^{99m}Tc-PENICILLAMINE-ACETAZOLAMIDE COMPLEX, A NEW RENAL SCANNING AGENT

S. Halpern, M. Tubis, J. Endow, C. Walsh, J. Kunsa, and B. Zwicker

Wadsworth Veterans Hospital, Los Angeles, California, and the University of California at San Diego, San Diego, California

Within the past 10 years numerous radiopharmaceuticals have been proposed as kidney scanning agents, all of which have advantages and disadvantages. The mercurials either deliver an unacceptable radiation dose to the patient or suffer from poor tissue penetration. ^{131}I-iodohippurate introduces artifacts into the scan and, like ^{113m}In compounds, is very inefficient for the ½-in. crystal of the Anger camera. Currently there are two ^{99m}Tc-labeled compounds that are being used for renal scanning. They are ^{99m}Tc-DTPA (*1*) and ^{99m}Tc-iron-ascorbic acid complex (*2*). The latter suffers from preparation problems and produces a high liver background which degrades the scan image if the serum creatinine exceeds 3.0 mg% (*3*), and the former depends entirely on glomerular filtration for its excretion, which may decrease scan quality if the glomerular filtration rate is diminished. These two compounds do, however, allow one to do simultaneous perfusion studies using the Anger camera, a procedure we feel to be valuable. We have incorporated ^{99m}Tc into a complex with acetazolamide, the theory being that a molecule largely excreted from the body by tubular secretion would result in higher target-organ-to-background ratios and thereby give more adequate scans in those patients with severe renal disease. Since the radionuclide incorporated would be technetium, perfusion studies would still be possible, thereby eliminating the necessity of using two radiopharmaceuticals for scan and perfusion.

Prior to our attempts to label acetazolamide with ^{99m}Tc we sought a more certain and efficient method for the reduction of pertechnetate which would give a "single valence" state for complexing and thus avoid the spectrum of rapidly interchanging valence states. We have noted that when pertechnetate is reduced by ascorbic acid and ferric chloride and other reducing agents there is a variable reoxidation to pertechnetate which makes labeling inefficient and under certain conditions impossible. What was clearly needed was a compound that would reduce the pertechnetate to a single valence state and hold it in that state until we needed it. We evaluated sodium borohydride for this purpose but found it unacceptable because of large amounts of insoluble technetium dioxide that were formed and did not react with other compounds. We decided to use a chelating agent to reduce the pertechnetate, and D-penicillamine was chosen for this purpose. The ^{99m}Tc-penicillamine complex was then used in the formation of ^{99m}Tc-penicillamine-acetazolamide complex (TPAC). The chemistry involved in this chelation will be the subject of a separate communication.

MATERIALS AND METHODS

All chromatography was performed on Whatman No. 1 paper using descending technique. The solvent used was n-butanol 4:acetic acid 1:water 1 v/v. Fluorescence was detected with a short-wave ultraviolet lamp (6 RP U.V.S.-11). The acetazolamide was supplied by Lederle Laboratories and the penicillamine by Merck, Sharp, and Dohme in a pure powder form. Autoclaving was performed in a 4-quart pressure cooker at 15-lb pressure. Pertechnetate eluate was used from molybdenum generators supplied by several different companies, with no discernible difference noted in the finished product. Imaging was performed on an Anger scintillation camera. Animal studies were performed on 30 albino mice, five New Zealand white rabbits, and ten mongrel dogs. Some of the rabbits and dogs were used for multiple studies. Twenty of the 30 mice were injected with 1.0 mg of TPAC in a 0.2-ml volume and were used to determine if high count ratios in the kidneys could be attained with the new com-

Received Feb. 19, 1971; revision accepted July 29, 1971.

For reprints contact: Samuel E. Halpern, Div. of Nuclear Medicine, University Hospital, 225 W. Dickinson St., San Diego, Calif. 92103.

pound. The results were affirmative. Following this, the rabbits were administered the same volume of TPAC as was used in the mice, and whole-body scintiphotos were obtained, which showed very high renal uptake of the radiopharmaceutical by 20 min postinjection.

