ALBUMIN MICROSPHERES FOR STUDY OF THE RETICULOENDOTHELIAL SYSTEM

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The spatial distribution of the reticuloendothelial system (RES) in man has been successfully visualized with gamma-emitting colloids such as ^{198}Au-colloidal gold, ^{99m}Tc-S-colloid, ^{113m}In-hydroxide, and ^{131}I-human serum albumin (HSA). Functional RES studies in man with these radioactive colloids, on the other hand, have not found widespread clinical application. Since individual batches of colloids vary in particle size and particle distribution, their use in functional RES studies is unsatisfactory.

The purpose of this paper is to describe a type of particle which (A) is rapidly removed from the vascular system by the RES, (B) has a particle size of approximately 1 micron, (C) has a narrow size distribution, (D) can be prepared in large batches which are stable for a long period of time, (E) can be labeled with short-lived radionuclides immediately before use, and (F) consists of an organic, nonantigenic substance, metabolizable within the body.

The last specification for the pharmaceutical agent is of particular importance since inorganic colloids remain in the liver and cannot be used in sufficient quantities to measure RES capacity.

After the introduction of metabolizable HSA microspheres in the size range of 12–44 microns for lung and circulation studies (*1*), the method of preparing these spheres was modified to yield smaller, approximately 1-micron HSA microspheres, which are selectively removed from the bloodstream by the RES (*2*). Several steps were added to the procedure for purification and narrowing the particle-size distribution.

METHODS

Preparation of HSA microspheres, 0.3–1 microns in size. The microspheres were prepared as follows:

1. 100 ml of USP cottonseed oil (Embassy's Lucky Boy) were mixed with 1 ml 25% HSA (USP albuminate, Hyland) and homogenized repeatedly by passing the suspension through a hand-operated homogenizer (C. W. Logeman Co., U.S. Pat. 2,064,402).
2. Another 100 ml of USP cottonseed oil were heated to 210°C in a 500-ml Erlenmeyer flask under continuous stirring with a motor-driven glass stirrer (approximately 1,700 rpm).
3. The homogenized albumin-oil was poured into the heated oil, the temperature adjusted to 175–185°C, and heating and stirring maintained for 10 min.
4. The suspension was cooled in an ice bath before being mixed with 200 ml of anhydrous diethyl ether (Fisher Scientific).
5. This was followed by centrifugation for 30 min at 3,000 rpm, 10–15°C.
6. The oil-ether phase was decanted from the precipitate. For complete removal of adhering oil, three successive washes with 100-ml ether were necessary.
7. The albumin spheres were suspended in absolute alcohol with the aid of an ultrasonic bath and the suspension kept in the refrigerator for 6–12 hr. Contaminants and clumps settled out and were removed. This alcohol washing and suspension step was repeated three times.
8. The albumin spheres were then suspended in diethyl ether and passed through a 14-micron Duralon filter (Millipore).
9. Working in a dustfree hood, the spheres were collected on a 0.22-micron Millipore filter, dried under ultraviolet light and transferred into preweighed, pyrogen-free 20-ml serum

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vials containing a 10 × 3-mm magnetic stir. Individual samples consisted of approximately 5 mg of dry material.

10. The final product was autoclaved for 20 min at 15 psi (250°F).
11. Random samples were checked for sterility and apyrogenicity.

A scanning electron micrograph of the dry product* (10,000 times magnification) is shown in Fig. 1. Complete dispersion of the spheres was achieved in normal saline-0.1% polysorbate 80 (Fig. 2).

Labeling of the HSA microspheres with ^{99m}Tc. The HSA microspheres were labeled with ^{99m}Tc using the technique by Stern, et al (*3*) and Cooper, et al (*4*):

1. A solution of reduced pertechnetate, containing 5 ml $^{99m}TcO_4^-$ eluate, 10 mg ascorbic acid, 5 mg $FeCl_3 \cdot 6H_2O$ and 1 ml saline 0.1% polysorbate 80, adjusted to pH 8.5, was sterilized by Millipore filtration and added to 5 mg of sterile dry microspheres.
2. After dispersion of the spheres by sonification, 0.5 ml of sterile, 0.25 *N* HCl was added (pH 1.8–2.0), followed by 10-min incubation under constant sonification at a waterbath temperature between 35 and 40°C. The pH was raised to 5.5 with 0.3 ml of 0.3 *N* NaOH.
3. The suspension was then boiled for 10 min in a 100°C waterbath, continuously stirring with a magnetic stir. The boiling step was found to be necessary to increase the stability of the ^{99m}Tc label.
4. For the removal of unbound $^{99m}TcO_4^-$, two different methods were used: (A) centrifugation of the preparation for 30 min in the original incubation vial (1,700 gm, 10–15°C) followed by withdrawal of the supernatant liquid and two successive washes with 5 ml saline-0.1% polysorbate 80; and (B) uptake of $^{99m}TcO_4^-$ by AG1-X8 anion exchange resin, chloride form, 50–100 mesh (Bio Rad). The microsphere suspension was passed through a fritted glass filter vial (22 mm, medium porosity, Wheaton Co.), containing 1 gm of washed, pyrogen-free, sterile resin. In order to recover 80–85% of the tagged microspheres, the resin had to be washed three times with 2 ml of saline-polysorbate 80 solution. The washes were added to the first resin eluate.
5. Finally, the suspension was sonicated for 5 min in an ice-bath sonicator and a sample checked for clumping under microscope.

