RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

Radiochemical Analysis of Tc-99m Human Serum Albumin with High-Pressure Liquid Chromatography: Concise Communication

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High-pressure liquid chromatography (HPLC) can be performed with an aqueous size-exclusion column to separate proteins or other macromolecules on the basis of molecular size. An HPLC system with a Spherogel-TSK SW column was modified to detect simultaneously uv absorption and radioactivity. Characteristic retention times (RT) were determined for pure human serum albumin (HSA) (RT = 17 min) and pertechnetate (RT = 28.5 min). When analysis was performed on Tc-99m HSA preparations, Tc-99m radioactivity was resolved into five different peaks, with RT ranging from 10.2 to 28.5 min. Less than 2% radioactivity was associated with the pertechnetate peak, whereas the remaining Tc-99m was protein bound. Most of the activity (90%) corresponded to the albumin peak, and 7% was bound to contaminants of high molecular weight with RTs of 10.2 and 14 min. Rapid separation of various radiochemical components differing in molecular size provides an improved basis for understanding the biodistribution of a Tc-99m HSA preparation. This technique would be useful for the preparation and analysis of various radiolabeled macromolecules such as enzymes, immunoglobulins, and other proteins.

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Size-exclusion gel chromatography using Sephadex G-25 was first used by Persson and Liden (*1*) for studying a method of labeling human serum albumin. Because this technique has greater potential than thin-layer chromatography for the separation of labeled proteins, chelates, and various technetium complexes, it had been recommended by Eckelman et al. (*2,3*) for use in the analysis of Tc-99m-labeled compounds. Subsequently, several artifacts of the method, including oxidation of technetium within the column and binding to the gel (*4,5*), have made this technique unsatisfactory for critical analysis. Competitive adsorption of Tc-99m to the support gel can be partly avoided by using the inert solid-phase Biogel P-10 (*6*). Other disadvantages of gel chromatography include the need for a large sample, prolonged fractionation time, and limited resolution. In search of a method without these problems, we evaluated high-pressure liquid chromatography (HPLC) with an appropriate size-exclusion chromatographic column.

Applications of HPLC for the analysis of Tc-99m agents have been reported using a weakly basic anion-exchange column (*7–9*) and a reverse-phase ultrasphere octadecylsilyl (ODS) column (*8,10*). Both of these columns separate various components of Tc-99m complexes, based either on net charge or on lipophilicity. An HPLC method for the separation of various components based on molecular size would be useful in the analysis of radiolabeled compounds.

Recently, a new line of aqueous size-exclusion spherogel columns has been introduced (*11*) and has shown promise for rapid separation of proteins and enzymes (*12,13*).

In this paper, we report the application of an HPLC methodology using a Spherogel-3000 TSK SW column

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for the radiochemical analysis of Tc-99m-labeled human serum albumin (HSA).

MATERIALS AND METHODS

Radiopharmaceuticals. A vial* containing 21 mg HSA and 0.23 mg stannous tartrate was reconstituted with 1 ml of sterile distilled water, after which 3 ml of generator-eluted* pertechnetate containing 10–660 mCi of Tc-99m was added. The mixture was incubated for 20 min before analysis.

HPLC apparatus and procedure. An HPLC system† consisting of a solvent metering pump (Model 110A), UV-VIS detector (Model 153), and sample-injection valve (Model 210) was used in an isocratic mode. A Spherogel-TSK SW (Grade G3000 SW) column† was connected to a sample-injection valve through a short precolumn of the same grade. The column tube is of stainless steel (7.5 × 600 mm), and packed with hydrophilic spherical and porous gel (particle size 10 ± 2 μ and protein molecular weight exclusion limit, 3×10^5 daltons). The size of the sample loop is 100 μl, and a 10–50 μl sample was injected into the column. The column was eluted at a flow rate of 1 ml per min with elution buffer, pH 7.0 (either 0.02*M* morpholinopropane sulfonic acid (MOPS) in 0.15*M* NaCl, or 0.1*M* phosphate buffer). The eluant from the column was monitored by a uv detector and a sodium iodide scintillation detector. The uv absorbance at 254 nm and the radioactivity were recorded on a dual-channel strip-chart recorder.

To determine the recovery of Tc-99m from the column, pertechnetate was injected into the column and eluant was collected as 1-ml fractions, which were counted in a well counter. Total radioactivity recovered was compared with a duplicate injectate.