Following this, five of the dogs were anesthetized with barbiturates, and an indwelling catheter was placed in the urinary bladder. Intravenous isotonic saline was used to hydrate the animal throughout the procedure to insure a urine flow of greater than 2.0 ml/min. Fifty milliliters of a 10% solution of inulin was then rapidly administered intravenously, followed immediately by 3 mCi of TPAC containing 10 mg of the radiopharmaceutical. After a 20-min equilibration period the bladder was emptied and urine was collected every 20 min in plastic containers and the volume determined. Ten milliliters of blood was drawn at the midpoint of each urine collection period in previously heparinized syringes and immediately centrifuged and the serum removed from the red blood cells. One milliliter of the serum was counted in a well counter, as was the same quantity of urine. These were used to compute the renal clearance of TPAC (*4*). The single-injection technique was chosen over the sustained-infusion technique for renal clearances because the radiopharmaceutical is always administered as a single intravenous bolus. A portion of the serum from each blood sample was electrophoresed along with a sample of the preinjection TPAC and $^{99m}TcO_4^-$ and autoradiographed.

The plasma proteins were then precipitated with 1 ml of 10% $Zn(OH)_2$, and the supernatant withdrawn. The precipitate was washed twice with the $Zn(OH)_2$ solution, and the precipitate and 1 ml of the supernatant were counted in the well counter. Care was taken to keep the geometry constant. These results were used to determine the percent of protein binding. Dialysis was performed on the serum of five dogs using the standard methods for this procedure. The red blood cells of five dogs were washed twice with 5 ml of normal saline and counted in a well counter to determine the binding of the radiopharmaceutical to the red blood cells. Plasma and urine inulin determinations were performed by the method of Roe et al (*5*).

Renal arteriovenous differences of TPAC were studied in two dogs using percutaneous catheterization of the renal artery and vein of one kidney. This procedure was also performed on two dogs through a surgical incision. The data from the two methods were virtually identical. Plasma disappearance curves were performed on three dogs following cross clamping of both renal arteries and on three dogs following the cross clamping of both renal arteries and the hepatic artery and vein. Blood samples in all animals were drawn at varying close intervals.

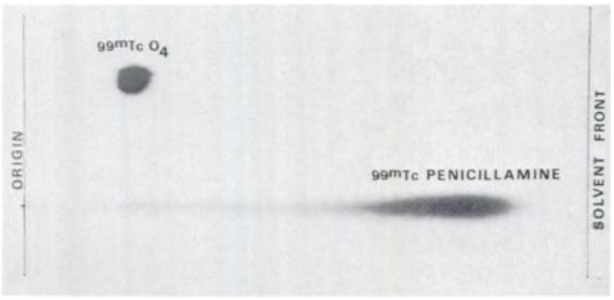

FIG. 1. Chromatogram showing $^{99m}TcO_4^-$ and ^{99m}Tc-penicillamine. Latter compound is virtually free of pertechnetate.

TABLE 1. PROCEDURE FOR PREPARING ^{99m}Tc-PENICILLAMINE-ACETAZOLAMIDE COMPLEX

Recommended amounts and concentrations of NaOH* for pH adjustment:

1. 1.0 ml 11.0 N NaOH
2. 4–5 drops 4.0 N NaOH
3. 1–2 drops 1.0 N NaOH
4. 1–2 drops 0.1 N NaOH

Procedure:

1. To a sterile, pyrogen-free, 15-ml centrifuge tube add 4.0 ml $^{99m}TcO_4^-$, and the following:
2. 60 mg D-penicillamine
3. 1.0 ml concentrated HCl (11.6 N)
4. 40.0 mg acetazolamide
5. Adjust the pH to 8.5 with NaOH*. Shake gently until the powdered chemicals go into solution
6. Autoclave 15 min at 15 lb pressure
7. Cool under running water and filter through a 0.22-micron Millipore filter.

The kidneys of two dogs and two rabbits were thinly sliced 60 min following the injection of TPAC and placed against photographic film and autoradiography was performed.