* Courtesy of 3M Company.

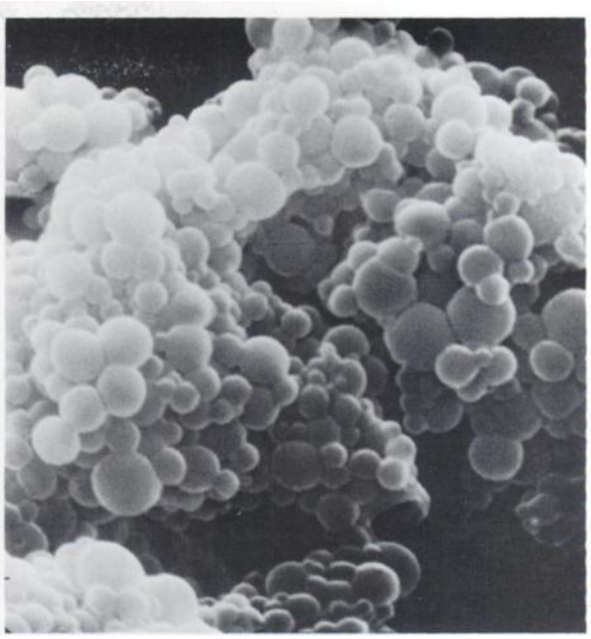

FIG. 1. Scanning electron micrograph (10,000 X) of HSA microspheres. After suspension of particles in acetone, preparation was dried which caused aggregation.

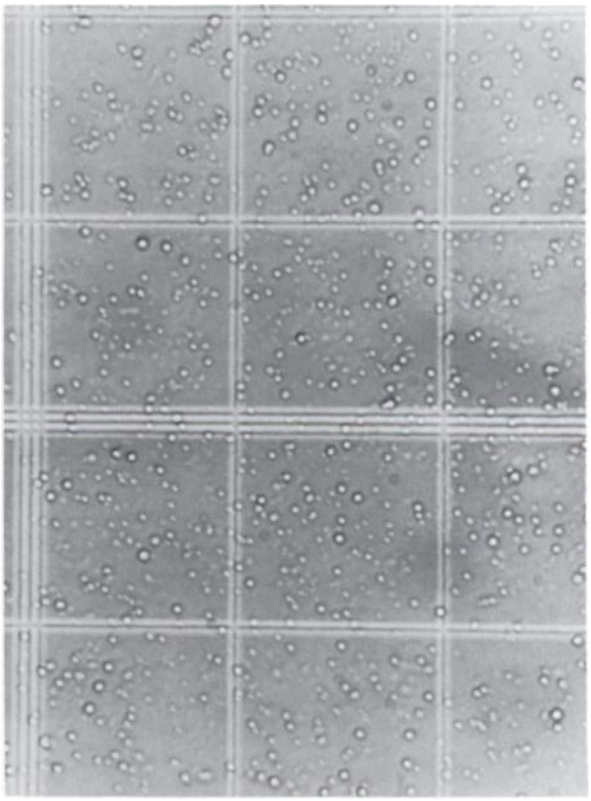

FIG. 2. HSA microspheres dispersed in saline-0.1% polysorbate 80, phase microscope photograph, 1,100 X.

Toxicity studies. Preliminary toxicity studies were performed in three animal species: three rabbits received doses of 0.5 mg/kg BW; one rabbit received three repeated injections of the same dose.

