by 4 minutes at 37 °C. The hydrolysis of the same concentration of technetium took considerably longer in normal undiluted human plasma, >15 minutes. However, the rate of hydrolysis in rodent plasma drops off to about zero after the second ester function has been cleaved. In human plasma the hydrolysis continued beyond the di-and tri-hydrolyzed products.

In addition, the distribution of cis and trans isomers obtained of the di-hydrolyzed species was inverted for the two enzyme systems. Figure 5.18 shows that after 40 minutes at 37 °C in human plasma the ratio of cis-to-trans isomers is 2.5:1.0. In the case of hydrolysis in rat plasma the ratio is 0.2:1.0.

Regardless of the exact mechanism involved, these data seemed to confirm the presence of two distinctly different enzymes in humans and rodents. The nature of their divergence is unknown. However, it does provide an explanation for the interspecies variation between mammals and rodents observed in the biodistribution of Tc(CPI)$_6$$^\text{+}$. Figure 5.19 confirms the rapid rate of hydrolysis observed in undiluted rat and mouse plasma. A HPLC chromatograph obtained after a 10-second incubation at 37 °C in human, rat, and mouse plasma shows virtually only di-hydrolyzed products for the rodents.
To confirm the difference in biological behavior of the mono-hydrolyzed species it was made, purified from starting materials, and tested by both Anger camera imaging in rabbits (Figure 5.20) and biodistribution in guinea pigs (Table 5.1). Neither species showed uptake into myocardial tissue as contrasted with the parent cationic compound. It appears the increased hydrophilicity or zwitter-ionic character prohibits uptake into heart muscle. This neutral species localized primarily in the liver of these animals and was also excreted through the hepatobiliary system as was the parent cation.

As a final check to make sure that rapid hydrolysis of the ester isonitrile technetium complexes was not occurring in the rabbit animal model, tests for hydrolysis in vitro at 37 °C were performed in rat, rabbit, and human plasma. Table 5.2 shows the complexes with various esters of methyl isocyanide and the percent of hydrolysis that occurred after 2 minutes at 37 °C. A good corelation was observed between human and rabbit plasma while significantly more hydrolysis had occured in rat plasma. This trend is also confirmed in Table 5.3 which compares the eight structural isomers of Tc(CPI)6+. In general more hydrolysis was observed in human plasma than in rabbit plasma. This seemed to confirm the rabbit as an optimistic screening model for these compounds. The largest difference in hydrolysis rates was observed for the ethyl ester analoges. Table 5.4 compares five ethyl ester isonitrile complexes. All of these species showed more hydrolsyis in humans than in rabbits.

It appears that enzymatic hydrolysis of these technetium complexes occurs quite readily in vivo and this conversion produces a significant change in the biological fate of the complex.
Table 5.1  Biodistribution data in guinea pigs comparing the organ localization of $^{99m}$Tc(2-carbomethoxlisopropyl isocyanide)$_6^+$ and its mono-hydrolyzed product. Note the factor of 10 decrease in heart uptake for the neutral zwitter-ion.
Comparison distribution in guinea pigs of $\text{Tc(cpi)}_6^+$ and its mono hydrolyzed neutral species.

<table>
<thead>
<tr>
<th></th>
<th>$\text{Tc(CNC(CH}_3)_2\text{COOCH}_3)^+$</th>
<th>$\text{Tc(CNC(CH}_3)_2\text{COOCH}_3)_5\text{(CNC(CH}_3)_2\text{COO})^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>% ID per Organ</td>
<td>% ID per Gram</td>
<td>% ID per Organ</td>
</tr>
<tr>
<td>Heart</td>
<td>1.25 ± 0.32</td>
<td>0.90 ± 0.20</td>
</tr>
<tr>
<td>Blood</td>
<td>1.40 ± 0.27</td>
<td>0.53 ± 0.08</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.91 ± 0.09</td>
<td>0.38 ± 0.11</td>
</tr>
<tr>
<td>Liver</td>
<td>13.3 ± 2.20</td>
<td>0.87 ± 0.29</td>
</tr>
<tr>
<td>Gut</td>
<td>34.4 ± 4.29</td>
<td>-----</td>
</tr>
<tr>
<td>Kidneys</td>
<td>10.4 ± 2.55</td>
<td>3.04 ± 0.78</td>
</tr>
<tr>
<td>Bladder</td>
<td>0.51 ± 0.35</td>
<td>-----</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.58 ± 0.10</td>
<td>1.01 ± 0.23</td>
</tr>
</tbody>
</table>
Table 5.2  In vitro hydrolysis for different esters of methylisocyanide in plasma after 2 minutes at 37 °C.
Hydrolysis of $^{99m}$Tc(CNC(CH$_3$)$_2$COOR in plasma in-vitro, after 2 minutes at 37°C.

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Rabbit</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl</td>
<td>(220%)</td>
<td>(65%)</td>
<td>(80%)</td>
</tr>
<tr>
<td>Ethyl</td>
<td>(207%)</td>
<td>(0%)</td>
<td>(43%)</td>
</tr>
<tr>
<td>n-Propyl</td>
<td>(262%)</td>
<td>(0%)</td>
<td>(2%)</td>
</tr>
<tr>
<td>iso-Propyl</td>
<td>(223%)</td>
<td>(0%)</td>
<td>(0%)</td>
</tr>
<tr>
<td>n-Butyl</td>
<td>(27%)</td>
<td>(5%)</td>
<td>(9%)</td>
</tr>
<tr>
<td>iso-Butyl</td>
<td>(118%)</td>
<td>(0%)</td>
<td>(0%)</td>
</tr>
<tr>
<td>tert-Butyl*</td>
<td>(26%)</td>
<td>(4%)</td>
<td>(5%)</td>
</tr>
</tbody>
</table>

