jnm/ RADIOCHEMISTRY AND RADIOPHARMACY

TECHNETIUM ^{99m}-PYRIDOXYLIDENEGLUTAMATE: A NEW HEPATOBILIARY RADIOPHARMACEUTICAL. I. EXPERIMENTAL ASPECTS

R. J. Baker, J. C. Bellen, and P. M. Ronai*

Institute of Medical and Veterinary Science, Adelaide, South Australia

The labeling of pyridoxal and the pyridoxylidene derivative of glutamic acid with ^{99m}Tc has been achieved by a simple autoclaving procedure. Technetium-99m-pyridoxylideneglutamate (^{99m}Tc-PG) shows marked biliary excretion with accumulation of radioactivity in the gallbladder and intestines of experimental animals. This compound has been extensively investigated with a view to its application in the diagnosis of biliary disorders in man by scintigraphy. Both scintigraphic and quantitative distribution studies showed that ^{99m}Tc-PG passed rapidly through the mouse liver with progressive accumulation in the gallbladder, allowing visualization of this organ within 10 min of injection. In 30 min over 40% of the injected dose was excreted into the intestine with an equivalent amount appearing in the urine; however, renal activity remained low. Scintigraphic studies in dogs showed results similar to those obtained in mice. Studies of the toxicity in three animal species indicated a wide margin of safety for ^{99m}Tc-PG in the dose proposed for diagnostic purposes in humans.

The formation of complexes or chelates of ^{99m}Tc with organic molecules is now an established method of modifying the biologic distribution of this useful radionuclide, creating new scope for the development of radiopharmaceuticals. Many of the components of living systems are molecules containing coordinating functions (*1*). These ligands hold the promise of a relatively low toxicity compared with molecules such as cyanide, pyridine, and other amines frequently employed in coordination chemistry to modify the properties of metal ions.

Pyridoxal, a member of the vitamin B_6 group, is one such ligand we have chosen to examine. This compound is known to be involved in enzyme systems catalyzing transamination reactions which can also be carried out nonenzymatically in the presence of transition metal ions (*2,3*). Condensation of the carbonyl group of pyridoxal and the amino group of amino acids may lead to the formation of Schiff-base ligands, some of which have been isolated in the solid state; e.g., potassium pyridoxylideneglutamate (*4*) and potassium pyridoxylidenealaninate (*5*) have been prepared from alcoholic solutions of the reactants. In aqueous solution pyridoxylideneglutamic acid was found to be relatively unstable (*4*) and this property applies to most pyridoxylideneamino-acid Schiff bases (*6*). The formation constant for pyridoxal-phosphate-amino-acid Schiff bases has been estimated (*7*) to be of the order of 10^3–10^4 and similar values are expected for the pyridoxalamino-acid compounds (*8*). However, the formation of a metal complex can stabilize the ligand in the Schiff-base configuration. Pyridoxylideneamino-acid metal complexes have been identified spectrophotometrically (*9,10*), isolated (*11,12*), and their structures have been determined by x-ray diffraction studies (*13,14*).

The preparation and biologic distribution of ^{99m}Tc compounds postulated as being the complexes of pyridoxal and pyridoxylideneglutamate (^{99m}Tc-PG) are described in this paper. The interesting and important property of the latter complex is its excretion in the bile of experimental animals, suggesting a potential for the diagnosis of biliary tract disorders in man by scintigraphy. The properties of ^{99m}Tc-PG have been investigated with this application in mind.

Received Dec. 30, 1974; revision accepted March 10, 1975.

For reprints contact: R. J. Baker, Div. of Nuclear Medicine, Institute of Medical and Veterinary Science, Frome Rd., Adelaide, South Australia 5000.

* Present address: Div. of Nuclear Medicine, Dept. of Radiology, University of Colorado Medical Center, Denver, Colo. 80220.

MATERIALS AND METHODS

Preparation of ^{99m}Tc-pyridoxylideneglutamate. Pyridoxal hydrochloride (Merck, Darmstadt, Germany) (54 mg) and monosodium glutamate monohydrate (British Drug Houses, Ltd., Poole, England) (50 mg) were weighed into a sterilized beaker and the desired activity of ^{99m}Tc-pertechnetate (usually 20–30 mCi) were added together with sufficient distilled water for injection to make the volume 3.0 ml. The solution was placed on the pH meter and 1 *M* sodium hydroxide was added with stirring until the pH was 8–8.5. After adjusting the volume to 4.0 ml, the bright yellow solution was transferred to a rubber-closed vial and membrane-filtered nitrogen passed through it for 5 min. Labeling was achieved by autoclaving for 30 min at 120°C. The various parameters of the reaction were studied in detail.

Preparation of ^{99m}Tc-pyridoxal. An analogous method to that described above was adopted with the omission of monosodium glutamate monohydrate as the only modification.

Analysis. The composition of the autoclaved reaction mixture was examined using electrophoresis on cellulose acetate membrane. The buffer (pH 8.6) was prepared by dissolving diethylbarbituric acid (1.84 gm) and sodium barbitone (10.4 gm) in 1.0 liter distilled water. The electrophoresis apparatus used is commercially available (Model U77, Shandon Scientific Co., Ltd., London, U.K.). The solution was applied to the center of the strip and electrophoresis carried out for 30 min at constant current (1.5 mA) after which the strip was dried, cut into 5-mm segments, and counted in a well scintillation counter.

