RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

Catabolism and Protein Binding of Tc-99m Pyridoxylideneglutamate

Anne-Line Jansholt, Kenneth A. Krohn, Robert C. Stadalnik*, Nathaniel M. Matolo, and Gerald L. DeNardo

Sacramento Medical Center of the University of California at Davis School of Medicine, Sacramento, California

Various Tc-99m-labeled compounds have been suggested as replacements for [I-131] rose bengal for imaging of the hepatobiliary system. Among such compounds are Schiff's bases, which are tin-free Tc-chelates easily prepared by 30-min autoclaving of an equimolar mixture of pyridoxal and an amino acid at pH 8.5. We have compared the properties of several Schiff's bases, including Tc-99m pyridoxylideneglutamate (Tc-PyG) with [I-131] rose bengal. Under conditions described, Tc-PyG can be prepared free of Tc-pyridoxal and with <2% TcO_4^- radiochemical impurity. Blood clearance and biliary excretion were studied in three animal models and in normal human volunteers. In all animal models, Tc-PyG initially cleared from the blood more rapidly than rose bengal, but a significant amount of Tc-PyG was excreted in the urine, this in contrast to [I-131] rose bengal which was almost completely excreted through the biliary system. Species differences were observed in the degree of urinary versus biliary clearance of Tc-PyG, with significantly greater urinary excretion in dogs than in monkeys and rabbits. Replacing glutamate with other amino acids did not significantly increase the blood clearance rate or decrease urinary excretion, so that Tc-PyG appears to be at least as good as any of the others studied. Tc-PyG was only 20% bound to plasma proteins, and electrophoretic and chromatographic studies did not reveal any in vivo changes of Tc-PyG before excretion in urine or bile.

J Nucl Med 19: 1036–1044, 1978

Iodine-131-labeled rose bengal has been the established radiopharmaceutical for evaluation of hepatocellular function and biliary excretion; however, images obtained with this agent are often of poor quality. Labeling with I-123 (*1*) would provide a better tracer, but since I-123 is not conveniently available, several attempts have been made to prepare a Tc-99m-labeled radiopharmaceutical for hepatobiliary imaging (*2–7*).

Our attention has focused on Tc-99m pyridoxylideneglutamate (Tc-PyG), first introduced by Baker et al. in 1974 (*5–8*). This radiopharmaceutical is cleared rapidly from the blood, followed by prompt passage through the liver and subsequent collection in the biliary tree and gallbladder (*8–12*). Unfortunately, a significant fraction is also excreted in the urine. Several clinical reports (*13–15*) have demonstrated that this agent is safe, effective, and constitutes a significant improvement over [I-131] rose bengal.

Pyridoxal forms Schiff's bases with amino acids in aqueous solution. These reactions are pH-dependent, and the formation of a metal complex can stabilize

Received Sept. 2, 1977; revision accepted Feb. 2, 1978.

For reprints contact: Anne-Line Jansholt, Dept. of Nuclear Medicine, Sacramento Medical Center—UCD, 4301 X St., Sacramento, CA 95817.

* Picker Scholar, James Picker Foundation.

the ligand in the configuration of a Schiff's base. Most of the work with these complexes has been with divalent cations that form bis complexes with Schiff's bases (*16–18*).

Important factors that determine biliary versus urinary excretion are molecular weight and the chemical structure of the molecule circulating in vivo. Organic anions of mol. wt. < 300 are excreted in the urine, whereas bile is the dominant channel of excretion with mol. wt. > 500 (*19*). Animal studies have shown a marked species difference in the extent of biliary versus urinary excretion for organic anions in the mol.-wt. range of 300–500 (*20*). Dogs appear to resemble rats, while monkeys and humans more closely resemble rabbits (*19*). This explains why certain agents appear to be promising hepatobiliary radiopharmaceuticals in dogs and rodents but prove less successful in humans.

In their work with radiographic contrast materials for alimentary-tract visualization, Lasser et al. (*21*) found that ability to bind to plasma proteins was a major determinant in directing a given material into the bile rather than the urine.

We report our studies of the preparation, radiochemical quality control, and in vivo clearance of Tc-PyG and some other Tc-labeled Schiff's bases of pyridoxal with different amino acids. Our initial work showed that Tc-PyG is a useful hepatobiliary imaging agent. In order to see whether an even better agent could be obtained by using other amino acids, we screened several complexes for blood clearance and degree of urinary versus biliary excretion in dogs. Rapid plasma clearance into the biliary system is the prime prerequisite for a successful biliary radiopharmaceutical. Because of the wide species differences in competitive biliary versus renal excretion, several animal models were used for clearance studies. After finding that Tc-PyG cleared as quickly as any of the other Schiff's bases studied, and in view of our extensive successful clinical experience with Tc-PyG, we chose it as the model compound for further studies of the plasma-protein binding and conjugation reactions of this class of radiopharmaceuticals.

MATERIALS AND METHODS

Preparation of radiopharmaceuticals. Schiff's bases of pyridoxal hydrochloride and the amino acids l-glutamic acid monosodium salt, l-lysine monohydrochloride, l-tyrosine, l-aspartic acid, glycine, l-leucine, and l-glutamine were prepared in a molar ratio of 1:1 dissolved in water for injection. The pH was adjusted to 8.5 ± 0.2 using sterile 1 *M* NaOH and diluted further with sterile water to a final concentration of 0.13 m*M*. Pyridoxylidenetyrosinate represented the only exception: this mixture was diluted to a final concentration of 0.02 m*M* at a pH of 8.5 in order to achieve complete dissolution. A 0.13-m*M* solution of pyridoxal hydrochloride at pH 8.6 was also prepared.