* tert-butylacetateisonitrile
+ results expressed as percent of technetium-99m
Table 5.3  In vitro hydrolysis, comparison of 8 structural isomers of $^{99m}$Tc(CPI)$_6^+$ in plasma after 2 minutes at 37 °C.
Hydrolysis of $^{99m}$Tc(esterisonitrile)$_6^+$ in plasma in-vitro,* after 2 minutes at 37°C.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Rat</th>
<th>Rabbit</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Ligand 1" /></td>
<td>CPI</td>
<td>(200%)</td>
<td>(65%)</td>
</tr>
<tr>
<td><img src="image2" alt="Ligand 2" /></td>
<td>BCPI</td>
<td>(185%)</td>
<td>(15%)</td>
</tr>
<tr>
<td><img src="image3" alt="Ligand 3" /></td>
<td>BCNI</td>
<td>(265%)</td>
<td>(6%)</td>
</tr>
<tr>
<td><img src="image4" alt="Ligand 4" /></td>
<td>MEG</td>
<td>(234%)</td>
<td>(45%)</td>
</tr>
<tr>
<td><img src="image5" alt="Ligand 5" /></td>
<td>EBA</td>
<td>(131%)</td>
<td>(2%)</td>
</tr>
<tr>
<td><img src="image6" alt="Ligand 6" /></td>
<td>EAI</td>
<td>(300%)</td>
<td>(64%)</td>
</tr>
<tr>
<td><img src="image7" alt="Ligand 7" /></td>
<td>NPG</td>
<td>(400%)</td>
<td>(366%)</td>
</tr>
<tr>
<td><img src="image8" alt="Ligand 8" /></td>
<td>IPG</td>
<td>(150%)</td>
<td>(0%)</td>
</tr>
</tbody>
</table>

* expressed as percent of $^{99m}$Technetium.
Table 5.4  In vitro hydrolysis of some ethylesteralkyl isonitrile complexes of technetium in rat, human, and rabbit plasma.
In-vitro hydrolysis of $^{99m}$Tc(ethylesterisonitrile)$_6^+$ in plasma after 2 min. at 37°C.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Rat</th>
<th>Rabbit</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Ligand 1" /></td>
<td>EGI</td>
<td>(209%)</td>
<td>(81%)</td>
</tr>
<tr>
<td><img src="image2" alt="Ligand 2" /></td>
<td>EAI</td>
<td>(300%)</td>
<td>(64%)</td>
</tr>
<tr>
<td><img src="image3" alt="Ligand 3" /></td>
<td>EBA</td>
<td>(123%)</td>
<td>(3%)</td>
</tr>
<tr>
<td><img src="image4" alt="Ligand 4" /></td>
<td>EMAI</td>
<td>(207%)</td>
<td>(0%)</td>
</tr>
<tr>
<td><img src="image5" alt="Ligand 5" /></td>
<td>EEG</td>
<td>(254%)</td>
<td>(20%)</td>
</tr>
</tbody>
</table>

Expressed as percent of technetium - 99m.
Figure 5.1  $^{99}$Tc NMR spectrum of a mixture of $^{99}$Tc(CPI)$_6^+$ and its six sequential hydrolysis products. Hydrolysis of each ester function causes a 5 ppm downfield shift of the $^{99}$Tc nucleus.
Figure 5.2  Fast Atom Bombardment Mass Spectrum (positive ion mode) of Tc(CNC(CH₃)₂COOH)₆⁺, the hexa-hydrolyzed product of Tc(CPI)₆⁺, demonstrating sequential H⁺ substitution by Na⁺.
Tc(CNC(CH₃)₂COOH)₆⁺ \text{m/z = 777}
Tc(CNC(CH₃)₂COONa)₆⁺ \text{m/z = 909}
Figure 5.3 Infrared spectrum (KBr pellet) of Tc(CNC(CH$_3$)$_2$COONa)$_6^+$, the hexa-hydrolyzed product of Tc(CPI)$_6^+$. 
Figure 5.4 Fast Atom Bombardment (positive ion mode) spectrum of a mixture of the mono-, di-, and tri-hydrolyzed products of $^{99}\text{Tc(CPI)}_6^+$. Note both the protonated and sodium salts of the carboxylic groups.
$^{99m}$Tc(CNC(CH$_3$)$_2$)$_5$ (CNC(CH$_3$)$_2$COOCH)$_5$ COO$^+$ m/z = 847
Figure 5.5 Reverse-phase HPLC chromatogram of a mixture of mono-, di-, and tri-hydrolyzed products of $^{99}$Tc(CPI)$_6^+$. The parent cation elutes last due to its greater lipophilicity.
HPLC of Hydrolyzed $^{99}$Tc(cpi)$_6^+$

Detector

UV absorption
214 nm 1.0 AUFS
1 cm path length

<table>
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<tr>
<th>Integration</th>
<th>RT</th>
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<tr>
<td>cation</td>
<td>2.0%</td>
<td>8.40 min</td>
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<tr>
<td>neutral</td>
<td>18.8%</td>
<td>8.15 min</td>
</tr>
<tr>
<td>dihydrolyzed</td>
<td>41.8%</td>
<td>7.70 min</td>
</tr>
<tr>
<td>trihydrolyzed</td>
<td>35.3%</td>
<td>7.43 min</td>
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Figure 5.6 $^{99}$Tc NMR spectra of $^{99}$Tc(CNC(CH$_3$)$_2$COO$^-$)$_6$$_{-}^5$, the hexa-hydrolyzed product of $^{99}$Tc(CPI)$_6^+$, as a function of pH at 22 °C in H$_2$O.
Figure 5.7  Plot of the $^{99}\text{Tc}$ NMR chemical shift of $^{99}\text{Tc}(\text{CNC}(\text{CH}_3)_2\text{COO})_6^{5-}$, the hexa-hydrolyzed product of $^{99}\text{Tc}(\text{CPI})_6^{+}$, as a function of pH. The $pK_a$ was calculated to be $2.95 \pm 0.05$. 
$^{99}\text{Tc}$ Chemical Shift of $\text{Tc(CNC(CH}_3)_2\text{COO}^-)_6$
Figure 5.8 $^{99}\text{Tc}$ NMR spectra for a mixture of the parent $\text{Tc(III)}$ complex and the six sequential hydrolyzed products as a function of pH.
$^{99}$Tc NMR Chemical Shifts as a Function of Charge

