SYNTHESIS AND RADIOLABELING OF TECHNETIUM RADIOPHARMACEUTICALS BASED ON N-SUBSTITUTED IMINODIACETIC ACID: EFFECT OF RADIOLABELING CONDITIONS ON RADIOCHEMICAL PURITY*

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SUMMARY

Prerequisite to the development of technetium-99m containing drug and biochemical analogs is the ability to synthesize radiochemically pure technetium chelates from mixtures of an appropriate chelating agent, pertechnetate, and various reducing agents. This paper reports the synthesis of a series of N-substituted iminodiacetates (IDA) in which the pKa of the imino nitrogen was varied from 5.0 to 8.7. The chelating agents were labeled with Tc-99m using the stannous reduction method at aqueous pH's of 4.0, 5.5 and 8.0 and in absolute methanol. The radiochemical purity of each chelate was examined by high pressure liquid chromatography, paper electrophoresis, paper chromatography, and tissue distribution studies. Aqueous radiolabeling conditions resulted in pure technetium chelates only when the pKa of the imino nitrogen was approximately 6. Methanolic labeling conditions resulted in pure radiochemicals for all N-substituted iminodiacetic acids provided the imino nitrogen had a pKa of greater than 6. Under non-aqueous conditions, however, the radiochemical purity deteriorated with time for all compounds in which the pKa of the imino nitrogen was greater than 7. These results indicate that only those IDA derivatives in which the *in vivo* nitrogen has a pKa of approximately 6 show a high degree of radiochemical purity when radiolabeled using stannous ion as the reducing agent.

Key Words: Radiopharmaceutical, Technetium-99m, Iminodiacetic Acid

INTRODUCTION

Understanding the complex interactions which dictate the biological fate of radiopharmaceuticals becomes exceedingly less

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difficult when single radiochemicals of high purity are available for study. Recent work has focused on the characterization of ^{99m}Tc-labeled radiopharmaceuticals as to their actual charge and molecular structure (1-4). Knowledge of these structural features together with proposed mechanisms of drug interactions with biological systems can aid greatly in the design of new imaging radiopharmaceuticals.

Earlier we proposed the incorporation of iminodiacetate (IDA) into drugs to create an agent capable of binding with ^{99m}Tc while retaining the biological actions characteristic of the parent drug (5). One such derivative, ^{99m}Tc-N-(2,6-dimethylphenyl)carbamoylmethyliminodiacetate (Tc-HIDA) has been previously reported to form a kinetically inert and thermodynamically stable bond with reduced technetium (3). A simple and convenient synthetic pathway for the preparation of N-substituted iminodiacetic acids utilizing a one-step nucleophilic displacement reaction has also been described (6). We now wish to report the synthesis of a series of IDA analogs in order to establish an optimum method of chelating with technetium and to determine the relationship between the basicity of the imino nitrogen and both radiochemical purity and chelate bond strength.

EXPERIMENTAL

Materials

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Proton NMR spectra were obtained from a Varian T-60 NMR spectrometer. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Paper electrophoresis was accomplished using a Beckman Durram type Model R-Series D Cell System and a .05M phosphate buffer as previously reported (3). Chromatograms were scanned with a Packard Model 7201 radiochromatogram scanner. Tissue samples were counted with a

Packard Model 2002 gamma counter. High pressure liquid chromatography (HPLC) separations were performed using a Waters Associates Model #M-6000A pump equipped with a Waters Associates μBondapak C_{18} column, UV detector and flow-through NaI(Tl) radiation detector. The flow rate of the mobile phase, .025M phosphate buffer (90%) and acetonitrile (10%), was 2 ml/min. The pKa measurements were performed on a Beckman pHasar I digital pH meter utilizing standard titration methods (7).

Synthetic Methods

N-phenyliminodiacetic acid (I) - Freshly distilled aniline (18.6 g, 0.2 mole) was added to an excess of chloroacetic acid (28.5 g, 0.3 mole). The solution was adjusted to pH 10 with 5N NaOH and refluxed for 24 hours. The mixture was then extracted with Et_2O (3 x 25 ml) and the aqueous layer acidified with conc. HCl to pH 3. The monosodium salt formed (15.0 g, 37.5% yield) was recrystallized from methanol, m.p. >300°C. NMR (δ) in methyl sulfoxide-d_6: 4.00 and 4.33 [4H, singlets, $N(CH_2)_2$], and 7.00 (5H, multiplet, aromatic).

Anal. Calcd for $C_{10}H_{10}NO_4Na$: C, 51.95; H, 4.33; N, 6.06. Found: C, 52.11; H, 4.46; N, 5.90.