Acute and chronic toxicity studies were performed in 20-gm albino mice. Each of ten mice received 1 mg TPAC in a 0.20-ml volume, and two were sacrificed after 2 weeks; all organs were observed and the kidneys removed for microscopic examination. The remaining eight mice were reinjected as above, and two more were sacrificed 2 weeks after the second injection and treated as above. The same routine was continued until the surviving two mice were sacrificed 10 weeks after the initial injection.

RESULTS

Figure 1 shows the results of a chromatogram of $^{99m}TcO_4^-$ and ^{99m}Tc-penicillamine. Note the almost total elimination of pertechnetate from the chelate. This compound can be made instantaneously in strong acid, or it can be prepared at pH 2.0 by boil-

ing for about 20 min. In either event, the complex migrates at an Rf of 0.68 (average). Furthermore, the environment can be changed from acid to slightly alkaline with only minute reversion to pertechnetate. The ^{99m}Tc-penicillamine was found to be without value as a renal scanning agent and was used to further complex the acetazolamide to form TPAC.

Table 1 describes the method used in the preparation of TPAC. The proportions of penicillamine and acetazolamide used gave the best results in making our compound. The pH adjustment is critical and should be in the range of 8.5–8.7 but should not exceed 8.7 as an upper limit because of excessive destruction of the acetazolamide and the formation of compounds that do not migrate on chromatography at the appropriate area. If difficulty arises in dissolving the acetazolamide, the pH of the solution can be raised above the 8.7 level, then brought back to the optimum pH with dilute acid. Slight warming also aids in dissolving the acetazolamide. The pH adjustment should be made using highly concentrated NaOH in order that volumes remain low. The water in the pressure cooker is heated to boiling before the radiopharmaceutical is put in to heat. The heating time of 15 min is measured from the moment that the gage indicates 15 lb of pressure. The vial is then cooled under running water, filtered through a 0.22-micron disposable Millipore filter into a sterile pyrogen-free vial and used for intravenous injection.

Figure 2 shows the results of a chromatogram of pertechnetate, TPAC, and a fresh nonlabeled preparation of acetazolamide. The acetazolamide was detected by UV light. There is an obvious difference in the Rf of the penicillamine chelate shown in Fig. 1 and the TPAC in Fig. 2. There is also a difference in the Rf of the TPAC and the nonlabeled acetazolamide, with the former migrating slightly faster than the latter. Fluorescent observation of the

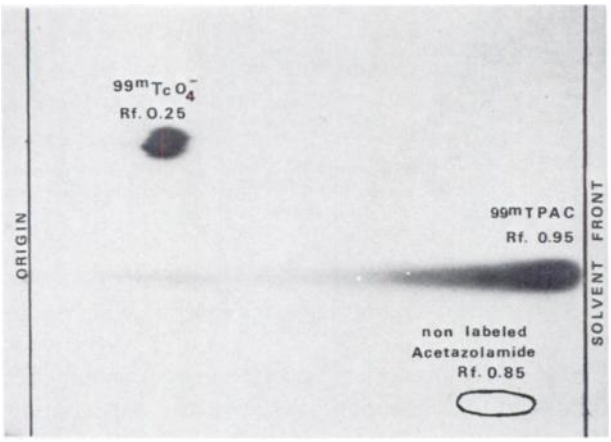

FIG. 2. Chromatogram of $^{99m}TcO_4^-$, TPAC, and nonlabeled acetazolamide. Latter was detected by UV fluorescent method.

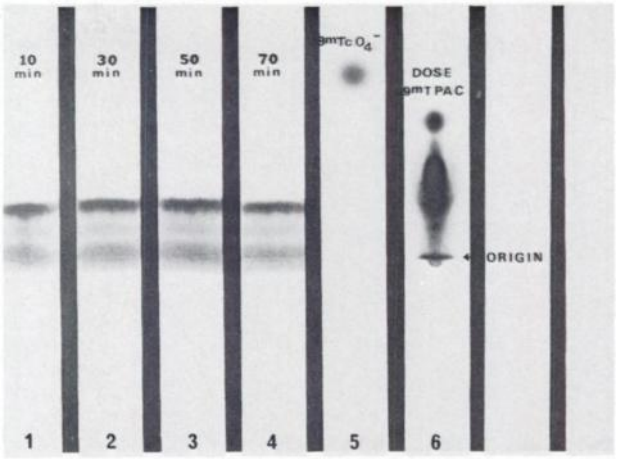

FIG. 3. Paper electrophoresis of serum at varying intervals postinjection TPAC. All radioactivity was associated with proteins, majority with albumin fraction.