Two-ml aliquots of the mixtures described above were filtered through 0.22-μm membrane filters directly into sealed, sterile vials. After nitrogen was bubbled through the solutions for five min, the vials were stored at 2–8°C. To label, sodium pertechnetate was added in a volume of 0.1 to 2 ml physiologic saline, and the solutions autoclaved at 121°C for 15, 30, or 60 min.

Radiochemical quality control. Electrophoresis, thin-layer chromatography (TLC), and high-pressure liquid chromatography (HPLC) were used for quantitative analysis of radiochemical purity (*22*). Samples of all Tc-labeled compounds, including labeled pyridoxal and sodium pertechnetate, were analyzed by electrophoresis at 250 V and 5 mA for 30 min using cellulose polyacetate electrophoresis strips and a pH 8.8 buffer of trimethylamine and 5,5′-diethylbarbituric acid and its sodium salt. Thin-layer chromatography on silica gel* was performed using acetone or 3:1 (v/v) acetonitrile and water as the mobile phase.

HPLC was performed on a 4 mm × 30 cm μBondapak C_{18} column† eluted with either 92:8 (v/v) acetonitrile and water or 4:1 (v/v) acetonitrile and 0.01 *N* acetic acid. These conditions were selected to give maximum peak-height/width ratios. The column inlet pressure was maintained at 1,500–2,000 psi using a single-piston pump with pulse damping; the resulting flow rate was 1.5–2 ml/min. The system provided for on-line injection of 25 μl of diluted samples of radiopharmaceutical. The eluate was monitored by a flow-through NaI(Tl) detector with a pulse-height analyzer connected to an analog ratemeter as well as to a digital-data output system to give a continuous graphic and digital record of the radioactivity profile eluted from the column. Any activity retained on the column was rinsed off with 1:1 (v/v) acetonitrile and water before the next sample was injected. A 4-mm × 30-cm μPorasil† column eluted with 3:1 (v/v) chloroform and methanol was also used in the same experimental configuration.

Clearance studies. Pyridoxal and pyridoxal-amino-acid Schiff's bases labeled with technetium by 30 min autoclaving were tested for blood clearance and degree of urinary versus biliary excretion in 12 mongrel dogs. In order to filter out biologic variability, six of the dogs were injected with both Tc-95m and Tc-99m preparations simultaneously. One dog received both [I-131] rose bengal and a Tc-99m-

labeled compound, while the remaining five dogs received only one preparation labeled with Tc-99m. Blood was collected from a leg vein at selected time intervals for up to 1 hr after i.v. injection of the radiopharmaceuticals. The bladder was emptied 1 hr after injection by means of a trans-urethral catheter. Serial scintiphotos of the abdomen (500 K counts) were obtained with a gamma camera at 5, 10, 20, and 60 min after injection.

Blood clearance and urinary excretion of Tc-99m PyG (30 min autoclave time) and [I-131] rose bengal were also compared in four New Zealand rabbits and in four normal Rhesus monkeys. Blood was sampled at selected time intervals for up to 2 hr, and the total urine was collected at 60 min after i.v. injection of the radiopharmaceuticals. The type and amount of anesthetic used and the amount of radiopharmaceuticals injected in the animal studies are summarized in Table 1.

Blood clearance of Tc-99m PyG autoclaved for 15, 30, or 60 min was studied in 24 normal human volunteers who had given their informed consent. Eight were studied with a preparation autoclaved for 30 min. An additional 13 subjects received preparations autoclaved for 15 min, and three received a preparation autoclaved for 60 min. Table 1 summarizes the amounts of radiopharmaceutical injected. Three milliliters of blood were withdrawn at selected time intervals up to 5 hr after i.v. injection of Tc-PyG. No urine samples were obtained in the human studies.

One-milliliter samples of whole blood and urine were counted in a well scintillation counter. Crossover corrections were applied to all data from dual-tracer studies, and percentages of injected dose in blood and urine were calculated from counting rates measured for appropriately diluted standards. Activity in total blood was estimated assuming a blood volume of 6.5% and 7% for female and male dogs, respectively—6% for rabbits, and 5.4% for monkeys. The blood volume for normal humans was estimated from a table based on the individual's height, weight, and sex (*23*).

Whenever multiple measurements were made, they are reported as mean ± 1 s.d. assuming *n-1* degrees of freedom. The Student's t-test was used to test the significance of differences ($p < 0.05$) between measured clearance rates of different radiopharmaceutical preparations.

Plasma-protein binding. The in vivo binding of Tc-99m PyG and [I-131] rose bengal by plasma proteins was measured in seven New Zealand rabbits after simultaneous injection of the radiopharmaceuticals. The amount of PyG injected was 4–5 mg/kg in three of the animals and 1 mg/kg in the remaining four. The amount of rose bengal was below 0.25 mg per kilogram body weight in all experiments. Minicon-B15 clinical sample concentrators‡ were used, containing a semipermeable membrane with a cut-off at 15,000 molecular weight, backed by a pad that absorbs water and membrane-penetrating solutes.