In vivo studies. The compounds were tested for biliary excretion using balb/c mice which were injected intravenously through the tail vein with 200–500 μCi, then anesthetized with sodium pentobarbitone (40 mg/kg) injected intraperitoneally. The mice were placed under the pinhole collimator of a scintillation camera and serial Polaroid pictures were taken. Pentobarbitone anesthesia was supplemented with ether, if required.

Quantitative distribution studies. Balb/c mice weighing 25–30 gm were injected in groups of four with 0.1 ml (approximately 75 μCi) of the preparation to be examined. At a specific time interval, the mice were killed by cervical dislocation and dissected. Urine was collected by keeping the injected live mouse in a 600-ml beaker containing tissue paper. Any urine expelled on killing the mouse was collected on filter paper and added to the beaker, which was counted after 48 hr in a large volume scintillation counter against a standard equal to the injected dose and distributed on tissue and filter paper in an identical beaker. It was necessary to add 100 ml water to each beaker and to mix well in order to achieve uniform counting geometry.

Dissected organs were collected in tared plastic counting tubes and weighed immediately after dissection was completed. Blood was collected by cardiac puncture and the gallbladder carefully dissected to avoid rupture and loss of contents. On the day following dissection all organs were counted against a standard of 1/25 dose in a well scintillation counter, but organs of high activity (>100,000 counts/10 sec) were recounted 48 hr after injection to avoid saturation of the counter. The tails were counted separately to check that none of the injected radioactivity was extravasated and where values exceeded 2% of the injected dose, they were subtracted from the total standard except at early time intervals where high blood activity was present.

To account for all injected activity, one mouse for each run was injected with a standard dose and killed after 2 min. This provided a standard for the carcasses, which were counted after 48 hr in the large volume counter. Large geometric variations were minimized by counting each carcass wrapped tightly in plastic in both upright and inverted positions, the mean result being used in calculations.

Reabsorption experiments. A female white rat weighing 200 gm was anesthetized with sodium pentobarbitone, supplemented with ether. A laparotomy was performed and a loop of duodenum was isolated by ligation on either side of the point of entry of the common bile duct, then 4.5 mCi of ^{99m}Tc-PG were injected through the tail vein. After 60 min, bile was aspirated from the loop of duodenum and a standard and two doses prepared by weight. Using a fine needle, the doses were injected into the duodenum of two recipient rats, prepared by laparotomy and ligation at the pylorus. The rats were maintained under ether anesthesia for 2 hr and blood samples collected from the tails at 15-min intervals. These samples were counted together with an aliquot of diluted bile standard in a well scintillation counter. At the end of the 2-hr period the distribution of radioactivity in the rats was examined using the scintillation camera.

Toxicity studies. The acute toxicity of PG was determined using male balb/c mice weighing 32–38 gm. After determination of the approximate toxic range using small groups of animals, five groups of ten mice each were injected intravenously through the tail vein. The PG was used in a fourfold concentration compared with the standard preparation without added ^{99m}Tc. The injection was made over a period of 1–2 min. Using a similar solution, the effects of doses 1,000 times that proposed for use

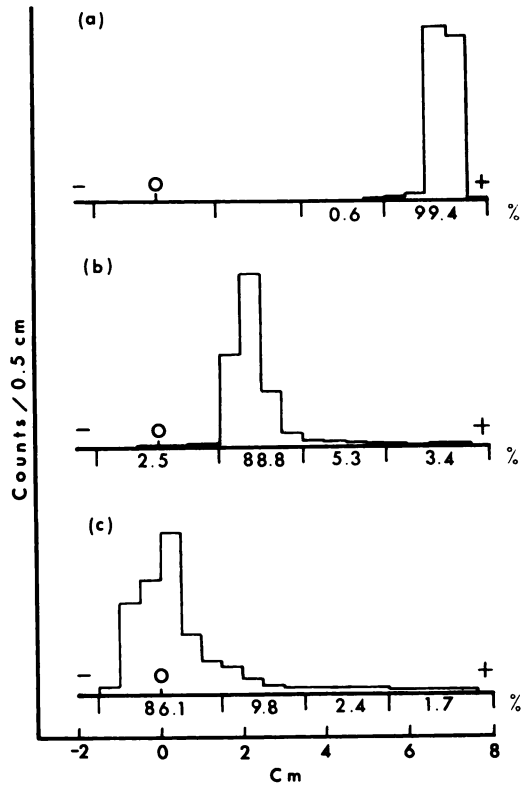


FIG. 1. Distribution of radioactivity on cellulose acetate membrane after electrophoresis. (A) ^{99m}Tc-pertechnetate, (B) ^{99m}Tc-pyridoxal, and (C) ^{99m}Tc-PG, showing percentage of total radioactivity in each fraction indicated.

in humans (i.e., 282 mg/kg) were examined in 20 balb/c mice, 13 white rats weighing 196 ± 19 gm, and 6 kittens weighing 889 ± 125 gm. The chronic effects of repeated doses of PG were determined in 15 balb/c mice given doses 1,000 times the proposed human dose. A total of ten doses was given to each mouse over 12 days, then five of the mice were dissected and the brain, heart, lungs, liver, gallbladder, stomach, intestines, pancreas, spleen, kidneys, and skeletal muscle were examined histologically in comparison with those of two normal mice.

