Synthesis and Biological Evaluation of Technetium-99m MAG_3 as a Hippuran Replacement

Alan R. Fritzberg, Sudhakar Kasina, Dennis Eshima, and Dennis L. Johnson

Department of Radiology, University of Utah School of Medicine, Salt Lake City, Utah

A new technetium-chelating agent based on a triamide monomercaptide tetradentate set of donor groups, mercaptoacetylglycylglycylglycine (MAG_3), was synthesized and evaluated. Chelation with ^{99m}Tc resulted in a single radiochemical product as expected. Studies in mice of [^{99m}Tc]MAG_3 indicated excretion rates faster than *o*-iodohippurate (OIH) both in normal and in probenecid treated animals. Specificity for renal excretion was essentially complete. Clearance studies in rats resulted in 2.84 ml/min/100 g for [^{99m}Tc]MAG_3, 2.17 for OIH, and 1.29 for [^{125}I]iothalamate. Extraction efficiencies were 85% for [^{99m}Tc]MAG_3, 69% for OIH and 39% for [^{125}I]iothalamate. Probenicid depressed the clearance both of [^{99m}Tc]MAG_3 and OIH at 25 and 50 mg/kg/hr, but to a greater extent with [^{99m}Tc]MAG_3. The greater effect is offset, however, by the larger fraction secreted by the renal tubular cells. The animal results suggest that [^{99m}Tc]MAG_3 may be a useful alternative to [^{131}I]OIH.

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A number of diamide disulfur (DADS) ligands for technetium-99m (^{99m}Tc) have been synthesized and evaluated as potential replacements for iodine-131 (^{131}I) *o*-iodohippurate (hippuran, OIH). Early chemical (*1,2*), biological (*3,4*), and clinical studies (*5*) of the parent complex, technetium-99m *N,N'*-bis(mercaptoacetyl)ethylenediamine ([^{99m}Tc]DADS) indicated that the ^{99m}Tc complex was a well-defined radiochemical and was excreted rapidly by the kidney in a manner consistent with tubular secretion. However, [^{99m}Tc]DADS was inferior to OIH in terms of specificity and rate of excretion in normals and especially in patients with elevated levels of creatinine. Derivatives of the parent ligand showed improvements with respect to specificity and rate of renal excretion, but only when functional groups were present that resulted in chelate ring stereoisomers (*6–9*). In general, the renal handling was shown to be different for the chelate ring stereoisomers and while changing the conditions of complex preparation changed the proportion of each, sufficient stereoselectivity for the desired epimer was not obtainable.

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For reprints contact: Alan R. Fritzberg, PhD, Dept. of Medicinal Chemistry, University of Washington, Seattle, WA 98195.

The results of structure distribution relationships suggested that optimal rate of renal excretion and specificity resulted from the core ligand system and an additional carboxylate group in a preferred stereochemical relationship. A means of obviating stereochemical isomers resulted from changing the core donor atoms from N_2S_2 to N_3S. Placement of the carboxylate group on the third amido nitrogen results in a single radiochemical product as long as no additional groups that introduce a second asymmetric center are present. The simplest ligand corresponding to this set of considerations, mercaptoacetylglycylglycylglycine (MAG_3) was synthesized and the ^{99m}Tc chelate prepared and biologically evaluated as a renal tubular function agent (Fig. 1). This paper presents the results of these studies.

MATERIAL AND METHODS

General

Elemental analysis was obtained commercially*. Proton magnetic resonance spectra were obtained on a 90 MHz instrument† and high performance liquid chromatography (HPLC) was done on 5-μ 4.6 × 250 mm octadecylsilyl columns‡.