Radiochemical purity of Tc-99m HSA preparations was also determined for comparison purposes using conventional chromatographic methods including: (a) paper chromatography in 85% methanol, (b) silica-gel thin-layer chromatography in 85% methanol, (c) gel chromatography with Biogel P-10 and with saline as an eluant, and (d) trichloroacetic acid precipitation (*14*). Tc-99m HSA was incubated with heparinized fresh human blood for 30 min to test for the stability of Tc-99m HSA preparation and transchelation of plasma components. At the end of this incubation, the blood was centrifuged and 50–100 μl of plasma was injected into the HPLC column.

RESULTS

With the above HPLC system, characteristic retention times (RT) were determined for HSA and TcO_4^-.

A control HSA vial was reconstituted with 1 ml water and 3 ml 0.9% saline (no Tc-99m activity). This nonradioactive preparation was injected into the HPLC system to obtain the uv absorbance pattern of HSA and stannous tartrate solution (Fig. 1). A standard elution radioactivity profile of the pertechnetate was also determined (Fig. 1). Single peaks with respective retention times of 17 min for HSA and 28.5 min for pertechnetate were obtained and confirmed in over 50 trials. More than 97% of the injected Tc-99m was eluted from the column. The uv absorbance of the unlabeled HSA preparation demonstrated two components of higher molecular weight, with retention times of 10.2 and 14.4 min

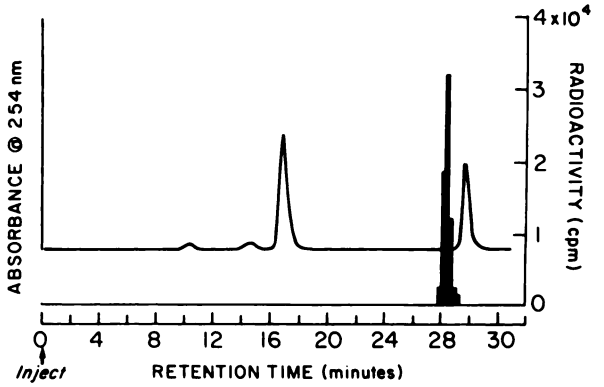

FIG. 1. Determination of retention times (RT) of HSA and free pertechnetate by aqueous size-exclusion HPLC. RT of pertechnetate is 28.5 min, determined by radioactivity elution profile of $^{99m}TcO_4^-$ (shaded). RT of HSA is 17 min, determined by uv absorbance of nonradioactive HSA preparation containing stannous tartrate.

When multiple samples from different vials of Tc-99m HSA were prepared and analyzed, the Tc-99m radioactivity was resolved into five different peaks with retention times 10.2, 14, 17, 24, and 28.5 min. A typical radiochemical analysis profile of a Tc-99m HSA preparation is shown in Fig. 2. Less than 2% of the injected activity was associated with the pertechnetate peak (28.5 min retention time) while the remaining Tc-99m activity is protein bound. Ninety percent of the injected activity corresponded to the albumin peak (17 min retention time), and 6% of the activity appeared with a retention time of 14 min. These results were obtained consistently

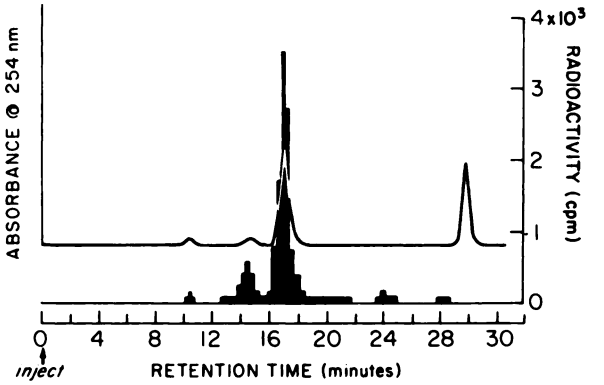

FIG. 2. HPLC radiochromatogram of Tc-99m HSA, with uv absorbance similar to unlabeled preparation. Tc-99m radioactivity profile shows five components with RT of 10.2, 14.4, 17, 24, and 28.5 min. Predominant peak of radioactivity corresponds to HSA peak with RT 17 min.

in 20 determinations. Similar results were found with both MOPS and phosphate buffer systems. There was no significant alteration of the radiochemical composition of the Tc-99m HSA preparation analyzed at 30-min intervals for 3 hr following preparation. Results are summarized in Table 1.