\begin{align*}
\text{pH} = 2.05 \\
\text{pH} = 2.55 \\
\text{pH} = 3.05 \\
\text{pH} = 3.75 \\
\text{pH} = 4.11
\end{align*}
Figure 5.9 Plot of difference in $^{99}\text{Tc}$ NMR chemical shift between the parent cationic $^{99}\text{Tc}$(CPI)$_6^+$ and the various sequentially hydrolyzed products as a function of pH. Measurements were made in 25% ethanol/D$_2$O.
$^{99}\text{Tc NMR Chemical Shifts as a Function of Charge}$

$$\Delta = \delta \left[ ^{99}\text{Tc(CN\text{\textsubscript{4}}\text{\textsubscript{2}}\text{O\textsubscript{2}}\text{Me})_{6}}^{\ominus} \right] - \delta \left[ ^{99}\text{Tc(CN\text{\textsubscript{4}}\text{\textsubscript{2}}\text{O\textsubscript{2}}\text{Me})_{6-n}}^{(\text{CN\text{\textsubscript{4}}\text{\textsubscript{2}}\text{O\textsubscript{2}}^{-})_{n}}}^{1-n} \right]$$

Harvard Medical School/MIT
Figure 5.10  Reverse-phase HPLC analysis of the random base hydrolysis of $^{99m}$Tc(CPI)$_6^+$ at pH 10.0 as a function of time.
Random Hydrolysis of Tc(cpi)₆⁺ by NaOH at pH 10.0
Figure 5.11  Scheme for theoretical prediction of isomer ratios in the sequential random hydrolysis of Tc(CPI)$_6$$. Each bar represents an isonitrile ligand in a fixed geometry.
Sequential Hydrolysis of Hexakis(esterisonitrile) Technetium(I)
Figure 5.12  Reversed-phase HPLC chromatograph of the products of random hydrolysis of $^{99m}$Tc(CPI)$_6^+$ by NaOH. The more hydrolyzed, hydrophilic compounds elute first.
Reversed Phase HPLC of Tc(cpi)$_6^+$ Random Hydrolysis by NaOH

<table>
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<tr>
<th>PEAK #</th>
<th>$R_t$ (min.)</th>
<th>PERCENT</th>
<th>CALC.</th>
<th>THEO.</th>
<th>Identity</th>
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<tr>
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<td>Penta. hyd.</td>
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<td>4 : 1</td>
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<tr>
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<td>4.2 : 1</td>
<td>4 : 1</td>
</tr>
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<td>4</td>
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<td>1.5 : 1</td>
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</tr>
<tr>
<td>5</td>
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<td>1.4 : 1</td>
<td>1.5 : 1</td>
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<td>6</td>
<td>7.50</td>
<td>8.53</td>
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<td>4 : 1</td>
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<td>3.2 : 1</td>
<td>4 : 1</td>
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<tr>
<td>8</td>
<td>8.10</td>
<td>14.00</td>
<td></td>
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</tbody>
</table>
Figure 5.13 Log(rate) versus log concentration for determination of reaction order of enzyme and substrate in the in vitro hydrolysis of Tc(CP)\textsubscript{6}^+, in vitro. Each point represents the mean of three experiments ± SEM.
Determination of Reaction Order for Enzymatic Hydrolysis of Tc(cpi)$_6^+$ in Human Plasma
Figure 5.14  Determination of order of reaction for the enzyme in the hydrolysis of Tc(CPI)$_6^{+}$ in rat plasma, in vitro. A slope of one equals first order. Each point represents the mean of 3 experiments ± SEM.
Hydrolysis of Tc(CPI)$_6^+$ in Rat Plasma

Log (Rate)

Log (Plasma)

slope = 1.21
n = 6
Figure 5.15  Hanes plot for the rate of $^{99m}$Tc(CPI)$_6^+$ hydrolysis in human and rat plasma, in vitro, as a function of $^{99}$Tc(CPI)$_6^+$ concentration. Each point represents the mean of five experiments ± SEM.
Hanes Plot for Rate of Tc(CPI)$_6^+$ Hydrolysis as a Function of Concentration in Human and Rat Plasma

Human

$K_m = 8.17 \text{ nM/L}$

$V_{max} = 0.629 \text{ nM/L/min}$

Rat

$K_m = 11.25 \text{ uM/L}$

$V_{max} = 2.82 \text{ uM/L/min}$

$[\text{Tc(CPI)}_6^+] = \text{nmoles/liter of Human Plasma}$

$\mu\text{moles/liter of Rat Plasma}$
Figure 5.16  Arrhenius plot for the log (rate) of hydrolysis of $^{99m}$Tc(CPI)$_6^+$ in human and rat plasma in vitro as a function of temperature. Each point represents the mean of five experiments ± SEM.
Arrhenius Plot for Hydrolysis of Tc(CP1)$_6^+$ in Human and Rat Plasma

Rat
- $E_a = 12,200 \text{ cal/mole}$
- $\Delta H^\ddagger = 11,600 \text{ cal/mole}$
- $\Delta S^\ddagger = -13.8 \text{ eu}$

Human
- $E_a = 13,400 \text{ cal/mole}$
- $\Delta H^\ddagger = 12,800 \text{ cal/mole}$
- $\Delta S^\ddagger = -10.6 \text{ eu}$
Figure 5.17 In vitro hydrolysis of $^{99m}$Tc(CPI)$_6^+$ in human and diluted rat and mouse plasma over time. Each point represents the mean of three experiments.
Tc(cpi)$_6$\textsuperscript{+} Hydrolysis vs Time at 37°C

- **Mouse Plasma**: 1/50 Dilution
- **Rat Plasma**: 1/50 Dilution
- **Human Plasma**: Undiluted