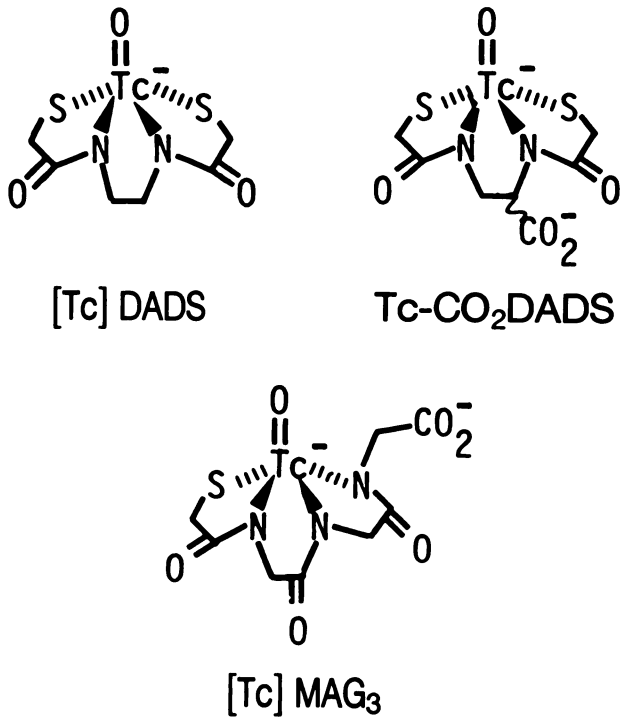

FIGURE 1
Structures of Tc-N_2S_2 (DADS) complexes and proposed structure of [Tc]MAG_3

Synthesis of Benzoylmercaptoglycylglycylglycine (Bz-MAG_3)

To a stirred solution of 2.50 g (0.013 mol) of glycylglycylglycine in 75 ml of 1.0 N NaOH in a 500-ml flask under nitrogen at 0°C, a solution of 13.0 g (0.115 mol) of chloroacetylchloride in 100 ml of ether was added dropwise from one addition funnel while simultaneously 100 ml of 1.0 N NaOH was added dropwise from another. After additions were complete the reaction mixture was stirred for 15 hr at 0°C. The mixture was then acidified with concentrated HCl while cooling. After stirring for an additional 30 min, the mixture was concentrated to one-third volume under reduced pressure at 40°C. The residue precipitated upon cooling in an ice bath. Isolation of the solid gave 2.75 g (78%) of chloroacetylglycylglycylglycine.

The crude chloroacetamido product (1.0 g, 0.0037 mol) was dissolved in 300 ml of anhydrous methanol under nitrogen. An equivalent of sodium thiobenzoate (prepared from 1.10 g (0.0076 mol) of thiobenzoic acid and dry methanol to which 175 mg (0.0076 mol) of sodium had been previously added in methanol) was then added in methanol. The reaction mixture was refluxed for 1.5 hr. After removal of solvent under reduced pressure, 2 N HCl was added with stirring. The solid residue was isolated by filtration and washed with chloroform. Crystallization from methanol gave 1.25 g (90%) of product: mp 195–196°C; nmr (DMSO-d_6) δ 3.73–3.80 (6 H, overlapping doublets, CH_2NHCO), 3.90 (2H, s, CH_2S), 7.65–8.05 (5H, complex, aromatic), 8.05–8.40 (2H, q, N*H*CO), and 8.40–8.65 (1H, t, N*H*CO), Anal. Calcd for $C_{15}H_{17}N_3O_6S$: C 49.05, H 4.63, N 11.44, S 8.72; Found: C 48.87, H 4.86, N 11.59, S 8.89.

Preparation of [^{99m}Tc]MAG

1. Dithionite Reduction. One milligram of Bz-MAG_3 was dissolved in 50 μl of 1 N NaOH. Then 1 to 2 ml of [^{99m}Tc]pertechnetate in generator saline containing the desired level of radioactive was added. Freshly dissolved sodium dithionite (1.0 mg in 20 μl of water) was added and the mixture heated for 2 min at 95°C. The preparation was then neutralized to pH 6 to 8 by addition of 1 N HCl. A major component (>80%) was found at about 4.0 ml elution volume on HPLC (ethanol:0.01*M* phosphate, pH 6.5, (5:95), flow rate 1.0 ml/min).

