Table III—Analysis of Typical Pharmaceutical Formulations

Sample	Type of Sample[a]	Amount Declared, %	Found, % of Declared: Blue Tetrazolium, Proposed Method	Blue Tetrazolium, USP XIX Method	Isoniazid	Phenylhydrazine
1	Cream	0.25	95.0, 97.8	91.2, 91.7	96.0, 95.6	91.5, 90.8
2	Cream	0.5	96.7, 101.4	96.6, 100.0	94.1, 97.7	93.8, 98.5
3	Cream	1	97.1, 97.6	99.2, 98.6	98.6, 99.6	96.1, 100.3
4	Gel	1	97.5, 99.9	97.5, 97.4	99.9, 97.9	99.5, 97.9
5	Lotion	0.25	97.1, 96.9	96.2, 97.2	92.8, 88.1	ND[b]
6	Lotion	0.5	100.0, 95.9	99.5, 96.3	ND[b]	101.2, 98.8
7	Lotion	0.5	99.8, 99.2	99.6, 99.2	100.9, 101.3	99.2, 100.4
8	Lotion	0.125	90.5, 89.0	92.4, 92.8	102.2, 99.3	97.9, 95.8
9	Lotion	0.25	95.4, 96.6	96.3, 98.0	98.5, 97.8	95.2, 96.9
10	Lotion	0.5	98.7, 99.0	102.4, 102.6	101.9, 101.2	102.9, 102.3
11	Lotion	1.0	103.2, 104.4	106.4, 106.6	101.1, 101.3	103.2, 103.2
12	Lotion	1.0	94.3, 92.6	94.4, 91.2	92.5, 91.0	93.5, 94.6
13	Ointment[c]	0.25	98.6, 97.3	94.9, 94.7	98.8, 98.0	97.5, 98.5
14	Suspension[c]	0.25	89.3, 91.2	87.9, 89.8	88.4, 87.8	86.9, 92.7
15	Suspension[c]	0.25	71.6, 73.6	73.3, 74.8	74.0, 72.3	72.3, 73.5
	Average		95.2	95.3	95.3	95.6

[a] The steroids were hydrocortisone in Samples 1–12 and prednisolone acetate in Samples 13–15. [b] ND = not determined. [c] Product also contained 10% sodium sulfacetamide.

by scanning against the solvent and calculating the corrected net absorbance as shown under *Experimental*.

Methylene chloride solutions undergo evaporative concentration from glass-stoppered volumetric flasks at a daily average rate of approximately 0.1%[9] (v/v). This result indicates that the same standard could be used for several days without significant change. In this study, however, the standards were used only on the day of preparation.

In some cases, the corticosteroid was so slowly soluble in methylene chloride that it was necessary to let it stand for 1 hr with periodic agitation or place it in an ultrasonic instrument for not more than 30 sec to ensure complete solution.

The proposed procedure for the analysis of corticosteroids with I is rapid and quantitative. In most cases, the reaction is complete within 15 min after addition of the tetramethylammonium hydroxide reagent and the formazans produced are stable for at least 90 min. Analysis of 15 different pharmaceutical products for corticosteroids by the proposed I procedure gave results that compared favorably with those obtained by three different analytical methods.

[9] Unpublished work.

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Preferential Localization of Radiolabeled Liposomes in Liver

GEORGE H. HINKLE *x, GORDON S. BORN, WAYNE V. KESSLER, and STANLEY M. SHAW

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*Present address: University of Oklahoma, Tulsa Medical College, Tulsa, OK 74114.

Abstract □ Liposome formulations were studied to achieve an efficient entrapment procedure for the production of liposomes of ^{99m}Tc-pentetic acid. The entrapment efficiency was studied by separation of the product using column chromatography. The particle-size range of the prepared liposomes was evaluated using electron microscopy. Entrapment techniques and separation procedures led to a liposome preparation with particles in the colloidal size range (0.001–0.5 μm). Dramatic differences in the organ distribution of the liposome preparation in mice were produced when different particle-size ranges were injected. Liposomes eluted in the first fraction after the void volume led to a maximum uptake by the liver and spleen 10 min after intravenous injection. Liposomes from pooled fractions provided less than half of the activity in the liver, as did the narrow size range liposome preparation.

Keyphrases □ Liposomes, radiolabeled—^{99m}Tc-pentetic acid, organ distribution in mice, effect of particle size □ Radiolabeled liposomes—^{99m}Tc-pentetic acid, organ distribution in mice, effect of particle size □ ^{99m}Tc-Pentetic acid liposomes—organ distribution in mice, effect of particle size □ Distribution, organ—^{99m}Tc-pentetic acid liposomes in mice, effect of particle size □ Particle size—^{99m}Tc-pentetic acid liposomes, effect on organ distribution in mice

Liposomes have been described as minute vesicles composed of lipid bilayers (1). Spontaneous formation of liposomes occurs when a combination of certain lipids is dispersed throughout an aqueous solution (2). Materials dissolved in the aqueous solution become trapped in the enclosed aqueous compartments, which form in an alter-

Table I—Organ Distribution of Pooled Fractions of Liposomes in Mice at Various Time Intervals