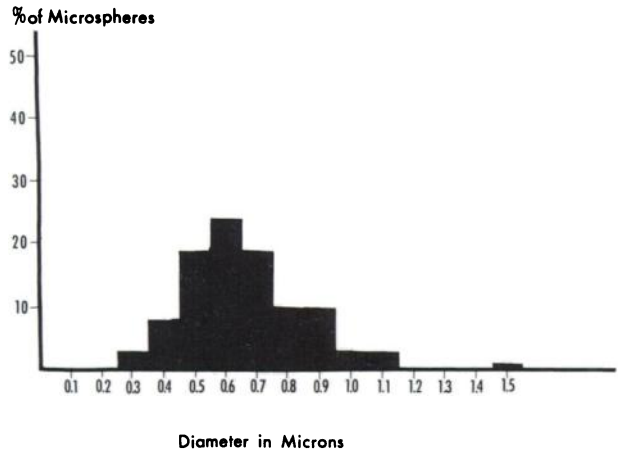

FIG. 3. Particle size and distribution of dry HSA microspheres.

TABLE 1. STABILITY OF LABELING

Microsphere preparation (No.)	Percent-free $^{99m}TcO_4^-$ (hr postprep.) 0	4	20
1	0.8	1.1	1.0
2	2.3	2.0	2.2
3	2.2	2.4	2.5
4	1.6		2.1
5	1.8		1.8

Three ICR mice were injected with 60 mg/kg BW doses. Doses of 0.04–4 mg/kg BW were given to 20 mongrel dogs. The animals were observed for a period of 3–4 weeks for signs of toxicity or death. An additional 80 ICR mice received 5–15 mg/kg doses and were checked for acute reactions.

Preparation of ^{99m}Tc-sulfur-colloid. Five-milliliters of generator-eluted $^{99m}TcO_4^-$ in saline were mixed with 1.5 ml of sterile buffered gelatin-thiosulfate solution containing 8.25 mg gelatin A, 1.5 mg of $K_2HPO_4 \cdot 3H_2O$ and 6 mg of anhydrous $Na_2S_2O_3$. Two milliliters of 0.25 *N* HCl were added and the solution heated for 10 min in a boiling water bath. After cooling, the pH was raised to 4.5–5.5 with 0.6 *M* sodium phosphate buffer (pH 7.7).

Distribution studies in mice. The 0.09-mg ^{99m}Tc-tagged spheres, suspended in 0.2-ml saline-0.1% polysorbate 80, were injected intravenously into the tail vein of ICR white mice. The animals were sacrificed 10 min–2 days after injection, the organ activities counted in a 3-in. well scintillation counter, and the percent of the injected dose calculated.

Blood clearance studies in dogs. Mongrel dogs were anesthetized and placed in supine position under a gamma camera connected with an image display and analysis (IDA) system. Trace doses of ^{99m}Tc-labeled microspheres (<0.2 mg/kg BW) or 0.2–1.0 ml of ^{99m}Tc-sulfur-colloid were injected intravenously into a vein of the fore limb. For the first minute after injection the data were recorded in the image display system as 5-sec digital frames; from 1 to 10 min after injection as 15-sec frames. Blood samples were taken from a hind limb vein at 1-min intervals from 1 to 8 min and at 10, 15, 20, and 30 min post-injection.

When sampling was complete, the images were replayed and sample regions of liver and heart selected in the optimal image frames. These two sample regions were delineated with a light pen, and the counts were then computed for each time interval.

The counts over the heart region and the counts of 1-ml blood samples were plotted on semilogarithmic paper as a function of time. The disappearance curve was divided into two components. The late, slow component (15–30 min for blood samples or the minimal counting rate over the heart) was sub-

TABLE 2. DISTRIBUTION OF ^{99m}Tc MICROSPHERES IN MICE (MEAN OF 5): % DOSE/ORGAN ± S.D.

Time after injection	Liver	Spleen	Lungs	Kidneys	Stomach	Blood
10 min	85.9 ±1.4	1.8 ±0.8	0.4 ±0.05	0.4 ±0.1	0.8 ±0.3	1.4 ±0.2
1 hr	80.2 ±1.8	1.1 ±0.4	0.4 ±0.05	0.5 ±0.1	3.1 ±0.7	1.3 ±0.2
2 hr	74.1 ±7.9	1.4 ±1.3	0.5 ±0.5	0.5 ±0.07	2.4 ±0.9	1.0 ±0.2
4.25 hr	73.2 ±3.1	1.0 ±0.4	0.3 ±0.04	0.6 ±0.2	2.7 ±0.9	0.9 ±0.2
17.5 hr	64.5 ±2.4	1.0 ±0.2	0.2 ±0.03	0.5 ±0.04	0.5 ±0.2	0.2 ±0.03
41 hr	53.1 ±2.9	1.0 ±0.7	0.1 ±0.01	0.4 ±0.02	0.07 ±0.02	0.08 ±0.06

tracted from the early, fast part of the curve. The first 2 min after injection were allowed for thorough mixing of the material in the vascular system. The straight part of the derived curve between 2 and 5 min was used to determine the half-life of clearance.