2. Exchange Method. Kits were prepared by dissolving Bz-MAG_3 with warming in a solution of sodium gluconate or other labile ligand of technetium at pH 5.5. Then under nitrogen, $SnCl_2 \cdot 2H_2O$ was added. Amounts were designed to result in 20 mg sodium gluconate or alternative ligand, 1.0 mg Bz-MAG_3, and 20 μg $SnCl_2 \cdot 2H_2O$. Kits were stored frozen or lyophilized. The ^{99m}Tc complex was prepared by adding 3 to 5 ml of [^{99m}Tc]pertechnetate in generator saline and heating at 95°C for 5 min. HPLC gave the same major component using the above-described condition as seen with Method 1. Radiochemical purity by HPLC for soluble forms and silica gel thin layer chromatography with saline as elution solvent was typically 95% or higher.

Iothalamate was radiolabeled with ^{125}I according to Kato et al. (*10*) and used as a glomerular filtration rate standard in renal clearance studies (*11,12*). High performance liquid chromatography (HPLC) analysis of recrystallized [^{125}I]isothalamate indicated greater than 99% of radiochemical purity.

Animal Studies General

Organ biodistribution studies were carried out in mice as previously described (*3,6*). Renal clearance studies and pharmacokinetic parameter determinations were carried out in rats. Radioactivity used for these studies were preparatively purified by HPLC before administration. Iodine-131 OIH was simultaneously administered in all studies and [^{125}I]iothalamate was also simultaneously administered as a glomerular filtration reference in rat studies. Analysis of radiochemical purity of OIH by HPLC resulted in values of 96.3 and 97.5% in two instances.

Biodistribution Studies

The time course of organ distribution was determined in groups of six female albino mice. Each was

injected with 0.10 ml (0.5 μCi) of the preparation. For comparison purposes, 0.2 μCi OIH was added to each injection. The mice were placed in metabolic cages for the collection of excreted urine. At indicated intervals after injection, the urethra was ligated and the mice killed with chloroform vapor. The organs were removed and counted in a dual-channel counter with correction from ^{131}I crossover into the ^{99m}Tc channel.

Rat Clearance Studies

Male Sprague-Dawley rats weighing between 250 and 350 g were used in the experiments. For a renal clearance study, the animal was anesthetized with ketamine hydrochloride 100 mg/kg intraperitoneally. A tracheostomy was performed and one jugular vein for infusions, one carotid artery for blood sampling and the bladder for urine collection were cannulated with PE 50 tubing. Two cannulas were placed in the jugular vein for infusion of the radiopharmaceuticals ([^{99m}Tc]MAG_3, [^{131}I]OIH, and [^{125}I]iothalamate) and for the replacement of intraoperative fluid, blood and urinary losses.

The glomerular filtration rate and the clearance of the radiopharmaceuticals were calculated from clearance periods obtained once steady-state blood levels were reached. Clearance values were determined by the standard UV/P relationship. Normal saline containing 10 μCi/ml of [^{99m}Tc]MAG_3, 2 μCi/ml of [^{125}I] iothalamate and 0.5 μCi/ml of [^{131}I]OIH was infused with a constant infusion pump at a flow rate of 20 μl/min. After an equilibrium period of 45 min and intravenous replacement of intraoperative fluid losses with normal saline, urine was collected under oil for three 10- to 15-min collection periods. Blood from the carotid artery was obtained at the midpoint of each clearance period. Hematocrits were measured from each blood sample obtained.

All samples were counted in a well counter with crosstalk correction for ^{131}I and ^{99m}Tc. The samples were recounted once ^{99m}Tc had been allowed to decay to background, and corrections for cross-talk between ^{131}I into the ^{125}I channel were made.

Rat Probenecid Studies

All animals were surgically prepared under the same conditions as those for the clearance value determinations. In addition, the left jugular vein was also cannulated with PE 50 tubing for the infusion of probenecid. After steady-state blood levels of the radiopharmaceuticals had been reached, two 10-min control collection periods were obtained. The probenecid infusion was started at dose levels of 10, 25, or 50/mg/kg/hr for 90 min. After the 90-min infusion period of probenecid three 10-min experimental clearance periods were obtained. Protein binding of the radiopharmaceuticals was also determined under 50 mg/kg/hr dose condition. The plasma sample was centrifuged in the Centrifree Micropartition System[§] for the determination of plasma protein binding. The micropartition system was centrifuged at 1,050 *g* in a swinging bucket centrifuge for 10 min. Samples of the serum and the ultrafiltrate were obtained and counted for radioactivity, with cross-talk corrections made for ^{125}I, ^{131}I, and ^{99m}Tc.