Percent of $^{99m}$Tc Hydrolyzed vs Time (minutes)
Figure 5.18  Overlay of two reversed-phase HPLC chromatographs of hydrolysis products of $^{99m}$Tc(CPI)$_6^+$ in rat and human plasma in vitro. Note the inversion of isomer ratios observed for the di-hydrolyzed species.
- Hydrolysis of $^{99m}Tc(cpi)_6^+$ in Rat Plasma
- Hydrolysis of $^{99m}Tc(cpi)_6^+$ in Human Plasma
Figure 5.19  Reversed-phased high performance liquid chromatographs of "Kit" preparations of $^{99m}$Tc(CPI)$_6^+$ after 10 second incubations with human, rat, and mouse plasma in vitro.
Invitro Hydrolysis of Tc(cpi)$_6^+$ in Plasma

Human Plasma
10 seconds at 37°

Rat Plasma
10 seconds at 37°

Mouse Plasma
10 seconds at 37°
Figure 5.20 Anger camera study of biodistribution kinetics in a rabbit for $^{99m}$Tc(CNC(CH$_3$)$_2$COOCH$_3$)$_5$(CNC(CH$_3$)$_2$COO$^-$)$^0$, the mono-hydrolyzed product of $^{99m}$Tc(CPI)$_6^+$. 
Reference:


CHAPTER 6

CELLULAR KINETICS OF $\text{Tc(CP\text{I})}_6^+$ IN CHICK HEART MONOLAYERS
**Introduction:**

Hexakis(2-carbethoxyisopropyl isocyanide)technetium(I), Tc(CPI)$_6^+$, has shown favorable in vivo characteristics for myocardial imaging in both animal studies and initial human trials. Experiments in rabbits have shown cardiac perfusion images of diagnostic quality that demonstrate a linear correlation with microsphere analysis of blood flow. (1) Planar, gated, and tomographic images of high technical quality have also been obtained after exercise and at rest in the human which correlate well with Tl-201 scintigraphy for identification of transient ischemia. (2) However, fundamental questions concerning the mechanism of myocardial localization, such as whether the Tc(CPI)$_6^+$ complex is accumulated in myocytes or localized to vascular and interstitial spaces, remain unanswered. Tc(CPI)$_6^+$ is a monovalent cation like $^{201}$Tl$^+$ and on this basis Deutsch has proposed that transmembrane uptake of similar complexes, mediated by a transport mechanism, may occur in a manner analogous to sodium pump-mediated $^{201}$Tl$^+$ uptake. (3,4) On the other hand, the lipophilicity of the complex may promote myocyte membrane binding. If so, is this binding non-specific or specific, thus implying a receptor-mediated process?

To establish the localization of Tc(CPI)$_6^+$ in muscle tissue, its behaviour relative to these other cations, and to assist in the further development of this class of compounds, cultured chick heart cells were used as a model system to characterize cellular uptake and washout kinetics. Heart cells in culture provided a unique system to study myocyte interaction with this compound unincumbered by the complexity of whole organ preparations. (5) Cellular uptake studies of monocations by Skulsim, (6) using human erythrocytes, have indicated that both K$^+$ and
Tl\textsuperscript{+} are recognized by the Na\textsuperscript{+}/K\textsuperscript{+} ATPase pump and that ion transport differed only in a greater affinity for Tl\textsuperscript{+} than K\textsuperscript{+}. These studies have also shown that Tl\textsuperscript{+} is interchangeable with K\textsuperscript{+} in stimulating Na\textsuperscript{+} efflux from erythrocytes. Based on such results it is generally accepted that Tl\textsuperscript{+} behaves physiologically as a K\textsuperscript{+} analog. Thus cellular uptake of 201\textsuperscript{Tl} is in a large part dependent on cellular metabolism and ATP content and could also vary depending on the availability of sodium pump binding sites. Gallagher et al\textsuperscript{(7)} have recently compared K\textsuperscript{+} and Tl\textsuperscript{+} with three technetium monocations Tc(dimethylphosphinoethane)\textsubscript{2}Cl\textsubscript{2}\textsuperscript{+}, Tc(t-butyl isocyanide)\textsubscript{6}\textsuperscript{+}, and Tc(isopropyl isocyanide)\textsubscript{6}\textsuperscript{+}, in human erythrocytes and neonatal rat myocytes. Uptake of all three of these technetium species was not altered by inhibition of the Na\textsuperscript{+}/K\textsuperscript{+} ATPase pump. Interestingly, the rates of uptake as well as the apparent localization in the "cytosol" or the cellular membrane differed greatly for these three compounds. These experiments have suggested that the technetium compounds tested did not behave as K\textsuperscript{+} analogues.

Correlation of radiochemical and in vivo studies with an understanding of the cellular mechanisms of Tc(CPI)\textsubscript{6}\textsuperscript{+} uptake should aid in the clinical application and interpretation of examinations performed with these agents. The data with Tc(CPI)\textsubscript{6}\textsuperscript{+} do not support the presence of a specific mechanism for binding. However, results do demonstrate preferential binding to myocyte cell membranes in a manner which correlates with lipophilicity of the complex and intensity of cardiac images.
Experimental:

**Tissue culture.** The techniques for producing monolayer cultures of spontaneously contracting chick heart cells from 10 day-old chick embryos disaggregated with trypsin have been described.\(^{(8,9,10)}\) Circular glass coverslips (25mm) served as the substrate for preparations used in binding and kinetic studies. Cells were grown for 3 days in media containing L-[\(4,5^{-3}\text{H}(N)\)]leucine which allowed normalization of each preparation to cell protein content as described.\(^{(10)}\) Non-contractile (fibroblast) preparations were produced from cells adhering to the culture dish surface following a differential cell enrichment procedure. \(^{(8)}\)