Our standard procedure was to add 0.5 ml distilled water and 0.2 ml plasma to each cell and immediately wash it with 0.5 ml water. When the volume of the solution had decreased to 10%, 1 ml water was added and mixed with the concentrate. This procedure was repeated once, and when the solution was again concentrated ten-fold, 1 ml water was added, mixed carefully with the concentrate, and the whole withdrawn completely with a Pasteur pipette. These solutions were counted for Tc-99m and I-131 activity in a well scintillation counter, as well as diluted standards of the initial plasma sam-

TABLE 1. EXPERIMENTAL CONDITIONS FOR BLOOD-CLEARANCE STUDIES

Animal model		Dose administered			
		Tc-PyG		[I-131] rose bengal	
(n)	Anesthesia	mg/kg	mCi	mg/kg	mCi
Dogs 13–25 kg (12)	Sodium pentobarbital 22 mg/kg i.v.	0.8–2.3	Tc-95m: 0.03–0.06 and/or Tc-99m: 0.8–2.0	0.08	0.20
Rabbits 3.7–5.1 kg (4)	Chlorpromazine 4 mg/kg i.m. followed by sodium pentobarbital 20 mg/kg i.v.	0.8–1.2	Tc-99m: 1.2–3.7	0.04–0.09	0.06–0.11
Monkeys 4.8–9.3 kg (4)	Ketamine 10 mg/kg i.v.	1.6–2.2	Tc-99m: 0.9–1.6	0.03–0.06	0.02–0.05
Normal humans 48–93 kg (24)	None	0.04–0.36	Tc-99m: 1.8–3.1	0.03*	

* Usual human dose.

ples. After correcting the counts for cross-over, the percentage of activity remaining after the concentration/filtration procedure was calculated. To find the true value for protein binding of Tc-PyG, it was necessary to correct for protein loss in the Minicon cells.

We initially established that [I-131] rose bengal was 100% bound to plasma proteins of mol. wt. > 15,000. This was done by comparing the loss of I-131 activity with the loss of protein during the filtration procedure. The amount of protein in the samples before and after filtration was measured by uv spectrophotometry at 280 nm. The amount of protein lost from each sample could therefore be calculated from the I-131 activity before and after filtration. It was assumed that the percentage of protein-bound radiopharmaceutical lost in the cells was the same for Tc-99m PyG as for [I-131] rose bengal. In order to see whether comparable plasma-protein binding data for cholescintigraphic radiopharmaceuticals could be obtained in vitro by this simple technique, another filtration experiment similar to the one described above was performed. One milliliter freshly collected plasma (EDTA anticoagulant) was incubated with 0.2 μl Tc-99m PyG and 1 μl [I-131] rose bengal in a 37°C water bath. The amounts of PyG and rose bengal used corresponded to 280 and 28 μg/kg respectively if one assumes 35 ml plasma per kilogram body weight.

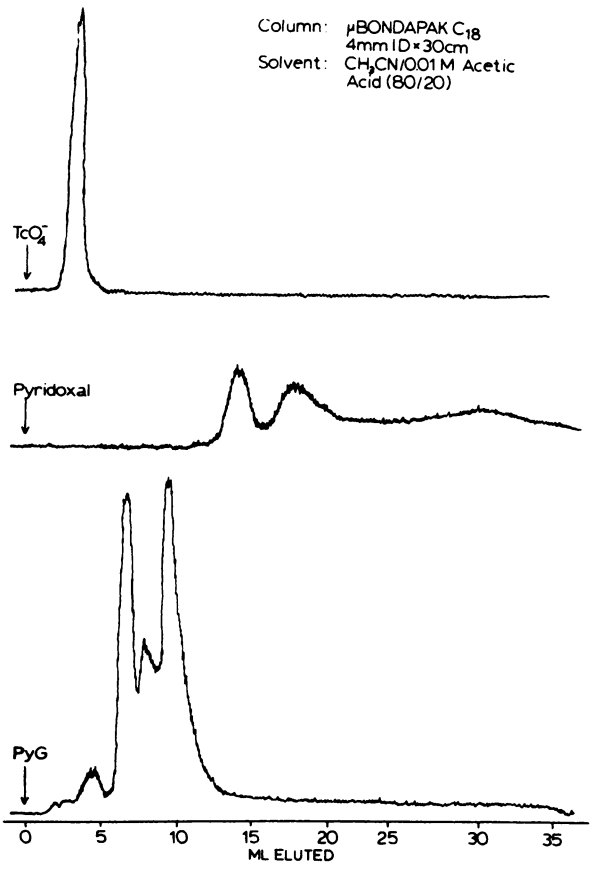

FIG. 1. High-pressure liquid chromatograms of Tc-99m-labeled PyG, pyridoxal and pertechnetate. Arrows indicate when samples were injected onto column.

Metabolic changes. Electrophoresis and chromatography separation, using three different mechanisms of chemical selectivity, were used to look for in vivo alteration of Tc-PyG before excretion through the liver and kidneys. The electrophoretic migration of plasma, urine, and bile samples obtained from dogs and rabbits injected with Tc-PyG were compared with that of the original Tc-PyG solution under the same conditions. PyG, pyridoxal, pertechnetate, and filtered blood, bile, and urine samples obtained from rabbits after injection of Tc-99m PyG were analyzed using the same experimental conditions described for radiochemical quality control.