Following incubation of Tc-99m HSA with fresh human blood for 30 min, an analysis of the plasma sample demonstrated 70–80% of the Tc-99m activity associated with the albumin peak (Fig. 3). The remaining Tc-99m activity was eluted in five different peaks, four of which corresponded to peaks observed with analysis of Tc-99m HSA. A fifth peak observed at 26.4 min had the maximum non-albumin-bound Tc-99m activity.

The results of chromatographic analysis of Tc-99m HSA by different methods are presented in Table 2. The amount of free pertechnetate in the Tc-99m HSA preparation varied from 1 to 7% depending upon the analytical method. Paper and silica-gel thin-layer chromatography in 85% methanol showed 3–6% free pertechnetate, whereas column chromatography and trichloroacetic acid precipitation showed 6–7% free pertechnetate.

DISCUSSION

Pertechnetate can be eluted from a Spherogel-TSK SW HPLC column as a single peak with a characteristic retention time of 28.5 min. More than 97% of the injected Tc-99m activity can be eluted from the column, in contrast with Sephadex gel columns which exhibit nonspecific binding of up to 78% of applied radioactivity (*6*).

The results in Table 2 show that the amount of free pertechnetate in the Tc-99m HSA preparation, as determined by the HPLC method, is less than 2% and is lower than the values for free pertechnetate determined by other conventional methods (Table 2). The Spherogel column separates the total Tc-99m-bound activity (98.5%) into four different components with retention

TABLE 1. RADIOCHEMICAL ANALYSIS OF Tc-99m HSA BY AQUEOUS SIZE-EXCLUSION HPLC

Peak No.	Retention time	Chemical species	% Tc-99m activity*
1	10.2 min	Not defined	0.89 ± 0.47
2	14.4 min	Not defined	5.77 ± 1.30
3	17 min	Albumin	90.63 ± 3.15
4	24 min	Not defined	1.30 ± 0.75
5	28.5 min	$^{99m}TcO_4^-$	1.44 ± 1.04

* N = 5.

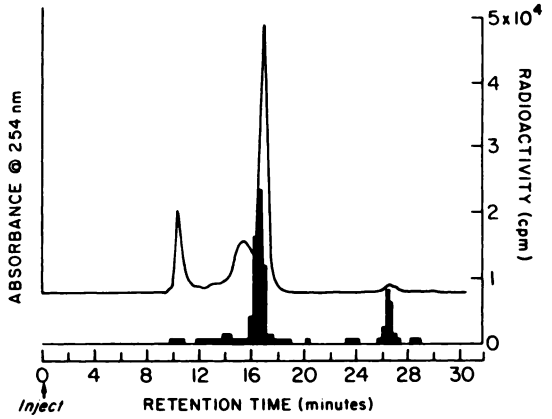

FIG. 3. HPLC radiochromatogram of a mixture of Tc-99m HSA and plasma. Radioactivity profile shows six components; 75% of radioactivity corresponds to HSA peak, while most of remaining radioactivity appears in a distinct peak with RT 26.5 min. This does not correspond to pertechnetate.

times ranging from 10.2 to 24 min, providing a more detailed radiochemical analysis of Tc-99m HSA preparations than can be obtained in a short time by more conventional analytical methods. Albumin binds 90% of the Tc-99m activity, whereas the components of higher molecular weight, with retention times of 10.2 and 14 min, bind about 7% of the Tc-99m. These high-molecular-weight components have not yet been characterized but may represent globulin contaminants of albumin, or perhaps polymers of albumin. After in vitro incubation with blood, Tc-99m HSA is relatively stable, with 70–80% of the Tc-99m activity still bound to albumin (Fig. 3). The peak at 26.4 min (Fig. 3) had the maximum non-albumin-bound Tc-99m activity. Based upon retention-time identification of the non-albumin-bound Tc-99m, this component is not free pertechnetate but appears to be a low-molecular-weight plasma component with a retention time of 26.4 min.

TABLE 2. DETERMINATION OF Tc-99m BINDING TO HSA

Method*	% Protein bound	% Free $^{99m}TcO_4^-$ †
1. Whatman, No. 1 & 85% methanol	96.89	3.11 (2.30–4.10)
2. Silica gel 85% methanol	94.19	5.81 (3.12–9.35)
3. Biogel, P-10 & 0.9% NaCl eluant	93.69	6.31 (5.86–7.63)
4. Trichloroacetic acid precipitation	92.90	7.10 (4.80–9.00)
5. HPLC Spherogel TSK-SW column	98.56	1.44 (0.87–3.00)

* N = 4.
† Mean and range.