The antigenicity of the preparation was studied in six guinea pigs given a loading dose of 9.9 mg intraperitoneally followed by subcutaneous doses (3.9 mg) each 48 hr for 2 weeks. Sterility and pyrogenicity tests were carried out by independent laboratories.

RESULTS

Electrophoretic behavior. The analysis of ^{99m}Tc-pyridoxal and ^{99m}Tc-PG by electrophoresis using cellulose acetate membrane yielded the results shown in Fig. 1. During a 30-min run ^{99m}Tc-pertechnetate migrated towards the anode to a distance of 6–7 cm while ^{99m}Tc-pyridoxal migrated in the same direction to a distance of 1.5–3.5 cm as determined by assaying the distribution of radioactivity along the strip. Under ultraviolet light the blue fluorescence due to pyridoxal was located at a distance of 1.5 cm from the origin.

The reaction product from $^{99m}TcO_4^-$ and pyridoxal together with a stoichiometric quantity of monosodium glutamate gave a different distribution of radioactivity. In this case the highest percentage of radioactivity was distributed about the origin, although each preparation appeared to contain some ^{99m}Tc-pyridoxal and a small additional band "X" of unknown origin but the percentage of free pertechnetate was generally low. In most preparations a small overlap with the neighboring ^{99m}Tc-pyridoxal band position could result in an error in the percentage of ^{99m}Tc-PG but the overlap is minimal and band positions are readily determined. The separation using this method was superior to that obtained with several chromatographic systems tried or with gel filtration using Sephadex G-25.

Preparative conditions. Further investigations into the conditions for maximum yield of ^{99m}Tc-PG were carried out using pyridoxal and monosodium gluta-

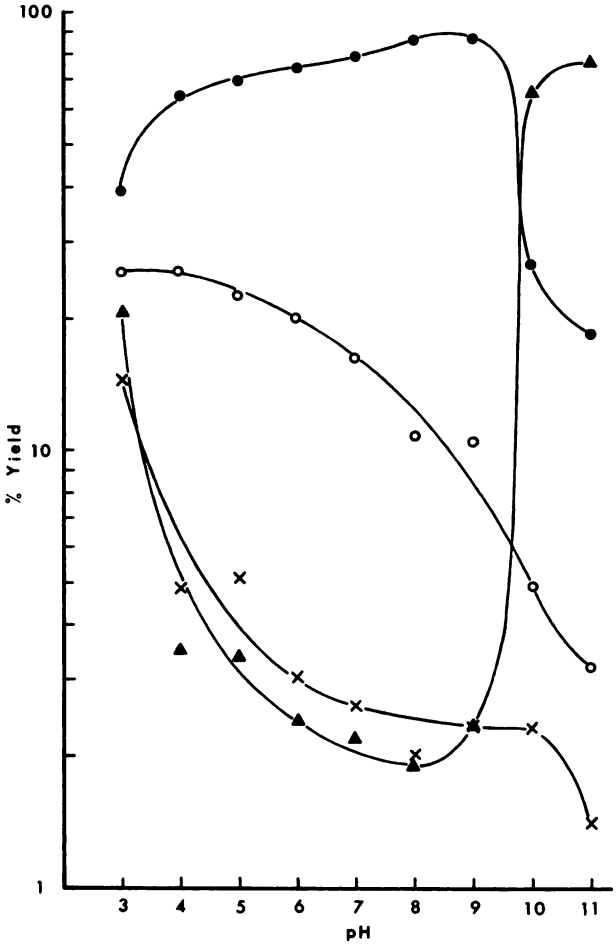


FIG. 2. Electrophoretic analysis of radioactivity in ^{99m}Tc-PG preparations in pH range 3–11: (●) ^{99m}Tc-PG, (○) ^{99m}Tc-pyridoxal, (X) band X, and (▲) $^{99m}TcO_4^-$.

TABLE 1. ELECTROPHORETIC RESULTS FOR ^{99m}Tc-PG AND ^{99m}Tc-PYRIDOXAL PREPARED UNDER VARYING CONDITIONS

Time* (min)	Concentration†	Conditions‡	% PG	% P	% X	% TcO_4^-
^{99m}Tc-PG						
10	1.0	N_2	45.3	14.3	8.8	31.6
10	1.0	Air	24.1	11.2	5.2	59.4
20	1.0	N_2	72.6	14.4	6.4	6.6
20	1.0	Air	68.3	12.1	5.1	14.5
30	1.0	N_2	82.9	11.7	3.9	1.4
30	1.0	Air	84.9	10.5	2.9	1.7
60	1.0	N_2	82.1	12.4	3.3	2.2
60	1.0	Air	87.2	10.0	1.7	1.0
30	0.8	N_2	80.5	11.7	5.8	2.0
30	0.8	Air	84.2	9.4	3.7	2.7
30	0.6	N_2	79.5	10.6	4.9	5.0
30	0.6	Air	82.2	7.4	4.0	6.4
30	0.4	N_2	48.4	7.3	4.8	39.5
30	0.4	Air	42.2	4.8	2.4	50.6
^{99m}Tc-pyridoxal						
20	1.0	N_2	1.3	87.7	6.3	4.7
20	1.0	Air	7.6	68.0	8.2	16.2
60	1.0	N_2	2.5	88.8	5.3	3.4
60	1.0	Air	23.8	64.4	4.9	6.8