RESULTS

Radiopharmaceutical development. Pyridoxal formed a Schiff's base with a number of different amino acids which, when autoclaved at a pH of 8.5 ± 0.2, chelated with technetium. Both electrophoresis and silica-gel TLC using acetone as solvent were useful for the estimation of the amount of free pertechnetate (which was usually <2%) in all pyridoxal-amino-acid complexes tested up to 24 hr after preparation. The two analytical methods gave comparable results. Labeled PyG and pyridoxal were inadequately separated using silica-gel/acetone TLC. Under the electrophoresis conditions used, PyG, pyridoxal, and pertechnetate moved approximately 1.5, 3.5, and 8 cm, respectively, but the first two peaks overlapped to such a degree that quantification of pyridoxal in PyG preparations involved unacceptable uncertainty. Thin-layer chromatography on silica gel using 3:1 (v/v) acetonitrile and water as the mobile phase showed several peaks with considerable overlap in the PyG preparations.

The best separation of pertechnetate and pyridoxal from the multiple peaks representing PyG was accomplished using a μBondapak C_{18} column eluted with 4:1 (v/v) acetonitrile and 0.01 *N* acetic acid. Typital elution patterns with this analytical system are illustrated in Fig. 1 for pertechnetate, PyG, and pyridoxal. Pertechnetate is eluted in the breakthrough volume. The multiple peaks representing PyG have elution volumes between 4 and 10 ml. The exact chemical structure causing the different peaks has not been identified but the elution profile was qualitatively and semi-quantitatively the same using pyridoxal supplied by four different manufacturers. Pyridoxal is eluted mainly in two broad peaks at 13–22 ml, although some tailing was seen

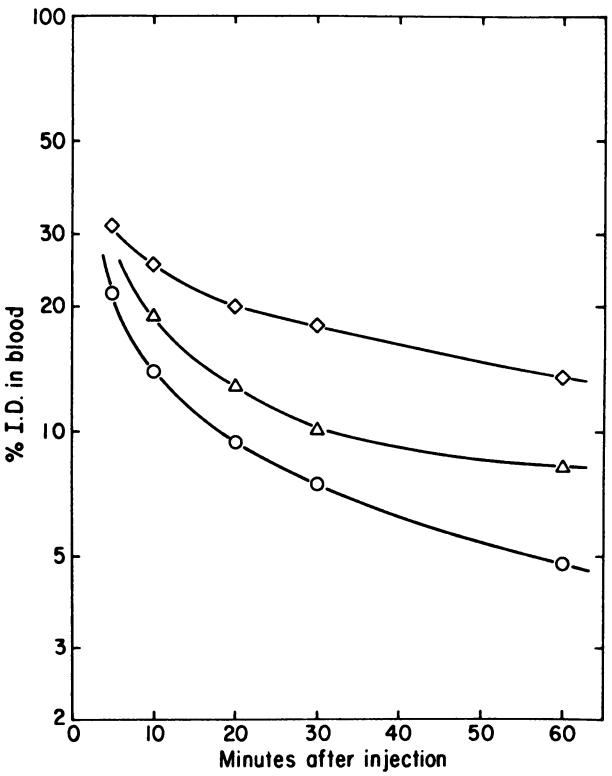

FIG. 2. Blood clearance of cholescintigraphic radiopharmaceuticals in normal dogs. ○ Average for Tc-pyridoxal - amino-acid complexes that cleared at the same rate (amino acids: glutamate, glutamine, leucine, aspartic acid and glycine); △ Tc-pyridoxylidenelysinate, ◇ Tc-pyridoxylidenetyrosinate.

with both PyG and pyridoxal. We found it convenient to rinse this off with 1:1 (v/v) acetonitrile and water before another sample was injected. For the Tc-99m PyG preparations this tailing represented 12 ± 4% of the injected activity, for Tc-99m pyridoxal the value was 32 ± 6%. The amount of free pertechnetate in Tc-99m PyG was routinely less than 2% by this method; the values obtained always compared well with the results from TLC and electrophoretic studies. Distinct pyridoxal peaks were never observed during analysis of Tc-PyG preparations, but very small amounts of pyridoxal may have been missed because of the tailing phenomenon. HPLC, using a μPorasil column eluted with 3:1 (v/v) chloroform and methanol, gave only one peak of radioactivity in the eluate with Tc-99m PyG. The retention volumes for pertechnetate (3.0 ml) or pyridoxal (6.4 ml) were not sufficiently different from that of PyG (7.8 ml) to make this a useful method for radiochemical quality control.