**Preparation of hexakis(carboxymethoxyisopropyl isocyanide)-technetium(I), \(^{99}\text{Tc}(\text{CPI})_6^{+}.** The no carrier added (NCA) \(^{99}\text{Tc}(\text{CPI})_6^{+} \) was prepared from \(^{99}\text{TcO}_4^{-} \) generator eluate using the neat ligand and sodium dithionite as the reducing agent as described in Chapter 3. To prevent possible toxic effects due to the presence of excess isonitrile in this reaction mixture, the \(\text{Tc(CPI)}_6^{+} \) was purified by loading onto a pre-wet Millipore SEP-PAK\textsuperscript{®} C-18 cartridge, and subsequently washed with normal saline (10 mL) followed by ethanol/water 40% (v/v), (8 mL). The remaining absorbed \(\text{Tc(CPI)}_6^{+} \) was then eluted with an ethanol/saline mixture 90% (v/v) (2 mL). This eluate was buffered with ammonium acetate (0.5 M, 0.5 mL) and tested for radiochemical purity by reverse phase HPLC and found to be \(>98\%\) in all cases. The eluate was tested for free isonitrile using a colorometric test adapted from Crabtree, et al.\(^{(11)}\)

**Experimental solutions.** Kinetic and binding studies were performed in control HEPES-buffered solution with the following composition: NaCl, (137 mM); KCl, (4.5 mM); MgCl, (0.5 mM); CaCl\(_2\), (0.9 mM); HEPES,
(4.0 mM); dextrose, (5.6 mM); pH 7.35 in air; 37 °C. Ammonia solution, for internal pH experiments, also contained 20 mM NH₄Cl. Where appropriate, buffered solutions contained the following (TRIS/HEPES/pH, respectively): 20 mM/5 mM/8.5; 10 mM/18 mM/7.5; 4 mM/25 mM/6.5. Ouabain (Sigma Chemical Co., St. Louis, MO), verapamil (Knoll Pharm. Co., Whippany, NJ), amiloride (Merck, Sharp and Dohme Research Laboratories, West Point, PA), and bumetanide (Hoffman-LaRoche, Inc., Nutley, NJ) were dissolved directly from solid powder into solutions.

**Cellular kinetic studies.** Monolayers of heart cells attached to glass coverslips were preincubated in HEPES-buffered solution, pH 7.5, for 1 minute, and then incubated in 40 mm Pyrex slides with the buffered solution containing Tc(CPI)₆⁺. After the desired uptake time period, coverslips were rapidly removed and rinsed for 8 seconds each in three 30 mL volumes of ice-cold HEPES-buffered control solution (2-4 °C). Control experiments confirmed clearance of >95% of extracellular radioactivity with this protocol. At the time of removal of the coverslips, a 200 μL aliquot of the Tc(CPI)₆⁺ loading solution was obtained for normalization of Tc(CPI)₆⁺ cellular uptake to extracellular isotope concentration. For washout experiments preparations were then returned to buffered Tc(CPI)₆⁺ free solution (37 °C). Immediately following the cold rinsing, cells were scraped off the coverslips and dissolved into 1.4 mL of a solution containing 1% sodium dodecyl sulfate and 10 mM sodium borate in glass scintillation vials.

Vials were assayed for ⁹⁹ᵐTc activity in a well-type NaI gamma counter (Omega-1 Multichannel Analyzer, Canberra Industries). Radioactive samples were then allowed to decay for 5-6 days, after which liquid scintillation techniques were used for quantifying ³H activity.
Correlation between $^3$H-Leucine and total cell protein was established with each preparation by a Louvy assay. Control experiments showed no detectable interference from Tc-99m decay product emissions in the $^3$H window. Blank glass cover slips showed that plateau level Tc(CPI)$_6^+$ uptake was no more than 9% of the total cell-associated counts.

**Biodistribution in neonatal chickens.** Newborn, 5-11 day old male chicks, ranging in weight from 45-86 grams, were injected without anesthesia via the central wing vein with 50 μCi of $^{99m}$Tc(CPI)$_6^+$ in 50 μL (25% ethanol/saline). After five minutes, chicks were sacrificed by ether inhalation, organs immediately dissected, weighed and counted in a Packard Auto Gamma 500C.

**Cellular fractionation.** Five day old male chicks were injected as above and sacrificed at 15 minutes post injection. The hearts were removed and immediately chilled to 4 °C in a buffer solution, PO$_4^-$, (10 mM); NaCl, (150 mM); pH 7.5. Each heart, weighing 0.20 grams, was minced on a tray over ice and homogenized in 3 mL of ice cold buffer in a teflon pestle homogenizer for 2 minutes at 3600 rpm. The homogenate was separated by differential centrifugation into "cell membrane fragments"/10 min @ 1K G, "mitochondria"/10 min @3K G, "microsomes"/3 hr @ 30K G; and "cytosol" using a Beckman model L3-50 ultracentrifuge (SW 50.1 Rotor) and a Sorvall RC-5B superspeed centrifuge (SS34 Rotor).(12)

**Miscellaneous.** Values are presented as the mean ± SEM unless indicated. Statistical significance was evaluated by the two-tailed Student's t test (13) or one-way analysis of variance (14) as indicated in text. Some curves were fit by a least-squares fitting procedure. Equilibrium calculations were used to estimate total technetium.(15)
Results & Discussion:

Confirmation of the chick cell model. Tc(CPI)$_6^+$ has demonstrated significant interspecies variation in myocardial uptake (Table 5.1, Chapter 5). (16) To help confirm the applicability of a chicken heart cell model, biodistribution experiments were performed in newborn, 5-11 day old male chicks (Table 6.1). Results showed that chick biodistribution compared favorably to those previously demonstrated in mammalian species. Cardiac localization of 4% of injected dose/gram tissue was well above that observed in lung and blood. These studies suggested that chick heart cells in culture would be a suitable model system.

Uptake of Tc(carbomethoxyisopropyl isocyanide)$_6^+$ into cultured heart cells. Tc(CPI)$_6^+$ cellular uptake in contractile cultured chick heart cells reached a plateau level after 20 minutes (Figure 6.1) with a half-time ($t_{1/2}$) for the uptake process of 4.1 ± 0.7 min (n=6). Half-time appeared to be independent of extracellular Tc(CPI)$_6^+$ concentration. "Saturable" binding is a criteria for "receptor" mediated uptake; however, it could also indicate a nonspecific equilibrium binding condition.