* Autoclaved at 120°C.
† Factor for weights of reactants given in experimental details.
‡ Nitrogen or air bubbled through rubber-closed vial for 5 min prior to autoclaving.

mate in constant quantities as indicated in the Materials and Methods section. Figure 2 shows the distribution of products of the reaction at pH values from 3.0–11.0. The maximum yield of ^{99m}Tc-PG was obtained at pH 8–9 and decreased rapidly above pH 9.5 or below pH 4.0. In acid conditions the yield of ^{99m}Tc-pyridoxal was in the order of 25% but this decreased as the pH was raised. The proportion of free pertechnetate decreased from 20% at pH 3.0 to less than 2% at pH 8.0, then rose rapidly above pH 9.0. Band "X" followed the pertechnetate content fairly closely except at high pH where it continued to decrease.

In another series of experiments, the effect of varying conditions at pH 8.0 was studied. In Table 1, results for increasing the autoclaving time (at 120°C) from 10–60 min are reported. This series was carried out under standard conditions using solutions from which oxygen was removed by purging with nitrogen or through which air was bubbled, both for 5 min, immediately before autoclaving. At the 10- or 20-min time intervals, the yield of ^{99m}Tc-PG was considerably reduced in the aerated solutions and the pertechnetate content was significantly increased. At the longer autoclaving times almost identical results were obtained in both nitrogen and air. Table 1 also shows that the concentration of reactants could be reduced to approximately three-fifths of the standard quantities used before a significant increase in free pertechnetate takes place, provided the total volume does not exceed 4.0 ml. The presence of air seemed markedly to affect the distribution of radioactivity in the ^{99m}Tc-pyridoxal preparation; as well as resulting in increased free pertechnetate, it also significantly increased the percentage of activity appearing at the origin.

In vivo distribution. In Fig. 3 the distribution of ^{99m}Tc-pyridoxal in the mouse is compared with ^{99m}Tc-PG and ^{99m}Tc-D-penicillamine as prepared by the method of Krishnamurthy, et al (*15*). With ^{99m}Tc-pyridoxal, marked blood-pool activity persisted throughout the study and considerable uptake in the kidneys and bladder was noted. Weak accumulation of activity in the gallbladder was seen later in the study. However, with ^{99m}Tc-PG the gallbladder was visualized within the first 10 min after injection and during the course of the study its image became much more intense than was observed with ^{99m}Tc-pyridoxal. In addition, more activity entered the intestine and, although considerable urinary excretion occurred, the radiopharmaceutical was not seen to accumulate in the kidneys to any great extent. Excretion through the mouse liver was more rapid than with ^{99m}Tc-D-penicillamine which showed a marked and persistent liver uptake before the gallbladder began to appear.

A more detailed comparison between the biologic properties of ^{99m}Tc-pyridoxal and ^{99m}Tc-PG is available in Tables 2 and 3, which show the quantitative distribution of these two compounds in mice at four

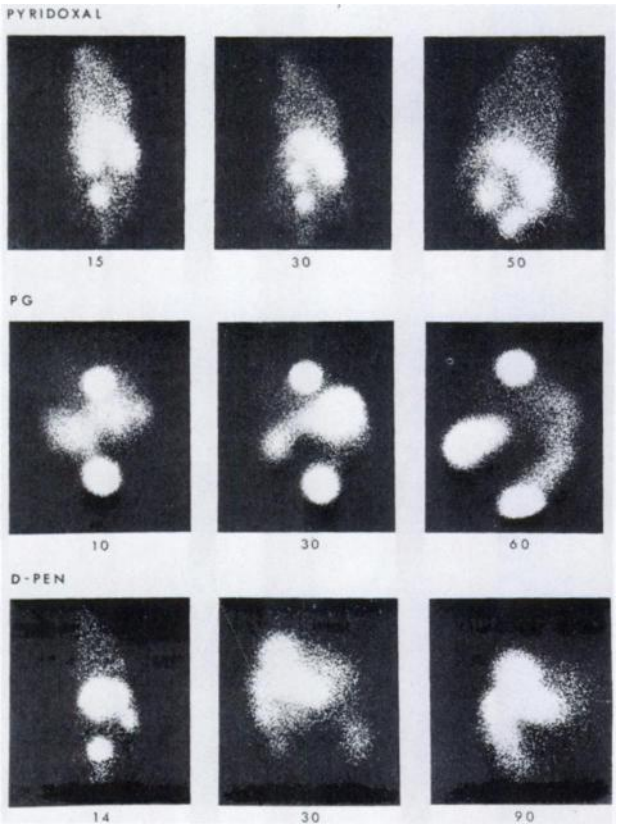


FIG. 3. Distribution of radioactivity in mice pictured with scintillation camera and pinhole collimator using ^{99m}Tc-pyridoxal, ^{99m}Tc-PG, and ^{99m}Tc-D-penicillamine. Numbers indicate time after injection in min.

time intervals. With ^{99m}Tc-pyridoxal approximately 40% was excreted in the urine within 15 min, increasing to 80% by 120 min. About 10% was excreted into the intestine within 60 min at which stage the concentration in liver and kidneys was low. The gallbladder showed some accumulation of activity, rising to a concentration of 45% of the dose per gram at 60 min, whereas ^{99m}Tc-PG showed a gallbladder concentration of 371% of the dose per gram at 15 min and 210% of the dose per gram at 60 min. By 30 min over 40% of the dose of ^{99m}Tc-PG was found in the intestine and thereafter this value remained relatively constant. The urine also contained a similar proportion of the injected dose. Most of the remaining activity was found in the liver with small amounts present in other organs.