Rat Extraction Efficiency Studies

All animals were surgically prepared under the same conditions as those for the clearance value determinations. After two control clearance periods a 0.5-ml blood sample was obtained from the renal vein and a 3-ml blood sample was obtained from the carotid artery. Hematocrits were measured from both samples. A 0.5-ml blood sample was saved for counting of radioactivity and the rest of the blood sample centrifuged to obtain a plasma sample. Plasma protein binding was determined as described above.

RESULTS

Chemistry

Chelation of reduced ^{99m}Tc by either dithionite in base or ligand exchange at pH 5.5 gave a predominately single radiochemical form as expected. Another product with a slightly shorter retention time on reversed phase HPLC was found to decrease in proportion as the amount of ligand decreased. It is thought that this component may be a bis ligand complex, since when isolated and heated, conversion to the main product was observed. The purity of chelation using stannous ion and a labile ligand such as gluconate was found to be highest when the concentration of stannous ion was reduced to 0.03 m*M*.

The rate of exchange was found to be complete in 5 min when heated at 95°C. When the temperature was 50° the exchange was 85% at 20 min and 98% complete at 1 hr. At room temperature exchange was 52% complete after 2 hr and 92% after 18 hr.

Biology

The biodistribution properties of [^{99m}Tc]MAG_3 complex were evaluated in mice (Table 1). Rapid renal excretion was apparent at the 10-min value, with [^{99m}Tc]MAG_3 found at 107% of [^{131}I]OIH. High specificity was also observed at 120 min with renal excretion accounting for essentially all radioactivity.

The ability of [^{99m}Tc]MAG_3 to compete for tubular secretion in the presence of the renal tubular transport inhibitor probenecid was also studied in mice (Table 2). At 10-min postinjection, renal excretion of [^{99m}Tc]MAG_3 was reduced from 80% in the urine to 65% while [^{131}I]OIH was reduced from 74 to 59%. Thus in mice, in contrast to [^{99m}Tc]DADS and

TABLE 1
Biodistribution Properties of [^{99m}Tc]MAG_3 in Mice*

Radiochemical	Blood	Kidneys	Liver	Stomach	Intestines	Urine
			10 min			
[^{99m}Tc]MAG_3	2.63 ±0.15	3.53 ±0.27	2.93 ±0.08	0.11 ±0.01	1.10 ±0.05	79.89 ±0.86
[^{131}I]OIH	4.06 ±0.25	2.21 ±0.11	1.84 ±0.13	0.47 ±0.05	0.97 ±0.04	74.44 ±0.79
			120 min			
[^{99m}Tc]MAG_3	0.03 ±0.00	0.06 ±0.03	0.08 ±0.02	0.02 ±0.01	1.20 ±0.11	98.46 ±0.72
[^{131}I]OIH	0.14 ±0.01	0.06 ±0.03	0.11 ±0.01	0.98 ±0.08	0.16 ±0.01	96.00 ±1.11

* Values are mean ± s.e.m. % injected dose for six mice at each time postinjection.

[^{99m}Tc]CO_2DADS-A, which were reduced by probenecid to 20% (*4*) and 74% (*6*) of OIH, respectively, [^{99m}Tc]MAG_3 still retained a slight competitive superiority to OIH.