Recently Wong et al. (*15*) have shown that size-exclusion HPLC with μ-Bondagel columns can be used for the detection of free pertechnetate in Tc-99m HSA preparations, but a combination of μ-Bondagel columns was necessary to separate free pertechnetate from Tc-99m-bound HSA. In this paper we have demonstrated that a Spherogel SW column separates various radiochemical components in Tc-99m HSA preparations with high resolution. In addition, the column does not appear to promote intracolumn oxidation or binding of Tc-99m to the gel within the column.

Separation of various radiochemical components differing in molecular size provides an improved basis for understanding the biodistribution of Tc-99m HSA preparations. By modifying a standard HPLC system to obtain simultaneous analysis of uv absorbance and radioactivity following column fractionation of the radiolabeled compound, a record is rapidly obtained, obviating the need for sample collection, counting, and calculation of results. This method of separation is useful for determination of optimal compounding conditions, monitoring preparation and storage, and providing data for assessment of biologic turnover and distribution for dosimetric considerations.

Routine analysis of clinical radiotracer preparations by HPLC is not currently recommended, nor is it necessary to obtain this level of precision in clinical quality control. The rapidity of analysis and ease of operation of the technique, however, would make it useful for this application.

FOOTNOTES

* Union Carbide, Tuxedo, NY.
† Beckman Instruments, Inc., Somerset, NJ.

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REFERENCES

1. PERSSON PBR, LIDEN K: Tc-99m labeled human serum albumin: A study of the labeling procedure. *Int J Appl Radiat Isot* 20:241-248, 1969
2. ECKELMAN WC, MEINKEN G, RICHARDS P: Chemical state of ^{99m}Tc in biomedical products. *J Nucl Med* 12:596-600, 1971
3. ECKELMAN WC, RICHARDS P: Analytical pitfalls with ^{99m}Tc labeled compounds. *J Nucl Med* 13:202-204, 1972
4. VALK PE, DILTS CA, MCREA J: A possible artifact in gel chromatography of some ^{99m}Tc chelates. *J Nucl Med* 14: 235-237, 1973
5. STEIGMAN J, MEINKEN G, RICHARDS P, et al: Gel chromatography in the analysis of ^{99m}Tc radiopharmaceuticals. *J Nucl Med* 15:318-319, 1974
6. BILLINGHURST MW, PALSER RF: Gel chromatography as an analytical tool for ^{99m}Tc radiopharmaceuticals. *J Nucl Med* 15:722-723, 1974
7. RUSSELL CD, MAJERIK JE: Determination of pertechnetate in radiopharmaceuticals by high-pressure liquid, thin-layer and paper chromatography. *Int J Appl Radiat Isot* 30: 753-756, 1979
8. SRIVASTAVA SC, BANDYOPADHYAY D, MEINKEN G, et al: Characterization of Tc-99m bone agents (MDP, EHDP) by reverse phase and ion exchange high performance liquid chromatography. *J Nucl Med* 22:P69, 1981
9. PINKERTON TC, HEINEMAN WR, DEUTSCH E: Separation of technetium hydroxyethylidene diphosphonate complexes by anion-exchange high performance liquid chromatography. *Anal Chem* 52:1106-1110, 1980
10. FRITZBERG AR, LEWIS D: HPLC analysis of Tc-99m iminodiacetate hepatobiliary agents and a question of multiple peaks. Concise communication. *J Nucl Med* 21:1180-1184, 1980
11. *Liquid Chromatography Applications Research Data Sheet.* T1B-B512, Beckman Scientific Instruments Division, Berkeley, California, 94710.
12. FUKANO K: Evaluation of new supports for high-pressure aqueous gel permeation chromatography: TSK-gel SW type columns. *J Chrom* 166:47-54, 1978
13. ROKUSHIKA S, OHKAWA T, HATANO H: High speed aqueous gel permeation chromatography of proteins. *J Chrom* 176:456-461, 1979
14. MCLEAN JR, WELSH WJ: Measurement of unbound ^{99m}Tc in ^{99m}Tc-labeled human serum albumin. *J Nucl Med* 17: 758-759, 1976
15. WONG SH, HOSAIN P, ZEICHNER SJ, et al: Quality control studies of Tc-99m labeled radiopharmaceuticals by high-performance liquid chromatography. *Int J Appl Radiat Isot* 32:185-186, 1981