Figure 4 shows scintiphotos of the distribution of ^{99m}Tc-PG in a dog. Intense uptake of radioactivity by the liver was seen clearly in the early phase of the study but this gradually decreased to a faint outline at about 60 min. The gallbladder commenced to accumulate activity by about 20 min and excellent pictures of this organ were obtained using the pinhole collimator. The dose of PG used in this study was 3.2 mg/kg, assuming complete formation from the reactants.

Toxicity studies. Table 4 shows the results obtained with groups of ten mice injected intravenously with doses increasing from 800 to 1,600 mg/kg. The lower doses up to 1,000 mg/kg were generally well tolerated (although one mouse died at 1,000 mg/kg). The LD_{50} was found to be approximately 1,400 mg/kg. At the higher dose levels, mice either convulsed and died immediately the injection was completed or remained depressed for several hours. Most mice affected in the latter manner recovered completely, although one animal died 60 min after

TABLE 2. ORGAN DISTRIBUTIONS OF ^{99m}Tc-PYRIDOXAL IN MICE AT VARIOUS TIME INTERVALS

	15 min		30 min		60 min		120 min	
Organ	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Blood	—	3.8 ± 1.1	—	2.9 ± 0.2	—	0.9 ± 0.1	—	1.5 ± 1.4
Heart	0.2 ± 0.0	1.7 ± 0.5	0.1 ± 0.0	1.1 ± 0.1	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.3 ± 0.1
Lungs	0.6 ± 0.1	3.3 ± 0.7	0.3 ± 0.1	2.4 ± 0.4	0.1 ± 0.0	0.8 ± 0.1	0.1 ± 0.0	0.6 ± 0.1
Liver	4.2 ± 1.3	2.9 ± 0.8	2.6 ± 0.3	2.5 ± 0.4	1.5 ± 0.2	1.0 ± 0.3	0.7 ± 0.1	0.7 ± 0.1
Gallbladder	0.5 ± 0.4	20.8 ± 1.6	0.6 ± 0.1	34.0 ± 5.9	0.4 ± 0.4	44.8 ± 10.6	0.9 ± 0.7	46.5 ± 12.9
Spleen	0.2 ± 0.0	1.0 ± 0.3	0.1 ± 0.0	0.7 ± 0.2	0.1 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.3 ± 0.0
Stomach	0.6 ± 0.2	1.3 ± 0.8	0.5 ± 0.0	1.5 ± 0.1	0.1 ± 0.0	0.2 ± 0.2	0.3 ± 0.3	1.0 ± 0.8
Pancreas	0.2 ± 0.0	1.2 ± 0.3	0.1 ± 0.0	0.9 ± 2.4	0.1 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.3 ± 0.1
Intestines	5.6 ± 0.8	1.7 ± 0.3	6.6 ± 1.5	2.7 ± 0.4	10.6 ± 1.7	3.1 ± 1.4	9.7 ± 1.6	3.7 ± 0.4
Kidneys	2.5 ± 0.3	7.4 ± 1.3	4.9 ± 2.4	12.9 ± 6.3	0.8 ± 0.2	2.2 ± 0.5	0.7 ± 0.0	1.7 ± 1.0
Brain	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Muscle	—	0.9 ± 0.3	—	0.7 ± 0.2	—	0.2 ± 0.0	—	0.2 ± 0.0
Bone	—	1.4 ± 0.3	—	1.2 ± 0.2	—	0.4 ± 0.1	—	0.4 ± 0.1
Tail	3.5 ± 1.2	—	2.3 ± 0.3	—	2.1 ± 0.4	—	1.0 ± 0.4	—
Urine	41.3 ± 3.7	—	54.3 ± 2.3	—	70.9 ± 3.5	—	79.7 ± 5.0	—
Carcass	46.0 ± 1.0	—	32.2 ± 2.4	—	11.0 ± 1.4	—	5.9 ± 0.4	—
Total	106.0 ± 2.5	—	105.0 ± 2.1	—	97.7 ± 0.8	—	99.0 ± 4.1	—

(a) % Dose per organ, (b) % Dose per gram. Mean results for four mice ± 1 s.d.