N-(2-bromobenzyl)iminodiacetic acid (II) - A solution of disodium iminodiacetate (2.8 g, 0.02 mole) in 40 ml H_2O and 2-bromobenzyl-bromide (4.0 g, 0.02 mole) in 60 ml EtOH was held at reflux for 24 hours. The mixture was evaporated to dryness, reconstituted in 25 ml of H_2O and extracted with Et_2O (3 x 25 ml). The pH of the aqueous layer was acidified to 3 with 1N HCl. The precipitate which formed was recrystallized from H_2O to give 2.1 g of product (31.6% yield), m.p. 182-184°C. NMR (δ) in methyl sulfoxide-d_6: 3.50 [4H, singlet, $N(CH_2)_2$], 3.97 (2H, singlet, aromatic CH_2N), and 7.43 (4H, multiplet, aromatic).

Anal. Calcd for $C_{11}H_{13}NO_4Br$: C, 43.71; H, 3.97; N, 4.64; Br, 26.49. Found: C, 43.44; H, 3.85; N, 4.55; Br, 26.56.

N-(2-phenethyl)iminodiacetic acid (III) - Chloroacetic acid (17.0 g, 0.18 mole) was dissolved in 50 ml 95% EtOH and placed in an ice bath. The pH was slowly adjusted to 5.0 using 6N NaOH. Phenethylamine (10.0 g, 0.09 mole) was added slowly. The pH was maintained at 9.5 by periodic addition of 6N NaOH while the mixture was stirred for 20 hours at room temperature. The solution was then evaporated to dryness and the residue dissolved in 20 ml H_2O. Concentrated HCl was added dropwise until the solution turned cloudy (pH 2.0-3.0). The precipitate which formed overnight was recrystallized from H_2O to give 6.5 g (33.2% yield), m.p. 171-174°C. NMR (δ) in methyl sulfoxide-d_6: 2.83 (4H, multiplet, CH_2CH_2), 3.50 [4H, singlet, $N(CH_2)_2$], and 7.17 (5H, singlet, aromatic).

Anal. Calcd for $C_{12}H_{15}NO_4$: C, 60.76; H, 6.33; N, 5.91. Found: C, 60.62; H, 6.48; N, 5.89.

N-benzoyliminodiacetic acid (IV) - A mixture of disodium iminodiacetate (15.3 g, 0.1 mole), benzoyl chloride (13.8 g, 0.1 mole), and 5% NaOH (100 ml) was stirred at room temperature for 18 hours. The precipitate which formed on acidification of the resulting mixture to pH 3 was collected, washed with Et_2O (50 ml) and recrystallized from H_2O to give 6.7 g (26%) of product, m.p. 84-86.5°C. NMR (δ) in methyl sulfoxide-d_6: 4.13 and 4.30 [4H, singlets, $N(CH_2)_2$], and 7.10 (5H, singlet, aromatic).

Anal. Calcd for $C_{11}H_{11}NO_5$: C, 55.69; H, 4.64; N, 5.91. Found: C, 54.81; H, 4.95; N, 5.63.

N-benzoylmethyliminodiacetic acid (V) - A solution of disodium iminodiacetate (15.3 g, 0.1 mole) in 5% NaOH (50 ml) and phenyl acetyl chloride (14.1 g, 0.1 mole) in 50 ml 95% EtOH was held at

reflux for 24 hours. The mixture was evaporated to dryness, reconstituted in 25 ml of H_2O and extracted with Et_2O (3 x 25 ml). The pH of the aqueous layer was acidified to 3 with conc. HCl. The precipitate which formed was recrystallized from MeOH to give 5 g (10% yield), m.p. 149-150°C. NMR (δ) in methyl sulfoxide-d_6: 3.57 [4H, singlet, $N(CH_2)_2$], 4.37 (2H, singlet, $COCH_2$), 7.80 (5H, multiplet, aromatic).

Anal. Calcd for $C_{12}H_{13}NO_5$: C, 57.37; H, 5.18; N, 5.58.
Found: C, 57.50; H, 5.29; N, 5.52.