Clearance studies performed in rats showed a superiority for [^{99m}Tc]MAG_3 over [^{131}I]OIH (Table 3). Extraction efficiencies were 85 and 69% for [^{99m}Tc]MAG_3 and OIH, respectively as determined by arteriovenous differences and were in agreement with clearance values. Interestingly, iothalamate which is a GFR reference compound was found to have an extraction efficiency of 39% in the rat in agreement with other data (*13*), considerably higher than the 20% value for glomerular filtration in humans (*14*). Technetium-99m MAG_3 was predominantly protein bound at 77% in rat serum while OIH was found to be 33% bound. Using the protein binding values to correct for the glomerular filtration contribution, the percentages extracted by the tubular cells can be estimated as 75 ± 1 for [^{99m}Tc]MAG_3 and 43 ± 3 for OIH in the rat.

The effect of probenecid on the renal clearance of [^{99m}Tc]MAG_3 and reference radiopharmaceuticals in rats is shown in Table 4. A small effect was seen on [^{99m}Tc]MAG_3 at 10 mg/kg/hr probenecid while the effect was negligible on OIH. At the higher dose levels, increased depression of clearance values for both [^{99m}Tc]MAG_3 and OIH were seen with a greater effect on [^{99m}Tc]MAG_3. Plasma protein binding values were decreased at the 50 mg/kg dose and were 46% for [^{99m}Tc]MAG_3 and 12% for OIH. Binding of iothalamate was 7% in controls and 5% at the 50 mg/kg/hr dose of probenecid. No significant effect was seen on iothalamate at all dose levels as expected for a filtration agent.

DISCUSSION

Previous work with diamide dimercaptide complexes of ^{99m}Tc in animals (*3,4*) and man (*5,15*) has established that a ^{99m}Tc complex could be prepared that was secreted by the renal tubular cells without retention. However, the optimal complexes in the series resulted in chelate ring stereoisomers that required HPLC purification. The purification step precluded development of an easily usable kit for wide distribution that would replace [^{131}I] *o*-iodohippurate and [^{99m}Tc]diethylenetriaminepentaacetic acid for renal function studies. The complex described in this study,

TABLE 2
Effect of Probenecid on Biodistribution and Renal Excretion of [^{99m}Tc]MAG_3 and [^{131}I]OIH at 10 min Postinjection in Mice*

Radiochemical	Blood	Kidneys	Liver	Stomach	Intestines	Urine
[^{99m}Tc]MAG_3	6.01 ±0.39	5.41 ±0.49	5.91 ±0.49	0.24 ±0.02	1.63 ±0.10	64.70 ±2.02
[^{131}I]OIH	7.00 ±0.47	3.59 ±0.42	3.70 ±0.33	0.75 ±0.03	1.99 ±0.14	59.21 ±1.96

* Values are mean ± s.e.m. % injected dose for six mice at 10 min postinjection. Probenecid dose was 50 mg/kg given 10 min prior to injection of radiochemicals.

TABLE 3
Renal Excretion Parameters of [^{99m}Tc]MAG_3 and Reference Radiopharmaceuticals [^{131}I]OIH and [^{125}I]Iothalamate

Radiochemical	Renal clearance (ml/min/100 g)*	Extraction efficiency (%)*	Percent protein bound†
[^{99m}Tc]MAG_3	2.84 ± 0.12	85 ± 1	77 ± 2
[^{131}I]OIH	2.17 ± 0.07	69 ± 3	33 ± 3
[^{125}I]Iothalamate	1.29 ± 0.05	39 ± 3	10 ± 2

* Values are mean ± s.e.m. for five rats with simultaneous administration of all radiopharmaceuticals.
† Values are mean ± s.e.m. for four rats.

[^{99m}Tc]MAG_3, is an N_3S ligand or a triamide monomercaptide. Placement of a substituent containing a carboxylate group, deemed useful in providing efficient renal secretion and high renal specificity (*6–8*), on an amide donor atom resulted in a single radiochemical product. A kit based on exchange of an initially formed labile ^{99m}Tc complex from stannous ion reduction has been developed that gives a preparation of high radiochemical purity and thus obviates the technical requirement of HPLC purification.