TABLE 3. ORGAN DISTRIBUTION OF ^{99m}Tc-PG IN MICE AT VARIOUS TIME INTERVALS

Organ	15 min		30 min		60 min		120 min	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Blood	—	1.2 ± 0.1	—	1.0 ± 0.1	—	0.3 ± 0.1	—	0.4 ± 0.0
Heart	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
Lungs	0.2 ± 0.0	1.1 ± 0.2	0.1 ± 0.0	0.8 ± 0.1	0.1 ± 0	0.4 ± 0.1	0.1 ± 0.0	0.4 ± 0.0
Liver	4.2 ± 0.7	3.5 ± 0.3	3.0 ± 0.5	2.6 ± 0.5	2.1 ± 0.4	1.5 ± 0.3	2.3 ± 0.2	2.1 ± 0.1
Gallbladder	2.8 ± 1.1	371.0 ± 109.0	1.8 ± 0.8	164.0 ± 38.0	3.2 ± 2.4	210.0 ± 119.0	2.5 ± 0.5	136.0 ± 43.0
Spleen	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
Stomach	0.6 ± 0.6	1.3 ± 1.3	0.2 ± 0.1	0.8 ± 0.2	0.2 ± 0.1	0.4 ± 0.2	0.4 ± 0.4	1.1 ± 1.1
Pancreas	0.1 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.4 ± 0.2	0.3 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
Intestines	32.7 ± 3.8	14.7 ± 1.9	42.5 ± 2.2	23.3 ± 3.2	39.3 ± 2.1	18.0 ± 3.3	42.3 ± 1.7	22.3 ± 2.3
Kidneys	0.8 ± 0.3	2.1 ± 0.9	1.4 ± 1.4	3.5 ± 3.3	0.6 ± 0.1	1.6 ± 0.2	0.5 ± 0.0	1.6 ± 0.1
Brain	0.0 ± 0.0	0.1 ± 0.0	0.0± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Muscle	—	0.3 ± 0.0	—	0.2 ± 0.1	—	0.1 ± 0.0	—	0.1 ± 0.0
Bone	—	0.6 ± 0.1	—	0.4 ± 0.0	—	0.2 ± 0.1	—	0.2 ± 0.0
Tail	1.6 ± 0.4	—	1.0 ± 0.2	—	0.7 ± 0.1	—	0.4 ± 0.1	—
Urine	43.7 ± 4.6	—	42.9 ± 0.9	—	47.8 ± 4.1	—	46.3 ± 4.6	—
Carcass	16.0 ± 1.9	—	10.4 ± 0.7	—	5.2 ± 0.7	—	5.3 ± 3.1	—
Total	103.0 ± 6.5	—	103.0 ± 1.1	—	99.5 ± 3.4	—	100.0 ± 4.2	—

(a) % Dose per organ, (b) % Dose per gram. Mean results for four mice ± 1 s.d.

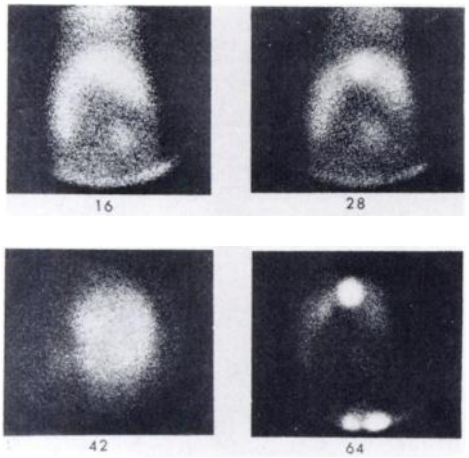


FIG. 4. Scintiphotos of distribution of ^{99m}Tc-PG in dog, showing anterior views at 16, 28, and 64 min after injection obtained with parallel-hole collimator. View at 42 min taken with pinhole collimator shows gallbladder.

TABLE 4. ACUTE TOXICITY DATA FOR ^{99m}Tc-PG IN MICE

Dose (mg/kg)	Number tested	Number dead	Percent mortality
800	10	0	0
1,000	10	1	10
1,200	10	0	0
1,400	10	5	50
1,600	10	8	80

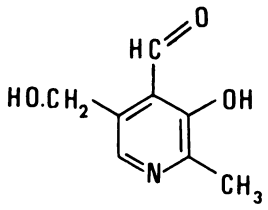
injection. This animal received 1,400 mg/kg. No adverse effects were noted in a group of 20 mice given 1,000 times the proposed human dose (i.e., 282 mg/kg) nor in groups of 13 rats and 6 cats given similar doses. None of the group of 15 mice given cumulative doses of 2,820 mg/kg in ten equal doses showed any reaction and the organs of five of these mice revealed no significant histologic difference from those of the controls. The preparation was found to be sterile, pyrogen-free, and did not produce an antigenic reaction when tested in guinea pigs.

Reabsorption experiments. When radioactive bile from a donor rat given ^{99m}Tc-PG was injected directly into the duodenum of recipient rats, the radioactivity appearing in the blood at 2 hr reached 0.02% of the dose per milliliter. No activity was observed in the bladder at this time when the rats were examined with the scintillation camera. Both the bile and urine of the donor rat were examined by electrophoresis and found to give almost identical results with the main proportion of radioactivity remaining at the origin (75%), compared with 84% for the original preparation. The ^{99m}Tc-pyridoxal content increased in both to 19% from 12% originally.