N-(2-phenethylcarbamoylmethyl)iminodiacetic acid (VI) - To nitrilotriacetic acid anhydride (10.0 g, 0.052 mole), prepared by the method of Burns (8), in pyridine (50 ml) was added 6.3 g (0.05 mole) phenethylamine and heated to 100°C for 30 minutes. The solution was reduced to a brown oil *in vacuo*. The oil was dissolved in 40 ml of 1.5N NH_4OH, treated with charcoal and filtered through a celite bed. Acidification of the filtrate with conc. HCl gave a white precipitate which was filtered and recrystallized from boiling water to give white crystals, 6.3 g (41.2% yield), m.p. 165-166°C. NMR (δ) in D_2O and NaOD: 2.87 (2H, triplet, J = 3.5Hz, phenyl CH_2), 3.17 (4H, singlet, $N(CH_2)_2$], 3.27 (2H, singlet, $COCH_2N$), 3.53 (2H, triplet, J = 3.5Hz, CH_2NCO), 7.27 (5H, singlet, aromatic).

Anal. Calcd for $C_{14}H_{18}N_2O_5$: C, 57.14; H, 6.12; N, 9.52.
Found: C, 57.57; H, 6.03; N, 9.43.

N-[1-(1-naphthyloxy)-2-hydroxypropyl]iminodiacetic acid (VII) - A solution of 1-(1-naphthyloxy)-3-chloro-2-propanol (700 mg, 3 mmole) and disodium iminodiacetic acid (600 mg, 3 mmole) in EtOH-H_2O (2:1) was held at reflux for 12 hours. The mixture was then evaporated leaving a yellow oil. The oil was taken up in 50 ml of H_2O and extracted with $CHCl_3$ (3 x 50 ml). The pH of the aqueous layer was adjusted to 3 with 1N HCl and the

precipitate which formed on cooling was recrystallized from H_2O to give 100 mg (10%) of VII: m.p. 149-151°C. NMR (δ) in methyl sulfoxide-d_6: 2.92 (2H, multiplet, $OCCH_2N$), 3.54 (4H, singlet, CH_2COO), 4.11 (3H, multiplet, OCH_2 and CHOH), and 7.63 (7H, multiplet, aromatic).

Anal. Calcd for $C_{17}H_{19}NO_6$: C, 61.26; H, 5.71; N, 4.20. Found: C, 60.94; H, 5.83; N, 4.01.

Labeling Procedure

The stannous reduction method for the formation of ^{99m}Tc chelates was used (9). A stock solution of stannous chloride was prepared by dissolving tin metal (20 mg) in concentrated HCl (0.1 ml). The resulting solution was diluted to 10 ml and stored at 4°C. In general, an aqueous solution of the appropriate ligand (10 mg) was adjusted to pH 5, 0.1 ml of the $SnCl_2$ stock solution was added, and the pH was then readjusted to 4.5, 7 or 8 prior to adding sodium ^{99m}pertechnetate. For chelate synthesis in non-aqueous solution a modified stannous reduction procedure was utilized. The ligand in this case was dissolved in absolute methanol. A stock solution of stannous chloride was prepared by dissolving 20 mg of anhydrous stannous chloride in 10 ml absolute methanol. A methanolic solution of $^{99m}TcO_4^-$ was prepared by extraction of basic $^{99m}TcO_4^-$ into methylethyl ketone. The solvent was evaporated and the $^{99m}TcO_4^-$ redissolved in absolute methanol. The normal sequence of chelation was then utilized using the same concentrations as in the aqueous synthesis. Tissue distribution studies were performed on various ^{99m}Tc chelates as previously described (5)

RESULTS AND DISCUSSION

As previously reported (4) HIDA was found to form a single bis radiochemical with Tc^{+3} and the resultant chelate, Tc-HIDA, was determined to undergo rapid hepatobiliary clearance.

Table 1 contains the HPLC retention time in minutes for Tc-HIDA as well as the retention times for similar N-substituted iminodiacetates. The pKa values for the imino nitrogen in each ligand are given. All compounds were chelated under aqueous conditions at a pH of 5.5.

TABLE 1.

Comparison of ^{99m}Tc Labeled N-Substituted Iminodiacetates at pH 5.5

Ligand	pKa Values	HPLC Retention Time in Min. (% Activity)
$C_6H_5CH_2CH_2N(CH_2COOH)_2$ (III)	8.7	2(26%), 5(52%), 22(22%)
$C_{10}H_7OCH_2CHOHCH_2N(CH_2COOH)_2$ (VII)	8.6	2(23%), 5.5(45%), 16(32%)
$Br\text{-}C_6H_5CH_2N(CH_2COOH)_2$ (II)	8.5	2(20%), 5(45%), 25(35%)
$C_6H_5COCH_2N(CH_2COOH)_2$ (V)	8.3	5(42%), 8(30%), 22(28%)
$(CH_3)_2C_6H_5NHCOCH_2N(CH_2COOH)_2$ (HIDA)	6.2	20(99%)
$C_6H_5CH_2CH_2NHCOCH_2N(CH_2COOH)_2$ (VI)	6.1	23(99%)
$C_6H_5CON(CH_2COOH)_2$ (IV)	5.1	2.5(10%)
$C_6H_5N(CH_2COOH)_2$ (I)	5.0	2.5(10%)