Clearance studies. Tc-pyridoxal - amino-acid complexes, as well as Tc-pyridoxal, showed rapid blood clearance in normal dogs but variable degrees of gallbladder accumulation versus renal excretion. After administration of Tc-99m PyG the liver could be seen within 5 min, and the common bile duct, gallbladder and duodenum could be visualized 10–15 min after injection. Equally good visualization was obtained with Tc-99m pyridoxylideneleucinate. Following administration of Tc-99m pyridoxal the kidneys showed up clearly within minutes, but the gallbladder was not well visualized until at least 30 min after injection. The Tc-pyridoxal - amino-acid complexes initially cleared more rapidly from the blood than [I-131] rose bengal (Table 2 and Fig. 3). No significant difference ($p < 0.05$) was found between properly prepared Schiff's bases of pyridoxal with glutamate, glutamine, leucine, aspartic acid, and glycine. Complexes with tyrosine and lysine cleared more slowly than those listed above (Fig. 2). The clearance rate for Tc-99m pyridoxal was similar to that of [I-131] rose bengal initially, but was significantly slower after 10 min (Fig. 3). The urinary accumulation at 1 hr after injection was 32 ± 5% for Tc-PyG (Table 2). For those complexes that cleared the blood at the same rate, the amount cleared through the kidneys by 1 hr was 36 ± 7% of the injected dose. Urinary accumulation of the Tc-pyridoxylidenelysinate complex was 42 ± 6% at 1 hr. In one dog injected with Tc-99m pyridoxal, more than 50% of the dose was in the urine within

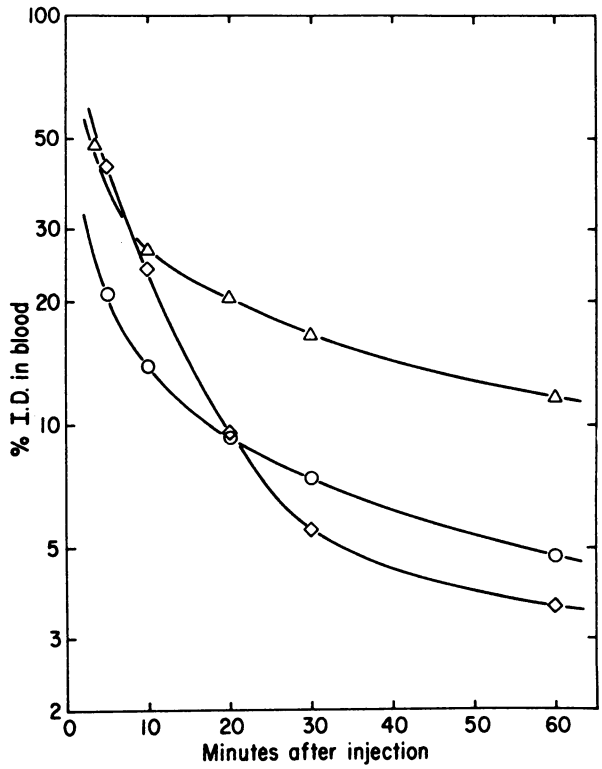

FIG. 3. Blood clearance of cholescintigraphic radiopharmaceuticals in normal dogs. ○ Average for Tc-pyridoxal - amino-acid complexes that cleared at the same rate (amino acids: glutamate, glutamine, leucine, aspartic acid and glycine); △ Tc-pyridoxal, ◇ [I-131] rose bengal.

TABLE 2. CLEARANCE OF CHOLESCINTIGRAPHIC RADIOPHARMACEUTICALS

Animal model	Blood clearance $t_{1/2} \pm 1$ s.d. of fast component (min) (% of injected dose)		60-min urinary excretion % injected dose ± 1 s.d.	
	Tc-99m PyG	[I-131] rose bengal	Tc-99m PyG	[I-131] rose bengal
Dogs	2.0 ± 0.5 (87.8 ± 0.6)	4.0 (91.5)	32 ± 5	0.4
Rabbits	2.0 ± 0.5 (90.2 ± 1.0)	4.5 ± 0.5 (87.7 ± 3.3)	20 ± 5	2.6 ± 2.5
Monkeys	2.0 ± 0.5 (92.8 ± 1.6)	3.0 ± 0.5 (94.4 ± 0.9)	17 ± 1	0.7 ± 0.5
Humans	2.0 ± 0.5 (84.9 ± 1.9)	—	—	—

1 hr. The renal excretion of all Tc-labeled Schiff's bases contrasted with that of rose bengal, where only 0.4% of the injected dose was in the urinary bladder at 1 hr.

Clearance measurements of Tc-99m PyG prepared with 30 min autoclaving were also done in rabbits, Rhesus monkeys, and normal humans. They showed blood-clearance rates comparable with those observed in dogs (Fig. 4). The initial plasma half-time was 2.0 ± 0.5 min and accounted for an average of 89 ± 3% of the injected dose in the four species studied. There were significant species differences, however, in the fractional urinary versus biliary excretion (Table 2).

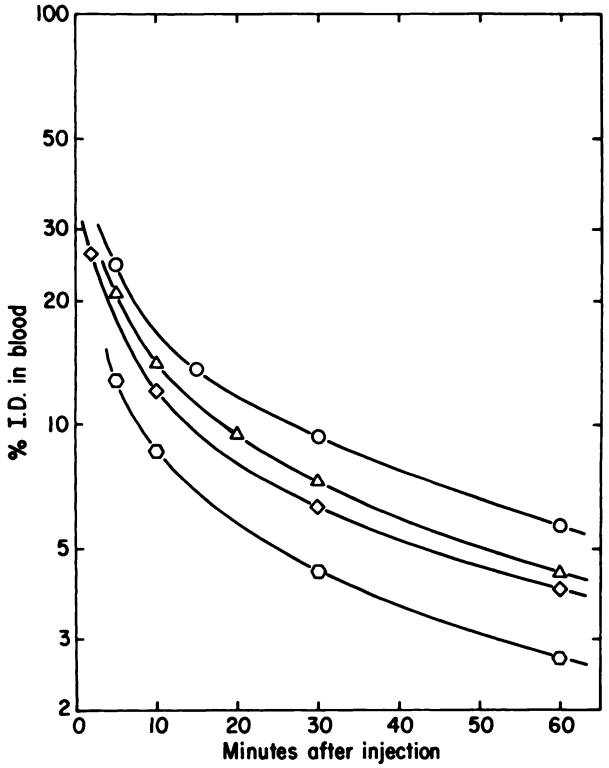

FIG. 4. Blood clearance of Tc-PyG in normals: ○ humans, △ dogs, ◇ rabbits, ○ monkeys.