For the evaluation of the rate of liver uptake, the recorded data in the selected liver region were subtracted from the maximum liver counting rate and plotted on semilogarithmic paper. The half-time was directly read from the resulting straight curve.

Study in human volunteer. HSA microspheres were labeled with ^{99m}Tc and separated from unbound pertechnetate by centrifugation. The 1.25-mg (0.02 mg/kg BW) or 1.4 $\times$ 10^9 particles with an activity of 2.7 mCi were injected intravenously. Blood samples were taken at intervals. A liver and spleen scan was performed 30 min after injection.

RESULTS

Particle size and distribution of dry HSA microspheres, as examined by electron microscopy technique, are shown in Fig. 3. In two different preparations the most frequently occurring diameter varied between 0.4 and 0.6 microns.

HSA microspheres were successfully labeled with ^{99m}Tc. The tagging efficiency, determined by paper chromatography in 85% methanol, ranged between 65 and 83%. The final product contained less than 2.5% free $^{99m}TcO_4^-$. The label was stable for up to 20 hr postpreparation when the suspension was kept under refrigeration (Table 1). Quality control of the final product included tests for (A) sterility, (B) apyrogenicity, and (C) toxicity. No adverse reactions to the drug were observed in mice, rabbits, dogs, and one human volunteer.

The distribution of ^{99m}Tc-labeled microspheres in white mice is given in Table 2. The extraction efficiency by liver and spleen amounted to 88%. The radioactivity in these organs decreased to 54% within a period of 2 days. The uptake of HSA microspheres by the lungs was less than 1%. A slight increase of radioactivity in the stomach between 10 min and 1 hr postinjection points to some in vivo release of free ^{99m}Tc from the labeled microspheres.

Figure 4 shows the time curves of blood disappearance and liver uptake for ^{99m}Tc microspheres in a dog. Comparative studies in dogs were performed with ^{99m}Tc microspheres separated from unbound $^{99m}TcO_4^-$ by centrifugation (four different preparations), with ^{99m}Tc microspheres freed from $^{99m}TcO_4^-$ by use of an anion exchange resin (four preparations), and with four batches of ^{99m}Tc-sulfur-colloid (Table 3).

The most precise clearance data (rel. s.d. = 4.4%) was obtained when ^{99m}Tc-HSA microspheres

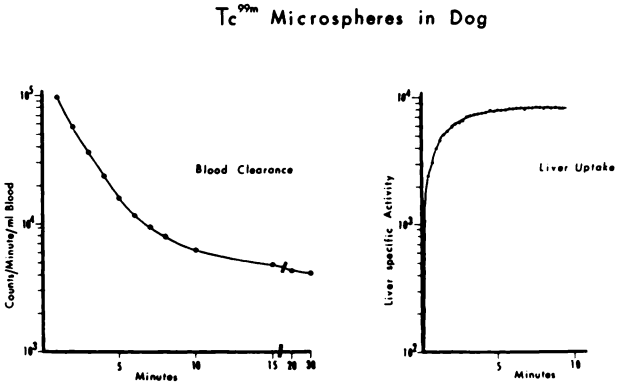

FIG. 4. Blood clearance and liver uptake time curves of ^{99m}Tc HSA microspheres, injected intravenously into dog.

TABLE 3. BLOOD CLEARANCE IN DOGS

^{99m}Tc microspheres (centrifuged)		^{99m}Tc microspheres (passed through resin)		^{99m}Tc-S-colloid	
Dog No.	$T_{1/2}$ (min)	Dog No.	$T_{1/2}$ (min)	Dog No.	$T_{1/2}$ (min)
5	0.9	9	1.2	10	1.7
5	0.9	10	1.4	10	1.4
6	1.1	10	1.3	10	2.4
7	0.6	10	1.4	10	1.2
8	1.1	10	1.3	11	1.6
		11	1.2	11	1.4
		11	1.3	11	1.6
		12	1.3	12	1.1
Mean:	0.9		1.3		1.6
s.d.:	±0.19		±0.06		±0.42
% rel. s.d.*:	21.31		4.4		26.9

* % rel. s.d. = s.d. expressed as percent of mean.

were used from which unbound pertechnetate was removed by anion exchange resin. The clearance half-time for these microspheres was longer (1.3 min) than that for microspheres which had been separated from free $^{99m}TcO_4^-$ by centrifugation (0.91 min). The ^{99m}Tc-sulfur-colloid, disappearing from the blood with an average half-time of 1.56 min, showed the widest variation (rel. s.d. = 26.9%).