TPAC shows that some breakdown of the acetazolamide has occurred in the preparation; however, the great majority of the fluorescence remains at the same Rf as the nonlabeled compound with a small amount detectable at one or two other areas on the paper strip. If the pH before heating is above 8.7, increasing amounts of radioactivity will migrate at an Rf of about 0.2 and some will remain at the origin. If the TPAC is allowed to remain at room temperature and chromatographed at 30-min intervals following its preparation, it will be seen that progressively more of the radioactivity migrates at the 0.94 Rf than in the freshly prepared material.

Table 2 shows the results of renal clearance of TPAC in dogs using inulin for comparison. The clearances were generally close with variations in results exceeding ±20% in only one of each of the studies. It is obvious that only a very small quantity of the total radioactivity is excreted in the urine as shown by the very low TPAC/IN clearance ratios. Determination of the protein binding reveals that 97% of the radioactivity is bound by the plasma proteins. The standard deviation of the protein binding was ±1%.

Figure 3 demonstrates the results of paper electorphoresis and autoradiography of four serum samples obtained at varying intervals postinjection of TPAC, and comparison strips of $^{99m}TcO_4^-$ and the preinjection TPAC. All of the TPAC is associated with the plasma proteins, the majority of it with the albumin fraction; however, some binding is noted in all the protein fractions. The degree of protein binding did not change with time.

The results of the TPAC arteriovenous differences are shown in Fig. 4. Plasma half-time of the radioactivity figured from the 10-min postinjection time

TABLE 2. TPAC CLEARANCE AND PROTEIN BINDING DATA. DEGREE OF PROTEIN BINDING WAS CONFIRMED BY DIALYSIS

Duration of collection period (min)	C^{99m}TPAC (cc/min)	Cln (cc/min)	$\frac{C^{99m}TPAC}{Cln}$	Percent precipitated with $Zn(OH)_2$	Percent not precipitated with $Zn(OH)_2$
20	1.5	104	0.014	97%	3%
20	1.4	118	0.019	97%	3%
20	1.1	82	0.013	97%	3%
20	1.6	127	0.013	97%	3%
20	1.9	110	0.017	97%	3%
20	1.83	104	0.018	97%	3%
20	1.78	65	0.0274	97%	3%
20	2.94	93	0.032		
20	1.3	96	0.014		
20	1.55	69	0.022		
20	1.4	50	0.028		

ranges from 40 to 60 min. These findings have been consistently noted in all dog studies. Of interest is the very small amount of radioactivity extracted on each pass through the kidneys. In the first 70 min postinjection, the radioactivity is accumulated by the kidneys at a much faster rate than in any time following this. These findings have been consistent. Only 5–8% of the radioactivity is extracted through the kidneys at the time of most rapid accumulation.

The changes in the plasma half-time disappearance on exclusion of the kidneys from the circulation is shown in Fig. 5. The plasma half-time becomes at least 90 min, and there is a flattening of the disappearance curve. Of interest of course is the fact that removal of radioactivity from the vascular compartment continued to occur. If the liver is excluded from the circulation and renal function left intact, one again sees prolongation of the plasma disappearance of TPAC. Thus there would appear to be multiple compartments into which TPAC can move.

A radiochromatogram of the urine following injection of TPAC revealed a broad spectrum of excretion products that do not resemble the chromatogram of the preinjection material. Areas of increased counting rate are noted; however, it does not appear that any significant amount of ^{99m}Tc is in the pertechnetate form. Under ultraviolet light several areas of fluorescence are seen. The majority of these areas are light blue in color; however, one area fluoresces purplish-brown and is the same hue as the preinjection material. The latter corresponds in Rf to an area noted for one of the breakdown products of the TPAC. The composition of these compounds is unknown. It is possible that they represent metabolism of TPAC by the kidneys.