Under radiolabeling conditions characteristic of instant kit formulations (pH 5.5) ligands I and IV did not form stable products since only 10% of the radioactivity eluted from the HPLC column. Paper chromatography with saline showed that most of the radioactivity remained at the origin. Both these compounds are very weak chelating groups due to the resonance electron removal by the benzene ring of the unshared amino electrons

making them unavailable as donor electrons for chelate formation (10).

Of the remaining ligands studied under these conditions, only those with pKa values of approximately 6 showed high radiochemical purity. Ligands with pKa values of 8-9 formed radiochemical mixtures. Phenethylcarbamoylmethyliminodiacetate (VI), pKa 6.1, formed a single, stable radioactive compound with reduced $^{99m}TcO_4^-$. Its paper electrophoresis migration distance of 3.1 cm and HPLC retention time are comparable with previous data reported for ^{99m}Tc-HIDA. However, three or more radioactive peaks were observed on HPLC analysis for all ligands in which the imino nitrogen had a pKa of greater than 8. Paper electrophoresis of these ^{99m}Tc chelates also showed mixtures of radioactive compounds. ^{99m}Tc-(III) gave three distinct peaks with migration distances of 0 cm, 2.5 cm, and 3.5 cm, whereas ^{99m}Tc-(V) and ^{99m}Tc-(II) gave unresolvable broad bands of radioactivity. Thus, it appears that those ligands with a pKa of greater than 8, under aqueous chelation conditions of pH 5.5, form multiple radiochemicals.

The effect of these multiple radiochemicals on in vivo distribution was evaluated in mice using ^{99m}Tc-(III) compounded at a pH of 5.5. Table 2 contains distribution data for the mixture of radiochemicals and for a pure compound eluted from the HPLC as peak #3 (retention time = 22 min). The in vivo distributions were seen to be substantially different with peak #3 possessing enhanced hepatobiliary clearance, clearly showing that the radiochemicals contained in the crude aqueous mixture are sufficiently different in structure to allow for the body to discriminate between them. This discrimination was further attested to by collection of the bile from a series of

mice which had been previously injected with the crude reaction mixture. Table 2 contains the tissue distribution found upon reinjection of biliary contents into another series of mice. The radioactive biliary contents had a tissue distribution substantially identical to that obtained with pure peak #3 and much different from that of the initial crude material.

TABLE 2.

Tissue Distribution Studies in Mice of ^{99m}Tc-(III)*

Sample	Crude Aq. Sol. pH 5.5	Peak #3	Reinj. Bile Aq. Sol. pH 5.5**
Blood	3.51	4.11	4.21
Liver	20.75	8.32	8.21
Kidney	7.96	3.31	3.45
Stomach	1.27	3.01	3.21
Spleen	0.14	0.05	0.01
Intestine	36.45	72.22	73.28

* % injected dose; all values 1 hour post-injection, mean results of 4 mice.
**For detailed methodology, see ref. 5.

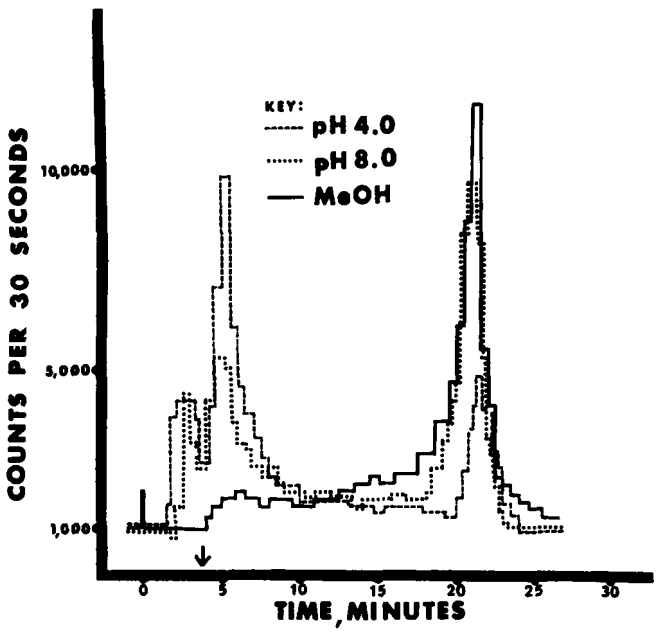
The enhanced purity of ^{99m}Tc. labeled VI and HIDA could be attributed to either the presence of the carbamoylmethyl group or to the reduced basicity of the imino nitrogen. Schwartzenbach et al (11) have shown that N-carbamoylmethyliminodiacetate can function as a tetradentate ligand with coordination through the amide oxygen. That such ligand participation could minimize hydrolysis appears unlikely since compound VII which possesses both a high pKa value and a hydroxy group capable of forming a third chelate ring (11) forms a mixture of radiochemicals when complexed with reduced technetium (Table I).