DISCUSSION

The reaction between ^{99m}Tc-pertechnetate and pyridoxal that occurs in solution on autoclaving under nitrogen yields a product behaving quite distinctly from $^{99m}TcO_4^-$ on electrophoresis. Although conclusive evidence is not available at this stage, the known properties of both pyridoxal and the element technetium would strongly suggest that the com-

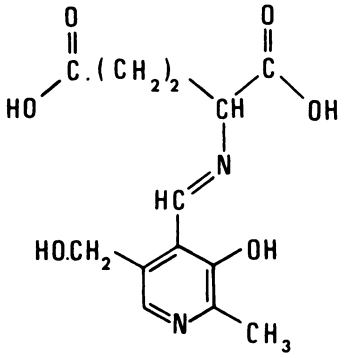
pound we have investigated is a ^{99m}Tc-pyridoxal complex. Pyridoxal has the formula:



The hydroxyl and aldehyde groupings provide a bidentate chelate function analogous to salicylaldehyde, a ligand known to form numerous metal complexes. It could also act as a simple monodentate ligand by coordination of the pyridine nitrogen atom. Recent radiopharmaceutical preparations have provided abundant evidence that ^{99m}Tc is able to form complexes with suitable ligands such as DTPA (*16*), pyrophosphate (*17*), etc., provided the system includes a reducing agent capable of producing technetium in a lower oxidation state than +7 as found in TcO_4^-. The lower oxidation state, probably +4 (*16*), is capable of combining with suitable ligands but the products should be kept under nitrogen or with a suitable excess of reducing agent to prevent atmospheric oxidation.

Apart from the chelating function, pyridoxal also provides a reducing function in the aldehyde group of the molecule. This action is a well-known property of aldehydes in general. Thus the combination of both these properties would suggest that a ^{99m}Tc-pyridoxal complex is formed.

The inclusion of L-glutamate in the reaction mixture produced a new ^{99m}Tc species, electrophoretically different from ^{99m}Tc-pyridoxal. Again the evidence is not conclusive but the most likely possibility is the formation of a ^{99m}Tc complex of the Schiff-base pyridoxylideneglutamate. This compound may act as a tridentate ligand:



Following ionization of the phenolic and carboxyl hydrogen atoms, coordination of these groups to a metal ion together with the imine nitrogen atom would result in the formation of a chelate containing fused five- and six-membered rings.

In general, the main impurity in the preparation is ^{99m}Tc-pyridoxal. The series of experiments carried out to optimize the yield of ^{99m}Tc-PG showed that the ^{99m}Tc-pyridoxal content of the preparation was usually 10–12%. Choosing a pH closer to 9.0 may reduce this value slightly (Fig. 2). However, the yield of ^{99m}Tc-PG begins to drop markedly at this point so that a pH close to 8.0 is preferred.

In vivo studies using the scintillation camera showed that ^{99m}Tc-pyridoxal behaved in a markedly different manner from ^{99m}Tc-PG in mice. With the latter compound, the presence of radioactivity in the intestine suggested a biliary excretory mechanism, particularly as the gallbladder was visualized in most cases. It is worth noting, however, that gallbladder uptake in mice cannot be considered a reliable phenomenon because in some instances we failed to observe uptake in an individual animal whereas its litter-mates gave good results with the same preparation of ^{99m}Tc-PG. In each case, however, normal intestinal activity was observed, indicating that biliary excretion was not restricted in the anomalous individual animals. Compared with ^{99m}Tc-D-penicillamine, ^{99m}Tc-PG appeared in the gallbladder and intestine much more rapidly. The former compound was found to require a period of 60–90 min before good-quality images of the mouse gallbladder could be obtained. In humans, a period of 3 hr has been recommended (*18*). The results of the quantitative distribution studies of ^{99m}Tc-pyridoxal and ^{99m}Tc-PG confirm the interpretation of the scintillation camera studies of these two compounds. One of the main differences in the biologic distribution is the higher percentage of ^{99m}Tc-pyridoxal excreted in the urine. The concentration of radioactivity in the gallbladder at 60 min is quite low when compared with ^{99m}Tc-PG, which was a factor of eight times greater by the end of the first 15 min. This result decreased over the successive time intervals, the erratic behavior being probably due to contraction of the gallbladder.

These results demonstrate a good potential for human use of ^{99m}Tc-PG, apart from the fact that nearly 50% is excreted in the urine of the mouse. This was not considered a major drawback since the distribution indicated that renal retention remained quite low, falling to less than 1% of the injected dose by 60 min. The presence of an enterohepatic circulation seems unlikely, at least in the rat, in view of the appearance of only minor amounts of activity in the blood or urine when bile containing excreted ^{99m}Tc-PG was injected directly into the duodenum of recipient rats. Although minor changes

occurred in the electrophoretic pattern of both bile and urine, it is likely that ^{99m}Tc-PG is excreted unchanged by both these routes although the presence of degradation products with similar electrophoretic behavior has not been excluded.

The results of sterility, pyrogenicity, and antigenicity studies and of toxicity studies in mice, rats, and cats indicate that ^{99m}Tc-PG is suitable for use in humans with a wide margin of safety. There was a low incidence of toxic effects up to 1,000 mg/kg. This dose represents 3,500 times the proposed human dose, assuming that one-quarter of the preparation (19.7 mg PG^- ion) is injected. Although no information is available on the biologic effects of PG, the starting materials or closely related compounds have been used in humans at dose levels many times that employed in the preparation of ^{99m}Tc-PG.