Figure 6 is an autoradiograph of a slice of dog kidney extirpated about 1 hr postinjection of TPAC.

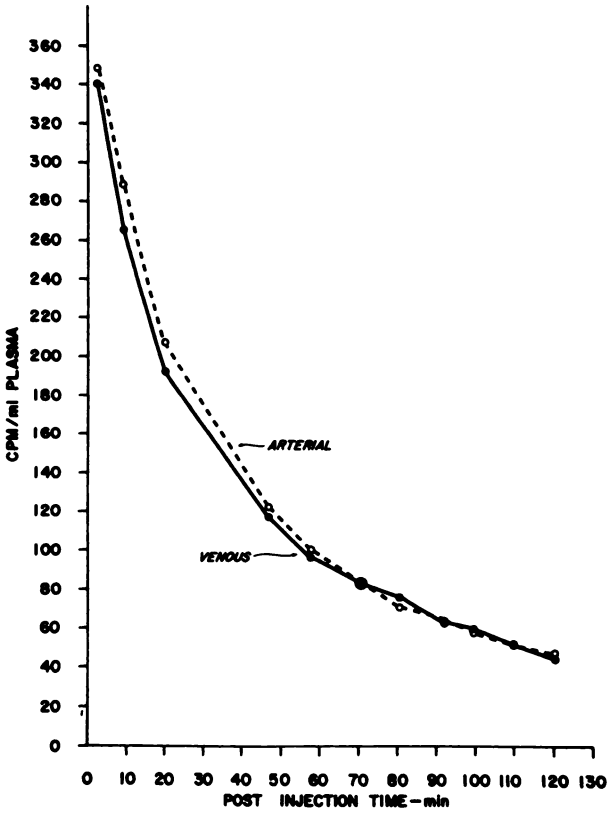

FIG. 4. Renal A-V differences of radioactivity at varying intervals following injection of TPAC. Most of extraction occurs within first 70 min.

Virtually all of the radioactivity is seen in the cortex of the kidney.

Figure 7 shows the results of gamma camera scintiphotography in a man with normal renal function. It is obvious that high-resolution scintiphotos can be obtained without background suppression by 40 min with virtually no liver interference. If rectilinear

scanning is done and background suppression used, excellent scans can be obtained by 15–25 min post-injection of TPAC.

TOXICITY STUDIES

There were no acute or chronic toxic reactions, and histological examination showed no morphological changes and no particulate matter in the lumen of the renal tubules. On the basis of 1-mg TPAC/20-gm mouse, the average human dose of 3 mCi/10 mg of TPAC/70-kg man represents 1/350th of the mouse dose, and on the basis of 5 mg/20-gm mouse, the human dose represents 1/1750th of the comparable mouse dose. Multiple studies were performed using TPAC in the same dog over a period of several weeks, and no alteration in the renal function of the dog was noted.

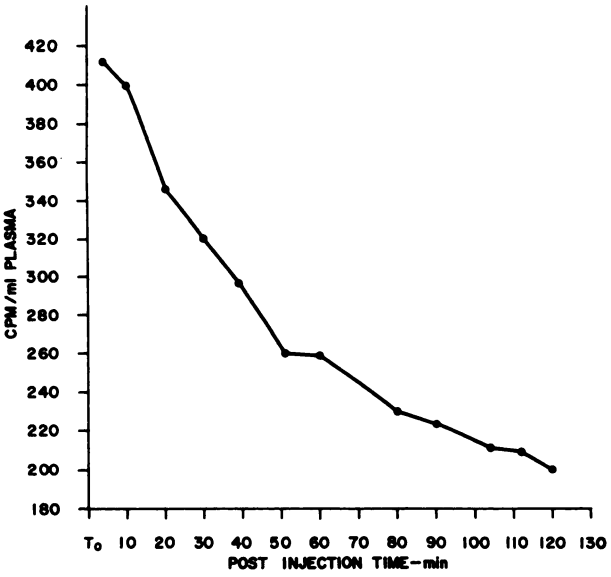

FIG. 5. Disappearance curve of TPAC from plasma following exclusion of kidneys from circulation. Note prolongation of plasma radioactivity as compared with Fig. 4.