Plateau level Tc(CPI)$_6^+$ uptake (binding) depended on external Tc(CPI)$_6^+$ concentration, [Tc(CPI)$_6^+$]$_0$, as shown in Figure 6.2. For concentrations of 10$^{-13}$ M to 10$^{-8}$ M, plateau level Tc(CPI)$_6^+$ binding was linearly proportional to [Tc(CPI)$_6^+$]$_0$ in the extracellular solution. In contrast to what might have been expected for an uptake mechanism requiring specific binding (i.e., a receptor), no concentration saturation was demonstrated. Assuming 4.04 x 10$^6$ cells/mg cell protein, (17) the range of plateau level binding demonstrated in this
experiment represented 10 to 3.3 x 10^5 molecules of Tc(CPI)_{6}^{+} bound per cell.

Equilibrium cell binding experiments were performed to confirm the non-specific nature of Tc(CPI)_{6}^{+} uptake. Contractile preparations were incubated in solutions containing equal amounts of tracer $^{99m}$Tc(CPI)_{6}^{+} with increasing amounts of carrier $^{99}$Tc(CPI)_{6}^{+} (Figure 6.3). No significant competitive displacement of $^{99m}$Tc(CPI)_{6}^{+} by carrier $^{99}$Tc(CPI)_{6}^{+} could be demonstrated.

Since Tc(CPI)_{6}^{+} is a monovalent cation,(1) several known inhibitors of cationic membrane transport in cardiac muscle were tested for their effect on 1 minute Tc(CPI)_{6}^{+} uptake (Table 6.2). Amiloride, an inhibitor of Na^{+}/H^{+} exchange,(18,19) showed a marginal inhibitory effect on Tc(CPI)_{6}^{+} uptake which was statistically insignificant by one-way analysis of variance. The Na^{+}/H^{+} exchange system is a pH adjustment mechanism for the cell. Because inhibition of this system could cause acidosis, the effect of pH on uptake was also examined, vide infra. Ouabain (which inhibits the Na^{+}/K^{+} ATPase),(5) bumetanide (which inhibits Na^{+}/K^{+}/2Cl⁻ co-transport),(20) and verapamil (which blocks slow Ca^{2+} channels)(21) also showed no significant effect on Tc(CPI)_{6}^{+} uptake. In summary, these results suggested that Tc(CPI)_{6}^{+} cellular uptake was unlikely to be through one of these known cationic membrane transport mechanisms.

One minute, initial rate, Tc(CPI)_{6}^{+} uptake was observed to be temperature-sensitive. Compared to control uptake at 37 °C (1.4 x 10^{-14} ± 1.3 x 10^{-15} moles Tc(CPI)_{6}^{+} (mg cell protein)^{-1} (min)^{-1}; (n=3), 22 °C and 4 °C uptakes were 4.4 x 10^{-15} ± 9.8 x 10^{-16} (n=3) and 3.9 x 10^{-15} ± 2.3 x 10^{-15} (n=3), respectively. Comparing experiments
performed at 37 °C to those at 22 °C yielded a $Q_{10}$ of 2.16$^{(22)}$ where $Q_{10} = (K_1 / K_2) \cdot 10^{(t_1-t_2)}$, suggesting that a highly complex biological process was unlikely to be mediating Tc(CPI)$_6^{+}$ uptake. This value corresponds to a calculated $E_a$ of about 14,000 cal/mole, from the integrated form of the Arrhenius equation.$^{(22)}$

Since tissue pH changes are known to occur during myocardial ischemia$^{(23)}$ and may effect uptake of a myocardial imaging agent, the effects of intracellular pH (pH$_i$) changes on Tc(CPI)$_6^{+}$ uptake were tested using the NH$_4$Cl "pre-pulse" technique.$^{(19)}$ Incubation of heart cells in NH$_4$Cl solution causes an intracellular alkalization during the first few minutes of exposure and subsequent return to control solution produces an intracellular acidification.$^{(19,24)}$ Tc(CPI)$_6^{+}$ uptake performed during the first minute of NH$_4$Cl exposure (alkaline pH$_i$) and during the first minute of return to control solution (acid pH$_i$) showed no significant difference from control Tc(CPI)$_6^{+}$ uptake (Figure 6.4A).

In addition to demonstrating the lack of pH$_i$ effect on Tc(CPI)$_6^{+}$ uptake, these data confirmed that the Tc(CPI)$_6^{+}$ was not transported into the cell on the Na$^+$ site of Na$^+/H^+$ exchange, since this exchanger has been shown to be strongly stimulated by intracellular acidification.$^{(19)}$

Large variations in external pH (pH$_0$), however, produced inhibition of Tc(CPI)$_6^{+}$ uptake particularly with alkalization (pH$_0$ = 8.5) (Figure 6.4B). No alkaline hydrolysis of the complex was observed below pH$_0$ 9.0 in control in vitro studies (22 °C). This may suggest that pH$_0$ could affect binding of Tc(CPI)$_6^{+}$ to the external membrane surface.

Washout of "bound" Tc(CPI)$_6^{+}$ from cultured heart cells. Following uptake to plateau levels in contractile cultures (30 min), Tc(CPI)$_6^{+}$ washout into isotope-free solution showed at least two components
(Figure 6.5). A slow component comprising approximately \(33 \pm 9\% \ (n = 4)\) of total Tc(CPI)\(_6^+\) content/mg cell protein had a \(t_{1/2}\) of 40 min and a fast component comprising the remaining Tc(CPI)\(_6^+\) content had a \(t_{1/2}\) of 4.5 min.