The biological evaluation of [^{99m}Tc]MAG_3 showed that it is slightly faster than OIH in mice and is essentially quantitatively excreted by the kidneys. Excretion of [^{99m}Tc]MAG_3 in mice in the presence of probenecid as an inhibitor of tubular secretion remained slightly faster than OIH in contrast to [^{99m}Tc]DADS and [^{99m}Tc]CO_2DADS. Studies in rats showed [^{99m}Tc]MAG_3 to have a higher renal clearance than OIH. Direct measurement of extraction efficiency resulted in a value of 85% for [^{99m}Tc]MAG_3 of which 75% is estimated to be by tubular secretion. This compares to 69% for OIH of which 43% is estimated to be due to tubular secretion. Probenecid affected the renal clearance of [^{99m}Tc]MAG_3 to a greater degree that OIH. However, estimates of the probenecid effect on the secretory component can better indicate the competitive affinity of [^{99m}Tc]MAG_3 and OIH for the tubular transport system. For [^{99m}Tc]MAG_3, 23% was nonplasma protein bound and thus the GFR component would be 0.23 ml/min/100 g and the secretory component 2.54–0.23 or 2.31 ml/min/100 g (Table 4). At 50 mg/kg/hr probenecid, nonplasma bound radioactivity was 54% and similar estimations give 0.39 ml/min/100 g for secretion. For OIH, 66% was nonbound in controls and the secretory component is thus estimated as 1.99 ml/min/100 g total −0.66 ml/min/100 g GFR or 1.33 ml/min/100 g secretion. At 50 mg/kg/hr probenecid 88% was nonbound and the secretory clearance is estimated as 0.35 ml/min/100 g. Thus, [^{99m}Tc]MAG_3 secretion was decreased from 2.31 to 0.39 ml/min/100 g or to 17% of control while OIH was decreased from 1.33 to 0.35 ml/min/100 g or 26% of control. These estimates indicate comparable affinity for the tubular secretion system under conditions that have reduced the function level to a small fraction of normal.

The results of these evaluation studies indicate that the simplest member of the N_3S ligand series results in a ^{99m}Tc chelate that not only is amenable to kit formulation and hence facile clinical use, but biologically appears to be handled more efficiently than radioiodinated hippurate.

Synthesis of [Tc]MAG_3 has been carried out with long lived ^{99}Tc. Structural studies involving crystallography and spectral methods are underway to confirm the structure of the complex. Other N_3S ligands for ^{99m}Tc have been synthesized to evaluate the effect of

TABLE 4
Effect of Probenecid on Renal Clearance of [^{99m}Tc]MAG_3 and Reference Renal Agents in Rats*

Dose	10 mg/kg/hr	25 mg/kg/hr	50 mg/kg/hr
		[^{99m}Tc]MAG_3	
Control	2.58 ± 0.11	2.41 ± 0.14	2.54 ± 0.15
Treated	2.21 ± 0.14	1.35 ± 0.09	0.94 ± 0.08
Percent of control	85 ± 3.0	56 ± 2.7	36 ± 2.0
		[^{131}I] *o*-iodohippurate	
Control	2.09 ± 0.05	1.91 ± 0.09	1.99 ± 0.07
Treated	2.05 ± 0.05	1.49 ± 0.09	1.24 ± 0.08
Percent of control	98 ± 2.0	78 ± 3.7	63 ± 3.7
		[^{125}I]Iothalamate	
Control	1.06 ± 0.03	0.99 ± 0.07	0.96 ± 0.05
Treated	1.12 ± 0.06	1.00 ± 0.06	1.01 ± 0.05
Percent of control	106 ± 3.7	101 ± 1.4	105 ± 1.8

* Values are mean ± s.e.m. for six rats for 10 mg/kg/hr dose and five rats at other doses. Units are ml/min/100 g.

changing the terminal amino acid and the form of anionic group. The syntheses and animal evaluation will be reported later.

FOOTNOTES

* Galbraith Laboratories, Inc., Knoxville, TN.

† Varian Associates, Inc., Palo Alto, CA (Varian EM-390 PMR Spectrometer).

‡ Beckman Instruments, Inc., Fullerton, CA (Beckman Ultrasphere).

§ Amicon Corp., Danvers, MA.

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