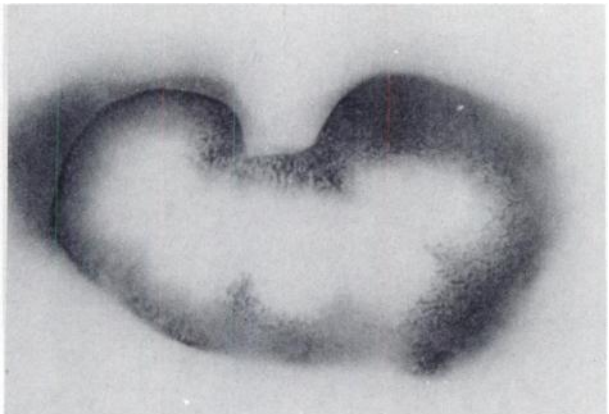

FIG. 6. Autoradiograph of dog kidney postinjection ^{99m}TPAC. Note absence of radioactivity in medulla.

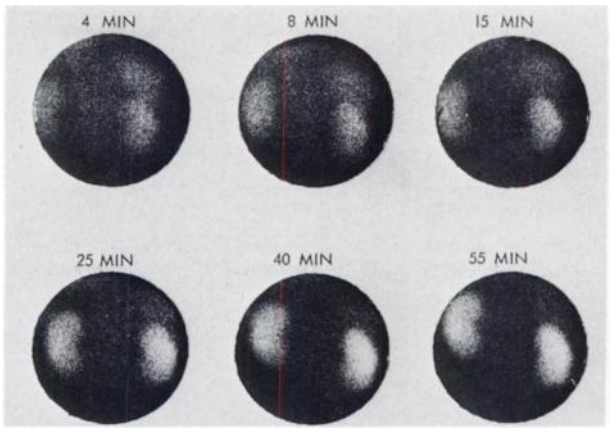

FIG. 7. Renal scan performed at varying intervals postinjection TPAC.

Scans also remained the same during this period of time. Studies in five rabbits using large doses of TPAC failed to reveal any evidence of toxicity.

DISCUSSION

From animal data and a very small human pilot study, we have concluded that TPAC is a potentially outstanding renal scanning agent. It presents many interesting features. To begin with, this is the first time a compound has been made using D-penicillamine as the agent for reduction of the pertechnetate. Following this, the technetium-penicillamine is complexed to acetazolamide. The exact mechanism of this complex and the chemical nature of the TPAC is presently unknown but is under investigation. Various sterioisomeric forms of penicillamine exhibit variable affinities for metals, and the chelates have more or less stability depending upon the metal. For D-penicillamine the decreasing order is Hg(II), PB, Zn, Co(II), Fe(II), and Mn(II) (*6*). Since ^{99m}Tc belongs to the same periodic group (7B) as Mn(II), it also probably chelates with D-penicillamine in the same manner, presumably by the sulfur and nitrogen atoms. The stability constants of the Mn complex indicate moderate stability while the presence of excess D-penicillamine lends additional stabilizing influence. We believe that part of the penicillamine is used to reduce the pertechnetate to a lower valence, which then chelates with penicillamine. The manner in which the ^{99m}Tc is chelated or complexed with acetazolamide is not known exactly, but acetazolamide in its deacetylated form contains a 1, 3, 4, thiadiazole-2-sulfonamide moiety which has nitrogen and sulfur atoms with potential chelating properties for the "reduced" ^{99m}Tc-penicillamine. The completed complex, TPAC, is approximately 97% protein bound regardless of the modality used to test