We compared preparations of Tc-99m PyG autoclaved for 15, 30, and 60 min in normal humans and detected no difference between preparations autoclaved for 30 min and those autoclaved for 60 min. The preparation that was autoclaved only 15 min had a 15% higher ($p < 0.05$) blood concentration at 15 min after injection, compared with the other preparations.

Plasma-protein binding. In rabbits that were injected simultaneously with [I-131] rose bengal and Tc-99m PyG, rose bengal was totally bound to plasma proteins within 2 min after injection. Only 17% Tc-99m PyG was bound to plasma proteins after injection of 4–5 mg PyG per kilogram body weight. When only 1 mg per kilogram was injected, the plasma-protein binding increased to 26% of the injected dose. Rose bengal was also totally bound in vitro, whereas the protein binding of Tc-99m PyG was found to be 11.5 ± 0.7% after 10 min incubation at 37°C with an amount of PyG corresponding to about 0.3 mg/kg body weight.

Metabolic changes. Electrophoretic studies of plasma, bile, and urine samples from dogs and rabbits injected with Tc-99m PyG showed in all instances one relatively wide radioactivity peak, with essentially the same migration for all samples as for authentic Tc-99m PyG. Chromatography using the μPorasil column eluted with 3:1 (v/v) chloroform and methanol likewise did not reveal any difference in the behavior of the radioactivity in bile samples compared with that of Tc-PyG. Reverse-phase HPLC using μBondapak C_{18} eluted with mixtures of acetonitrile and water or dilute acetic acid was the most sensitive method for detecting changes in the radioactivity profile of plasma, bile and urine as compared with the initial Tc-99m PyG preparation. Any conjugation product would be more water-soluble than Tc-PyG and would therefore elute before the unchanged peaks. Figure 1 shows that there are several milliliters of column eluate before the

first major Tc-PyG peak. It is in this region, where TcO_4^- is known to elute, that we looked for metabolic alterations of Tc-PyG. The fraction of radioactivity eluted before the first major Tc-PyG peak was 5.7 ± 1.3%, so we could potentially detect the presence of ≧ 3% conjugation. Bile samples were always free of $^{99m}TcO_4^-$ or any other Tc-99m-labeled compound eluting before Tc-99m PyG. Although the relative heights and widths of the peaks in the injected material were somewhat different from the corresponding parameters in the plasma, bile, and urine specimens, no earlier peaks representing different chemical structures were observed, indicating absence of chemical modification in Tc-99m PyG prior to excretion.

DISCUSSION

The desirability of replacing [I-131] rose bengal with an agent having better physical characteristics for cholescintigraphy has been apparent for some time. Several Tc-99m-labeled compounds have been suggested. Our investigations and others have shown that in experimental animals several pyridoxal - amino-acid complexes labeled with Tc-99m are cleared rapidly from the blood by both liver and kidneys, and that a major fraction is excreted in the bile. Van Heertum et al. (*24*) compared Tc-99m pyridoxylideneleucinate (PyL) with Tc-99m PyG and a number of other prospective cholescintigraphic agents in a baboon model, and found that PyL had the highest and earliest bile concentration of the agents tested. In a follow-up report (*11*), however, this finding was not confirmed. We found that of the pyridoxal - amino-acid complexes studied in dogs, Tc-99m PyL had rapid blood clearance and gave excellent images of the gallbladder 15–20 min after injection. However, the difference in clearance between this agent and Tc-99m PyG lacked statistical significance.

Several clinical studies (*13–15,26*) have established the advantages of Tc-99m PyG over [I-131] rose bengal as a radiopharmaceutical for hepatobiliary imaging. Furthermore, as a cholescintigraphic agent it has characteristics at least as good as those of any of the other Tc-99m compounds currently in general clinical use. In normal humans the common bile duct, gallbladder, and duodenum are seen 15–30 min after injection, and renal retention does not interfere with interpretation of the scans (*13,14*). Our blood-clearance results for Tc-PyG compare favorably with those reported for Tc-99m HIDA (*7,11,26*) and Tc-99m Bioquin-7CA (*27*), although these agents are excreted in the urine of dogs and mice to a lesser extent than Tc-PyG. Extensive experience with these tracers in humans has not yet been reported. The rapid liver clearance and gallbladder accumulation of Tc-PyG represent advantages over agents such as Tc-99m penicillamine (*28*) and Tc-99m dihydrothioctic acid (*4*), since these tracers require a waiting period of at least 1 hr before the normal gallbladder can be visualized well.