The two components demonstrated on Tc(CPI)\(_6^+\) washout experiments from contractile preparations could reflect either two subcellular compartments in the preparation or represent the two cell types known to be present in contractile preparations, i.e., contractile myocytes and non-contractile fibroblasts.\(^{5,10}\) To approach this question, non-contractile (fibroblast) preparations were grown in culture and evaluated using identical conditions for Tc(CPI)\(_6^+\) uptake and washout as that used for contractile cultures. These preparations yielded a washout curve best approximated by a single component of washout from contractile preparations (Figure 6.5). Thus, it is possible that the slow washout component from contractile preparations represents washout of Tc(CPI)\(_6^+\) from contaminating fibroblasts in the preparations.

In addition, Tc(CPI)\(_6^+\) uptake was allowed to occur to plateau levels at various extracellular concentrations and then washout experiments were performed for 30 minutes to estimate slow compartment binding. This demonstrated that the compartment loaded in a manner also directly proportional to the initial extracellular Tc(CPI)\(_6^+\) loading concentration (data not shown). Therefore, fibroblasts in the preparation also showed no evidence of concentration saturation.

The concentrations of extracellular Tc(CPI)\(_6^+\) used in this study on cellular binding appear to be within the clinically relevant range. In current clinical imaging protocols, 5 mCi Tc(CPI)\(_6^+\) (specific activity \(\sim 1 \times 10^{-9}\) moles of total \(^{99}\)mTc(CPI)\(_6^+\) and \(^{99}\)Tc(CPI)\(_6^+\) /mCi) are injected
into patients. (2) Assuming a bolus dilution to about 0.5 liters of blood for initial volume-of-distribution, Tc(CPI)\(_6^+\) extracellular concentration would be typically 2 \times 10^{-11} \text{ M/liter}. Of course, if Tc(CPI)\(_6^+\) were distributed additionally into the extracellular spaces, its concentration would be several-fold lower. Nevertheless, these estimates are well within the linear bounds of the experimental series illustrated in Figure 6.2. These results also correlate with flow studies in isolated perfused heart preparations which show Tc(CPI)\(_6^+\) uptake to be directly proportional to regional flow. (1)

Despite the apparent non-specific partitioning of Tc(CPI)\(_6^+\) into heart cell membranes, some selectivity for heart tissue was demonstrated in these experiments. When both contractile and non-contractile fibroblast preparations were equilibrated with Tc(CPI)\(_6^+\) loading solution at the identical specific activity, contractile preparations showed 3 to 4-fold increase in binding. In addition, assuming similar compartment sizes, analysis normalized to cell protein also indicated that the myocyte Tc(CPI)\(_6^+\) binding compartment contained 3-4 times the activity found in the fibroblast compartment.

Independent confirmation of the subcellular localization was obtained by cell fractionation techniques of in vivo localized Tc(CPI)\(_6^+\). Following exposure to \(^{99m}\text{Tc(CPI)}\(_6^+\) for 15 minutes, fractionated cells showed localization of 63% of the activity within the cell membrane fragment, 8% within the mitochondria, 3% within the microsomes, and 26% in the cytosol. Examination of the membrane fraction under the light microscope confirmed the absence of intact cells. Subcellular distribution was not altered by addition of sodium dodecyl sulfate, sucrose, or triton X-100 to the buffer solutions prior to homogenizing.
The selectivity of Tc(CPI)_{6}^{+} uptake into myocytes over fibroblast cells is apparent in the clinical images. Supposedly nonspecific binding to lung fibroblasts accounts for the prolonged lung activity observed with the Tc(t-butyl isocyanide)_{6}^{+} compound. This lower non-specific uptake in fibroblasts produces the large heart/lung ratio of accumulation for this Tc(CPI)_{6}^{+} complex. The nature of the myocyte's higher affinity for this lipophilic species has not been established. It is conceivable that certain lipid components of myocyte cell membranes impart favorable characteristics for lipid partition of this Tc(CPI)_{6}^{+} complex, compared to other cell types.

**Summary:**

Cultured chick heart cells are a physiologically stable myocardial preparation with a loose extracellular matrix which minimizes extracellular diffusion delays. This property in combination with the lack of vascular endothelial or neural elements provides a simple system advantageous for the evaluation of cellular kinetics of tracers without the complexities of whole tissue preparations.\(^{(5)}\)

This study has characterized cellular kinetics as well as selectivity of binding of Tc(CPI)_{6}^{+} to myocytes. Tc(CPI)_{6}^{+} is a highly lipophilic complex with overall monovalent cation charge.\(^{(1)}\) Unknown was whether the lipophilicity or monovalent charge of the complex would dominate in any mechanism of cellular uptake of binding.

Tc(CPI)_{6}^{+} cellular uptake was found to reach a plateau with \(t_{1/2}\) of 4.5 minutes. Several known inhibitors of cationic membrane transport in heart tissue were tested and shown to have no significant effect on Tc(CPI)_{6}^{+} uptake. Increasing extracellular Tc(CPI)_{6}^{+} concentration linearly increased plateau levels of Tc(CPI)_{6}^{+} uptake (binding). Carrier
$^{99}$Tc(CPI)$_6^+$ did not compete for tracer $^{99m}$Tc(CPI)$_6^+$ binding. Furthermore, cell fractionation experiments demonstrated Tc(CPI)$_6^+$ to be closely associated with the cell membrane.