Similar results were observed in studies using a gamma camera and analysis system for external monitoring (Table 4). The ^{99m}Tc microspheres proved to be less variable than ^{99m}Tc-sulfur-colloid. The disappearance rates measured over the heart area and the half-time values for liver uptake were not significantly different from clearance data obtained by analyzing blood samples.

The blood clearance half-time for microspheres in the human study was 1.4 min. The scan performed 30 min after injection showed clear delineation of liver and spleen.

TABLE 4. COMPARISON OF BLOOD CLEARANCE DATA (BLOOD SAMPLES) WITH DISAPPEARANCE RATES OVER HEART AND ACCUMULATION RATES OVER LIVER, OBTAINED BY EXTERNAL MONITORING (MEAN OF 8 DETERMINATIONS)

		Clearance from blood		
		Blood	Heart	Uptake in liver
^{99m}Tc microspheres (passed through resin)	Half-time (min):	1.3	1.3	1.1
	±s.d.:	±0.06	±0.10	±0.10
	% rel. s.d.*:	4.4	7.9	8.9
^{99m}Tc-sulfur-colloid	Half-time (min):	1.6	1.4	1.3
	±s.d.:	±0.42	±0.24	±0.38
	% rel. s.d.*:	26.9	17.8	28.8

* % rel. s.d. = s.d. expressed as percent of mean.

DISCUSSION

Blood disappearance rates and hepatic blood-flow data in the literature vary widely depending on the types of colloids (*5*). When using one particular type of colloid, accurate results can only be obtained when controls and tests are performed with the same batch of colloidal agent (*6*). Because of limited shelf life of radioactive materials, this approach cannot be taken in all instances. ^{99m}Tc-sulfur-colloid, readily available in most diagnostic isotope laboratories, has been suggested for use in liver blood-flow studies (*7,8*). According to the data presented in Tables 3 and 4, ^{99m}Tc-sulfur-colloid is probably not a suitable radiopharmaceutical for assessment of RE function because of great variability in day-to-day preparations.

Measurement of hepatic blood flow and phagocytic capacity with a metabolizable HSA aggregate labeled with ^{125}I or ^{131}I has been described by several authors (*6,9–11*), but the preparation of successive batches of colloidal heat-denatured albumin with uniform particle size is quite difficult. DiLuzio and his group employed an ^{131}I-labeled lipid emulsion for the evaluation of RE function (*12,13*).

In our present study we have tested a particulate material, HSA microspheres, labeled with ^{99m}Tc immediately before injection for the use in functional RES studies. Blood clearance data in dogs obtained with microspheres, tagged with ^{99m}Tc on successive days, revealed good reproducibility. The average blood clearance half-time for trace amounts (<0.2 mg/kg) of ^{99m}Tc microspheres in dogs was shorter (1.4 min*) than the half-time for the same quantity of ^{131}I-labeled aggregated albumin (2.5 min) (*6*). This may be explained by the difference in particle size: Taplin (*14*) measured the particle size of heat-treated HSA ^{131}I suspensions at 10–20 millimicrons as compared to an average particle size of 500–700 millimicron for HSA microsphere preparations. Although we have not studied this aspect ourselves, our data are in agreement with Taplin's findings that larger particles are cleared faster from the blood than smaller size particles (*5*).

Labeling of HSA microspheres with ^{99m}Tc can easily be performed in less than 1 hr in a routine radiopharmaceutical laboratory. The preparation can be adapted to "kit" form. Short-life isotopes other than ^{99m}Tc can be used for tagging. HSA microspheres containing a small amount of iron hydroxide were labeled with ^{113m}In according to the method of Zolle, et al (*1*). The tagging yield for ^{113m}In amounted to 85–95%. The behavior of these particles in the RES is presently under investigation.

SUMMARY

An improved method for the preparation of HSA microspheres with an average diameter of 0.5–0.7 microns and narrow particle-size range, suitable for the study of the RES, has been described. No toxic effects towards the new drug were observed in animals. Nonradioactive HSA spheres could be stored for more than 10 months. Labeling of the spheres with ^{99m}Tc was carried out under sterile conditions immediately before use. The tagging efficiency was 65–83%, and the labeled product was stable for up to 20 hr. When injected intravenously, about 90% of the ^{99m}Tc microspheres were extracted by the liver. Blood clearance studies in dogs showed small variability in day-to-day preparations. The advantage of this new drug over the widely used ^{99m}Tc-sulfur-colloid is that it can be used for the assessment of RES functions in addition to scanning the distribution of the RES.

ACKNOWLEDGMENT

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* Determined as the time required for the counting rate to fall to half of the value measured at 2 min after injection.

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