Technetium-99m PyG has proved to be a radiopharmaceutical that is reliable and simple to prepare. It consistently contains less than 2% $^{99m}TcO_4^-$ up to 24 hr after preparation, which indicates that air oxidation subsequent to labeling is not the problem that it is with many tin-reduced agents. With the current composition and reaction conditions, blood-clearance rates in normal humans were the same for Tc-99m PyG solutions autoclaved for 30 and 60 min, but 15 min of autoclaving resulted in a slower blood clearance rate. We use 30 min autoclaving as our routine procedure.

Our studies showed very rapid blood clearance for Tc-PyG in humans, dogs, rabbits, and monkeys. The fast component ($t_{1/2}$ = 2.0 ± 0.5 min) of the clearance curve accounted for 85–95% of the injected dose. We did, however, observe species differences in the amount of Tc-PyG excreted in the urine. The amounts excreted in the urine of rabbits and monkeys within 60 min were 20 ± 5 and 17 ± 1%, respectively, and in dogs a urinary excretion of 32 ± 5% was observed; the last value was significantly ($p < 0.01$) greater than the other two values. Kubota et al. (*9*) found 17 ± 4% of a similar preparation in the urine of rabbits at 60 min, while Baker et al. (*8*) observed 48 ± 4 % urinary excretion in mice after injection of Tc-99m PyG containing 10–12% Tc-99m pyridoxal.

The ratio of biliary to urinary excretion of Tc-PyG in humans might be increased if more favorable reaction conditions (such as pH, molar ratio, and autoclaving time) could be found to increase the proportion of compounds with high molecular weight in the preparation. A dimer with two PyG complexes per atom of technetium would have a molecular weight of 735, while a complex in which pyridoxal:glutamate:technetium = 1:1:1 would have a molecular weight of only 417. A change in the molar ratio of pyridoxal and glutamate, substitution of pyridoxal with another aldehyde, or addition of some metallic cation carrier, are some of the possible variations that might lead to a compound with higher biliary versus urinary excretion.

The major fraction of Tc-PyG circulates in the blood as a low-molecular-weight species, with only about 20% bound to plasma protein. Thus this radiopharmaceutical is not directed toward a biliary detoxification pathway because of protein binding,

and thus prevented from renal filtration as is the case with [I-131] rose bengal and many radiographic biliary agents.

At the present time, neither the valence state of Tc-99m in the pyridoxylideneglutamate complex, nor the overall structure of the Schiff's base, is known. It has been postulated that the aldehyde group on pyridoxal reduces the pertechnetate to give Tc-99m in a lower valence state as a positively charged ion that can readily form coordination complexes with electron-rich Schiff's base ligands (*8*). Reduction of Tc(VII) using excess $SnCl_2$ has shown that Tc(IV) is the most likely state under most conditions, but Tc(III) and Tc(V) have also been observed in the course of some experiments (*29*). The tetravalent state has also been suggested as being most probable when kethoxal-bis(thiosemicarbazone) is labeled with Tc-99m (*30*). Detailed analytical studies will be necessary to define the oxidation state of technetium in Tc-PyG and related complexes.

Our radiochemical quality-control analysis for Tc-PyG revealed a complex product spectrum. We have not attempted to identify the chemical structure associated with each chromatographic peak. We have shown that the chromatographic elution profile is constant when different sources of reagents are used. Furthermore, plasma and bile specimens obtained after injection of Tc-PyG into rabbits, and analyzed by electrophoresis and normal and reverse-phase HPLC, demonstrated the same radiochemical composition as the injected Tc-PyG. Urine specimens demonstrated a slightly higher amount of TcO_4^-. Presence of this radiochemical impurity in the injected material will always cause more urinary-bladder activity. Metabolism or chemical conjugation of Tc-PyG resulting in a more water-soluble structure would be detected by an early peak on reverse-phase HPLC, a region where 5.7 ± 1.3% of the radioactivity eluted. We conclude that an upper limit for conjugation is therefore about 3%, and conjugation is most likely nil.

In summary, Tc-PyG is not extensively bound to plasma proteins and is not conjugated in the liver, but is apparently excreted directly and unmetabolized. Although Tc-99m PyG has become a valuable tool in the clinical evaluation of hepatobiliary function, much remains to be learned about the chemistry and physiology of this type of compound. This information may, in turn, prove valuable for the development of better hepatobiliary imaging agents.

FOOTNOTES

* Eastman Chromatogram Sheets 6000 Silica Gel.
† Waters Associates.
‡ Amicon Corp., Lexington, Mass.

REFERENCES

1. Serafini AN, Smoak WM, Hupf HB, et al: Iodine-123-rose bengal: An improved hepatobiliary imaging agent. *J Nucl Med* 16: 629–632, 1975

2. Tubis M, Krishnamurthy GT, Endow JS, et al: Tc-99m-penicillamine, a new cholescintigraphic agent. *J Nucl Med* 13: 652–654, 1972

3. Eikman EA, Cameron JL, Colman M, et al: Radioactive tracer techniques in the diagnosis of acute cholecystitis. *J Nucl Med* 14: 393, 1973 (Abst)

4. Tonkin AK, DeLand FH: Dihydrothioctic acid: A new polygonal cell imaging agent. *J Nucl Med* 15: 539, 1975 (Abst)

5. Baker RJ, Bellen JC, Ronai PM: ^{99m}Tc-pyridoxylideneglutamate: A new rapid cholescintigraphic agent. *J Nucl Med* 15: 476, 1974 (Abst)