for protein binding. No more than 4–8% is extracted on a single pass through the kidney, the extraction becoming less with the increase of time. Seventy minutes following injection very little in the way of renal A-V differences can be found; therefore one would expect the best scans to be obtained about 70 min postinjection of TPAC, and such appears, from the renal study performed, to be the case. When inulin and TPAC clearances are simultaneously measured in the dog, the former is found to be cleared 50 times as fast as the latter. The high degree of protein binding could account for this. Our postulate that large amounts of TPAC would be excreted from the body by tubular secretion does not appear to be the case. While tubular mechanisms may contribute to its removal, only small amounts are extracted from the plasma by a single pass through the kidney. The exact renal mechanism for the handling of the radioactivity that is excreted is presently unknown; however, it appears that there is a delay in the excretion of that amount of radioactivity that is extracted by the kidney, thereby allowing high target-to-background ratios and excellent renal scans. If the kidneys are excluded from the circulation prior to the injection of the TPAC, the plasma disappearance curve becomes flatter and the plasma half-time longer. The same is noted if the liver is excluded from the circulation, with the kidneys remaining intact. Thus it appears that TPAC moves into multiple compartments, which is of interest since the compound is so highly protein bound. Autoradiographs of the kidneys show the great majority of the radioactivity to be concentrated in the renal cortex and only small amounts to be in the renal medulla. Histological studies failed to show any material within the tubular lumen, which leads one to believe that the material is still within renal tubular cells. Thus it seems reasonable to postulate either tubular secretion of some or all of the nonprotein-bound compound with minimal amounts being secreted into the tubular lumen, or filtration at the glomerular level with tubular reabsorption in the proximal nephron. Our animal studies reveal this compound to be nontoxic in both acute and chronic experiments. In the human pilot studies so far performed, there have been no reactions. Pyrogen testing has always been negative. Comparison studies of TPAC, ^{131}I-iodohippuran, ^{197}Hg, and ^{99m}Tc-DTPA have shown TPAC to be equally as good as any of these in imaging normal kidneys, and invariably better in cases of severe renal disease. High-resolution scintiphotos have been obtained with clearances of creatinine in a range of 3–5 cc/min. Thus it would appear from early work that TPAC is an outstanding kidney scanning agent.

SUMMARY

Technetium-99m-penicillamine-acetazolamide complex (TPAC), a new renal scanning agent, is prepared by the reduction of pertechnetate by penicillamine followed by complexing of this compound with acetazolamide. Plasma half-time disappearance of the compound ranges from 45 to 60 min in a dog with normal kidneys. If the kidneys are excluded from the circulation, half-time disappearance is prolonged to 90 min. TPAC is 97% protein bound to all classes of plasma proteins, the majority, however, being associated with the albumin fraction. Minute amounts are removed from the circulation by the kidney by either glomerular filtration and tubular reabsorption or by tubular extraction and delayed excretion. Outstanding scan images can be obtained in patients with very severe renal disease.

REFERENCES

1. HAUSER W, ATKINS HL, NELSON KG, et al: ^{99m}Technetium DTPA: A new radiopharmaceutical for brain and kidney scanning. *Radiology* 94: 679–684, 1970

2. HARPER PV, LATHROP KA, HINN GM, et al: Technetium-99m iron complex. In *Radioactive Pharmaceuticals*, AEC Symposium Series Conf-651111, 1966, pp 347–351

3. WINSTON MA, HALPERN SE, WEISS ER, et al: A critical evaluation of ^{99m}Tc-Fe-ascorbic acid complex as a renal scanning agent. *J Nucl Med* 12: 171–175, 1971

4. LANDOWNE M, ALVING A: A method of determining the specific renal functions of glomerular filtration, maximal tubular excretion (or reabsorption) and "effective blood flow" using a single injection of a single substance. *J Lab Clin Med* 32: 931–942, 1947

5. ROE JH, EPSTEIN JH, GOLDSTEIN NP: A photometric method for the determination of inulin in plasma and urine. *J Biochem* 178: 839–845, 1949

6. DOORNBOS DA, FABER JS: Studies on metal complexes of drugs D-penicillamine and *n*-acetyl-*d*-penicillamine. *Pharm Weekblad* 99: 289–309, 1964