These data suggest that Tc(CPI)$_6^+$ uptake is dominated by its lipophilic properties and probably involves non-specific partitioning into the cell membrane. This would make Tc(CPI)$_6^+$ an ideal myocardial perfusion imaging agent since cellular uptake would depend on perfusion (delivery) alone. Unlike $^{201}$Tl$^+$ imaging, which depends on perfusion as well as cellular uptake (extraction), which in turn depends in part on cell metabolism and theoretically on available cell binding sites (sodium pump sites), Tc(CPI)$_6^+$ imaging would appear to depend only on delivery of the complex to the extracellular spaces of myocardial tissue.
Table 6.1  Biodistribution data for $^{99m}$Tc(CO)6+ in male, 5-7 day old chicks.
### TABLE 6.1

**BIODISTRIBUTION OF $^{99m}$Tc(CPI)$_6^+$ IN 5-11 DAY OLD MALE CHICKS**

<table>
<thead>
<tr>
<th>Organ</th>
<th>% ID/organ</th>
<th>% ID/gm</th>
<th>Heart/Organ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1.3 ± 0.2</td>
<td>4.0 ± 1.3</td>
<td>---</td>
</tr>
<tr>
<td>Blood</td>
<td>5.4 ± 1.5</td>
<td>1.6 ± 0.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Lung</td>
<td>1.1 ± 0.4</td>
<td>2.3 ± 0.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Liver</td>
<td>22.0 ± 2.0</td>
<td>11.0 ± 3.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Intestines</td>
<td>11.9 ± 2.3</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Kidneys</td>
<td>5.4 ± 1.4</td>
<td>12.0 ± 4.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Muscle</td>
<td>13.0 ± 3.0</td>
<td>0.6 ± 0.2</td>
<td>7.0</td>
</tr>
<tr>
<td>Gallbladder</td>
<td>4.3 ± 2.2</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SD (n=7) of the percent injected dose (ID) per organ and per gram 5 minutes post injection. Total recovery of activity in all cases was between 78 and 89 percent. Heart/organ ratio is derived per gram.
Table 6.2 Effect of various metabolic inhibitors on uptake of Tc(CPI)$_6^+$

into chick heart cells.
**TABLE 6.2**

EFFECT OF VARIOUS DRUGS ON Tc(CPI)$_6$\(^+\)

UPTAKE IN CULTURED HEART CELLS

<table>
<thead>
<tr>
<th>Drug</th>
<th>1 min Tc-99m CPI Uptake (%) control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiloride [10(^{-4})M]</td>
<td>76 ± 4% (N = 4)</td>
</tr>
<tr>
<td>Amiloride [10(^{-3})M]</td>
<td>79 ± 13% (4)</td>
</tr>
<tr>
<td>Ouabain [10(^{-4})M]</td>
<td>90 ± 8% (4)</td>
</tr>
<tr>
<td>Ouabain [10(^{-3})M]</td>
<td>98 ± 10% (4)</td>
</tr>
<tr>
<td>Rumetanide [10(^{-5})M]</td>
<td>101 ± 31% (4)</td>
</tr>
<tr>
<td>Verapamil [10(^{-6})M]</td>
<td>106 ± 27% (4)</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM (number of determinations) expressed as percent of control 1 minute uptake. Comparison between all values for $^{99m}$Tc(CPI)$_6$\(^+\) uptake showed no significant differences based on one-way analysis of variance.(14)
Figure 6.1 Tc(CPI)$_6^+$ uptake in contractile cultured chick heart cells.

Coverslips with cells were incubated in HEPES-buffered solution containing 13 µCi/mL of $^{99m}$Tc(CPI)$_6^+$ for various times before washing in ice-cold isotope-free solution. Each point represents the mean ± SEM of 3 determinations each. Solid line was drawn by eye assuming a saturable process with $t_{1/2}$ of 4.5 minutes.
Figure 6.2 Plateau level Tc(CPI)$_6^+$ uptake (binding) dependence on extracellular Tc(CPI)$_6^+$ concentration at constant specific activity. Loading solutions were prepared by addition of increasing volumes of the same $^{99m}$Tc(CPI)$_6^+$ stock solution to control solution. Solvent volume was adjusted to maintain constant total volume. Each point represents the mean ± SEM of three preparations each incubated for 20 minutes at the indicated total Tc(CPI)$_6^+$ extracellular concentration. Closed and open symbols represent different cultures.
Figure 6.3 Equilibrium binding of $^{99m}$Tc(CPI)$_6^+$ (20 minute incubation) as a function of carrier $^{99}$Tc(CPI)$_6^+$ concentration. Load solutions were prepared by addition of equal aliquots of $^{99m}$Tc(CPI)$_6^+$ with increasing aliquots of $^{99}$Tc(CPI)$_6^+$ Total extracellular Tc(CPI)$_6^+$ is indicated on abcissa.
Figure 6.4 One minute uptake of Tc(CPI)$_6^+$ in contractile cultured chick heart cells as a function of temperature. Each point represents the mean ± SEM of 3 determinations at each temperature. Asterix (*) indicates room temperature and ice water respectively.
Figure 6.5 Effect of intracellular pH (pH$_i$) changes (A) and extracellular pH (pH$_o$) changes (B) on 1 minute Tc(CPI)$_6^{+}$ uptake in cultured heart cells. Alkaline pH$_i$ changes: $^{99m}$Tc(CPI)$_6^{+}$ uptakes were performed concurrently upon switching preparations to control solution containing 20 mM NH$_4$Cl which typically alkalizes pH$_i$ by ~0.4 pH units. Acid pH$_i$ changes: $^{99m}$Tc(CPI)$_6^{+}$ uptakes were performed concurrently upon switching preparations into control solution following a 15 minute pre-incubation period in 20 mM NH$_4$Cl solution. This typically acidifies pH$_i$ by ~0.4 units. External pH changes: Preparations were pre-rinsed in HEPES/TRIS buffered solutions of the indicated pH$_o$ for one minute to clear extracellular solution spaces prior to $^{99m}$Tc(CPI)$_6^{+}$ uptake. Values are mean ± SEM of 4 determinations each. Only pH$_o$ 8.5 was significantly different from the appropriate control value.
Figure 6.6 Tc(CPI)$_6^+$ washout in contractile (o) and noncontractile (*) preparations. All preparations were incubated for 20 minutes in loading solution containing 63 µCi/mL of $^{99m}$Tc(CPI)$_6^+$ of identical specific activity and switched to Tc(CPI)$_6^+$-free control solution for washout. Each point represents a different preparation. Solid lines were fit by a least squares curve fitting procedure and graphical curve peeling which yielded the following equations: fibroblast preparations $y = 14e^{-0.017t}$; contractile preparations $y = 79e^{-0.16t} + 22e^{-0.017t}$. A second series of experiments confirmed the two-component washout from contractile preparations.
References:


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