Large doses of monosodium glutamate have been used for the treatment of hepatic coma, up to 25 gm being infused intravenously (*19*). The quantity injected in a proposed diagnostic dose of ^{99m}Tc-PG prepared according to the method given (6 mCi in 1.0 ml) is equivalent to 9.8 mg of glutamic acid. This would raise the plasma glutamate level in a 70-kg man to approximately 11.2 mg/liter, which is a 44% increase over the mean fasting level of 7.8 mg/liter (53 ± 16 μmole/liter) (*20*). Earlier reported plasma glutamate levels appear to be erroneously high due to breakdown of glutamine which is normally present at about 15 times the concentration of glutamate (*21*). Pyridoxine, one of the interconvertible forms of vitamin B_6, has been used intramuscularly in humans in doses as large as 1 gm as the hydrochloride salt (*22*) while pyridoxal hydrochloride has been found to have an LD_{50} of 390 mg/kg when injected intravenously in the mouse (*23*). This is more toxic than we have observed if the pyridoxal content of the PG preparation is considered and may be due to acidosis induced by the hydrochloride salt used in that study.

In summary, ^{99m}Tc-PG appears to be a suitable agent of low toxicity for use in humans for the investigation of biliary tract disorders.

ACKNOWLEDGMENTS

The authors wish to thank the University of Adelaide Anti-Cancer Foundation and the Institute of Medical and Veterinary Science for financial assistance during this project.

We are indebted to D. A. Purcell of the Institute of Medical and Veterinary Science for the histologic examinations.

REFERENCES

1. WILLIAMS DR: Metals, ligands and cancer. *Chem Rev* 72: 203–213, 1972

2. METZLER DE, IKAWA M, SNELL EE: A general mechanism for vitamin B_6-catalyzed reactions. *J Am Chem Soc* 76: 648–652, 1954

3. SNELL EE, FASELLA PM, BRAUNSTEIN A, et al (eds): *Chemical and Biological Aspects of Pyridoxal Catalysis.* New York, Macmillan, 1963

4. BRANDENBERGER H, COHEN PP: Ein Beitrag zum Studium des Reaktionsmechanismus der biologischen Transaminierung. *Helv Chim Acta* 36: 549–554, 1953

5. HEYL D, HARRIS SA, FOLKERS K: The chemistry of vitamin B_6. VI. Pyridoxylamino acids. *J Am Chem Soc* 70: 3429–3431, 1948

6. MATSUSHIMA Y, MARTELL AE: Pyridoxal analogs. IX. Electron absorption spectra and molecular species in methanol solution. *J Am Chem Soc* 89: 1322–1330, 1967

7. MATSUO Y: Formation of Schiff bases of pyridoxal phosphate. Reaction with metal ions. *J Am Chem Soc* 79: 2011–2015, 1957

8. MATSUO Y: Pyridoxal catalysis of non-enzymatic transamination in ethanol solution. *J Am Chem Soc* 79: 2016–2019, 1957

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

10. MATSUSHIMA Y: Reactions of pyridoxal, amino acids and metal ions in methanol. *Chem Pharm Bull (Tokyo)* 16: 2143–2150, 1968

11. BANKS BEC, DIAMANTIS AA, VERNON CA: Transamination. Part II. The non-enzymatic reactions between pyridoxamine and pyruvic acid and between pyridoxal and alanine. *J Chem Soc* 4235–4247, 1961

12. DAVIS L, RODDY F, METZLER DE: Metal chelates of imines derived from pyridoxal and amino acids. *J Am Chem Soc* 83: 127–134, 1961

13. WILLSTADTER E, HAMOR TA, HOARD JL: Stereochemistry of the manganese(II)-pyridoxylidenevaline chelate. *J Am Chem Soc* 85: 1205–1206, 1963

14. WATERS JM, WATERS TN: Vitamin B_6-metal ion-amino acid intermediates. *Proceedings of the XII International Conference on Coordination Chemistry*, Freeman HC, ed, Sydney, Science Press, 1969, pp 195–196

15. KRISHNAMURTHY GT, TUBIS M, ENDOW JS, et al: ^{99m}Tc-Penicillamine—a new radiopharmaceutical for cholescintigraphy. *J Nucl Med* 13: 447, 1972

16. ECKELMAN WC, MEINKEN G, RICHARDS P: The chemical state of ^{99m}Tc in biomedical products II. The chelation of reduced technetium with DTPA. *J Nucl Med* 13: 577–581, 1972

17. ECKELMAN WC, REBA RC, KUBOTA H, et al: ^{99m}Tc-pyrophosphate for bone imaging. *J Nucl Med* 15: 279–283, 1974

18. KRISHNAMURTHY GT, ENDOW J, TUBIS M, et al: ^{99m}Tc-Penicillamine cholescintigraphy: comparison with oral cholecystography. *J Nucl Med* 14: 418–419, 1973

19. MCDERMOTT WV, WAREHAM J, RIDDELL AG: Treatment of "hepatic coma" with L-glutamic acid. *N Engl J Med* 253: 1093–1102, 1955

20. GUTMAN AB, YÜ T-F: Hyperglutamatemia in primary gout. *Am J Med* 54: 713–724, 1973

21. PAGLIARA A, GOODMAN AD: Pitfalls in the determination of plasma glutamate. *N Engl J Med* 279: 1402, 1968

22. TODD RG (ed): *Extra Pharmacopoeia: Martindale.* London, The Pharmaceutical Press, 1967, p 120

23. KRAFT HG, FIEBIG L, HOTOVY R: Pharmacology of vitamin B_6 and its derivatives. *Arzneim Forsch* 11: 922–929, 1961