The determinant of radiochemical purity would therefore appear to be the basicity of the imino nitrogen. Compounds II, III and V which have pKa values in excess of 8 are substantially protonated at the radiolabeling pH of 5.5, possessing less than .16% of the added ligand (L) in the L^{-2} form. In contrast, compound VI and HIDA have 20 and 17%, respectively, of the added

ligand in the L^{-2} form. The hundred-fold reduction in the concentration of L^{-2} in compounds II, III and V favors dissociation of the chelate and increases the relative concentration of competing hydroxide ions. This hypothesis was evaluated by varying the radiolabeling pH from 5.5 to 4.0 and 8.0. In addition, the radiolabeling was conducted in absolute methanol. Figure 1 contains the HPLC radioactive elution profiles of ^{99m}Tc-(III) compounded at pH values of 4 and 8 and in methanol. The elution volume for free TcO_4^- is indicated by the arrow. As predicted, an increase in the amount of the radioactive compound having a 22 minute retention time under HPLC was observed as the pH of the reaction mixture became more basic. However, a single radiochemical could not be obtained by elevating the pH of the reaction mixture, presumably due to the increased hydroxide concentration. Similar elution profiles were obtained for ^{99m}Tc-(II) and ^{99m}Tc-(V).

FIGURE 1.

HPLC Elution Profile of ^{99m}Tc-Phenethyliminodiacetate



Chelation using methanol as the solvent yielded a relatively pure radioactive compound for all ligands in which the pKa of the imino nitrogen was greater than 6. If the pKa was greater than 8, however, these non-aqueous preparations became unstable after a maximum storage time of two hours, after which multiple radioactive peaks were again observed upon HPLC analysis.

The effect of radiolabeling methodology on tissue distribution is illustrated in Table 3 which contains tissue distribution data obtained by injecting the aqueous or non-aqueous chelates of ^{99m}Tc-(III) or aqueous ^{99m}Tc-(VI) into mice.

TABLE 3.

Tissue Distribution Studies in Mice*

Organ	^{99m}Tc-(III) pH 5.5	^{99m}Tc-(III) Methanol**	^{99m}Tc-(VI) pH 5.5
Blood	3.51	2.17	0.79
Liver	20.8	6.02	7.5
Kidney	8.0	3.22	0.35
Stomach	1.27	0.51	0.84
Spleen	0.14	0.02	0.06
Intestine	36.4	70.0	88.9

* 1 hour post-injection; % injected dose.
**Injected immediately after preparation.

The ^{99m}Tc chelate prepared in methanol has an *in vivo* distribution similar to that obtained for pure peak #3 (Table 2). This is in agreement with electrophoretic and HPLC data in which the two radiochemicals were seen to behave identically. In addition, ^{99m}Tc-(VI) was also found to exhibit marked hepatobiliary clearance (Table 3) and high radiochemical purity (Table 1).

The chromatographic and distribution data for the methanolic preparation of (III) and for the phenethylcarbamoylmethyliminodiacetate (VI) correspond well with that obtained for ^{99m}Tc-HIDA which has previously been shown to exist as an anionic bis

complex with the technetium being in the +3 oxidation state. It appears that such bis complexes can be prepared radiochemically pure from N-substituted iminodiacetates through aqueous reaction conditions when the pKa of the imino nitrogen is approximately 6 or through the use of a methanolic reaction media when the pKa is greater than 8. Even when the methanolic reaction is employed, the radiochemical purity is transient. This would indicate that such radiochemicals would have to be used shortly after preparation and imaged very shortly after injection. Thus, IDA should be synthetically incorporated into drug analogs in such a fashion as to reduce the electron density on the imino nitrogen from a pKa value of 8 to one of approximately 6. For this purpose, the carbamoylmethyliminodiacetate appears to be a better ligand than iminodiacetate itself.

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