6. Lin TH, Khentigan A, Winchell HS: A Tc-99m-labeled replacement for I-131 rose bengal in liver and biliary tract studies. *J Nucl Med* 15: 613–615, 1974

7. Loberg MD, Cooper M, Harvey E, et al: Development of new radiopharmaceuticals based on N-substitution of iminodiacetic acid. *J Nucl Med* 17: 633–638, 1976

8. Baker RJ, Bellen JC, Ronai PM: Technetium-99m-pyridoxylideneglutamate: A new hepatobiliary radiopharmaceutical. I. Experimental aspects. *J Nucl Med* 16: 720–727, 1975

9. Kubota H, Eckelman WC, Poulose KP, et al: Technetium-99m-pyridoxylideneglutamate, a new agent for gallbladder imaging: Comparison with I-131-rose bengal. *J Nucl Med* 17: 36–39, 1976

10. Trumper Y, Weininger J, Lubin E: Tc-99m-pyridoxylideneglutamate kit: A rapid cholescintigraphic agent. *Int J Nucl Med Biol* 3: 45–48, 1976

11. Wistow BW, Subramanian G, Van Heertum RL, et al: An evaluation of Tc-99m-labeled hepatobiliary agents. *J Nucl Med* 18: 455–461, 1977

12. Chiotellis E, Subramanian G, McAfee JG: Preparation of Tc-99m labeled pyridoxal-amino acid complexes and their evaluation. *Int J Nucl Med Biol* 4: 29–41, 1977

13. Ronai PM, Baker RJ, Bellen JC, et al: Technetium-99m pyridoxylideneglutamate: A new hepatobiliary radiopharmaceutical. II. Clinical aspects. *J Nucl Med* 16: 728–737, 1975

14. Matolo NM, Stadalnik RC, Krohn KA, et al: Biliary tract scanning with Tc-99m pyridoxylideneglutamate: A new gallbladder scanning agent. *Surgery* 80: 317–321, 1976

15. Stadalnik RC, Matolo NM, Jansholt A-L, et al: Technetium-99m pyridoxylideneglutamate (P.G.) cholescintigraphy. *Radiology* 121: 657–661, 1976

16. Eichhorn GL, Dawes JW: The metal complexes of vitamin B_6 and Schiff's base derivatives. *J Am Chem Soc* 76: 5663–5667, 1954

17. Davis L, Roddy F, Metzler, DE: Metal chelates of imines derived from pyridoxal and amino acids. *J Am Chem Soc* 83: 127–134, 1961

18. Leussing DL, Huq N: Pyridoxal-glycinate complexes with some divalent metal ions. *Anal Chem* 38: 1388–1392, 1966

19. Smith RL: *The Excretory Function of Bile.* London, Chapman and Hall, 1973, pp 14–16 and 76–93

20. Hirom PC, Milburn P, Smith RL, et al: Species variations in the threshold molecular-weight factor for the biliary excretion of organic anions. *Biochem J* 129: 1071–1077, 1972

21. Lasser EC, Farr RS, Fujimagari T, et al: The sig-

nificance of protein binding of contrast media in roentgen diagnosis. *Am J Roentgen* 87: 338–360, 1962

22. KROHN KA, JANSHOLT AL: Radiochemical quality control of short-lived radiopharmaceuticals. *Int J Appl Radiat Isot* 28: 213–227, 1977

23. Medotopes Handbook, Princeton, E. R. Squibb and Sons, Inc., 1973, pp 26–27

24. VAN HEERTUM RL, SUBRAMANIAN G, THOMAS FD, et al: Comparative evaluation of Tc-99m labeled hepatobiliary agents with I-131 rose bengal. *J Nucl Med* 16: 577, 1975 (Abst)

25. BROWN RG, GILDAY DL, ASH JM: Tc-99m pyridoxylideneglutamate: Quality control and clinical aspects in pediatric nuclear medicine. *J Nucl Med* 17: 555, 1976 (Abst)

26. RYAN J, ISIKOFF M, NAGLE C, et al: The combined use of Tc-99m HIDA and ultrasound in differential diagnosis of jaundice. *J Nucl Med* 17: 545, 1976 (Abst)

27. FRITZBERG AR, LYSTER DM, DOLPHIN DH: Tc-99m Bioquin-7CA, a potential new hepatobiliary scanning agent. *J Nucl Med* 17: 907–910, 1976

28. KRISHNAMURTHY GT, TUBIS M, BLAHD WH, et al: Cholescintigraphy with Tc-99m penicillamine. *Radiology* 115: 201–204, 1975

29. STEIGMAN J, MEINKEN G, RICHARDS P: The reduction of pertechnetate-99 by stannous chloride—I. The stoichiometry of the reaction in HCl, in a citrate buffer and in a DTPA buffer. *Int J Appl Radiat Isot* 26: 601–609, 1975

30. YOKOYAMA A, TERAUCHI Y, HORIUCHI K, et al: Technetium-99m-kethoxal-bis(thiosemicarbazone), an uncharged complex with a tetravalent Tc-99m state, and its excretion into the bile. *J Nucl Med* 17: 816–819, 1976