Minutes after Injection	Blood (1 g)	Liver	Kidneys	Urinary Bladder and Urine	Spleen	Small Intestine	Lungs	Heart
1	23.5[a] ± 2.3	35.3 ± 3.1	2.6 ± 0.1	0.5 ± 0.3	2.8 ± 0.9	2.2 ± 0.8	1.7 ± 0.3	0.7 ± 0.2
3	19.8 ± 0.4	37.9 ± 1.7	3.5 ± 0.4	4.1 ± 0.6	2.7 ± 0.4	2.1 ± 0.5	1.0 ± 0.6	0.3 ± 0.3
5	17.6 ± 3.5	43.8 ± 7.4	3.2 ± 0.9	4.8 ± 2.6	2.4 ± 0.1	1.7 ± 0.2	0.7 ± 0.1	0.2 ± 0.02
10	11.0 ± 0.9	41.6 ± 0.9	1.9 ± 0.2	9.6 ± 0.9	3.0 ± 0.6	2.1 ± 0.3	0.8 ± 0.05	0.2 ± 0.03
15	10.1 ± 3.7	44.1 ± 5.3	1.9 ± 0.3	10.7 ± 7.9	3.2 ± 1.3	1.8 ± 0.4	0.6 ± 0.1	0.1 ± 0.05
30	4.9 ± 0.9	37.9 ± 3.7	2.9 ± 0.5	22.1 ± 3.6	2.0 ± 0.1	3.2 ± 0.3	0.4 ± 0.1	0.09 ± 0.01
60	1.7 ± 0.2	29.8 ± 1.3	1.4 ● 0.3	50.1 ± 11.5	2.1 ± 0.5	2.1 ± 0.3	0.2 ± 0.09	0.06 ± 0.03

[a] Percentage of injected dose per organ or per gram ± *SD*. Mean of four mice.

nating, concentric fashion with the lipid bilayers. In addition, lipid-soluble materials may be incorporated in the formed liposomes if added to the lipids forming the bilayer structures. These properties make liposomes ideal carriers of drugs, enzymes, and other biologically important compounds (3–11).

The components of the formed liposomes, the nature of the material entrapped in the aqueous compartment, and the size of the structures all contribute to the initial degree of entrapment of the aqueous material and the stability of the preparation (1–7, 12–14). The biological distribution of the entrapped materials follows that of the liposome carrier (2–11). The direction of liposomes to specific target cells *in vivo* by altering the surface or structure of the liposomes is being studied (15–17).

Phagocytosis of small diameter liposomes by the Kupffer cells of the liver occurs within minutes after intravenous injection (9–11, 16–18). The phagocytic removal of liposomes by the reticuloendothelial system hinders their use as drug carriers to afford specific delivery of medically important agents to sites of action other than the reticuloendothelial system. However, this property has been used to obtain diagnostic information about the liver and spleen and may lead to the use of liposomes as a delivery system for radiopharmaceuticals (19–23).

After a study of the preparation of radiolabeled liposomes [^{99m}Tc-pentetic acid[1] (^{99m}Tc-I)] and their uptake in mice was completed in this laboratory, a similar study was reported (23). The maximum uptake by the liver reported was less than 45%. The present study shows that greater than 90% uptake can be obtained by control of liposome size. Two different lipid combinations and different sonication times were evaluated. Although some results show little difference from those already reported (23), it is important to describe a procedure for the preparation of radiolabeled liposomes of the correct particle size that achieves maximum localization in the liver.

EXPERIMENTAL

Preparation of Liposomes of ^{99m}Tc-I—The effect of the inclusion of oppositely charged amphiphiles in the liquid composition on the entrapment of ^{99m}Tc-I into the liposomes was studied using two formulations. Negatively charged liposomes were prepared using 15.75 μmoles of dimyristoyllecithin[2], 4.5 μmoles of cholesterol[2], and 2.25 μmoles of dicetyl phosphate[2] (a 7:2:1 molar ratio). Positively charged liposomes contained 2.25 μmoles of stearylamine[3] in their formulation, replacing the dicetyl phosphate.

The procedure for the preparation of liposomes was identical for both negative and positive formulations. The lipids were weighed, dissolved in chloroform, and combined. The combined lipid solution was transferred to a 10-ml pear-shaped flask, and the chloroform was removed by rotary evaporation under reduced pressure. The open flask containing the thin layer of lipid on its walls was transferred to a desiccator and placed under vacuum for 1 hr to remove any remaining chloroform.

In-house kits, as described by Eckelman and Richards (24), for the rapid preparation of ^{99m}Tc-I were prepared. ^{99m}Tc-Labeling of I was carried out while the pear-shaped flask remained in the desiccator under vacuum.

One milliliter of the freshly prepared ^{99m}Tc-I solution was added to the pear-shaped flask, and the thin lipid film was dispersed by vortex mixing for 10 min at high speed. The flask was placed in a lead shield and allowed to stand at room temperature for 2 hr. This step allowed the formation and swelling of the liposomes, in which the aqueous ^{99m}Tc-I solution became entrapped between the lipid bilayers. The flask was then placed in a water bath sonicator[4], which generated frequencies of 50–55 kHz and sonicated for varying lengths of time depending on the experiment being performed.

To prevent oxidation and hydrolysis of the lipids due to heat generated by the sonication procedure, the temperature of the water was maintained below 20° by the addition of crushed ice to the sonication bath. A second 2-hr interval of standing at room temperature was used after sonication to allow swelling and continued liposome formation.

A sample of the liposome (^{99m}Tc-I) preparation was placed on a 1.5 × 30-cm column of a gel chromatography medium[5] equilibrated with isotonic sodium phosphate buffer (pH 7.05) eluant. Forty fractions were collected, each having a volume of 2 ml. To determine the degree of liposome entrapment of the ^{99m}Tc-I, a 10-μl aliquot of each fraction was counted in an automatic γ-counter. A second elution of the column after the addition of 0.1% hydrogen peroxide was performed to remove any hydrolyzed technetium. These counts were added to the counts of the free ^{99m}Tc-I species after decay corrections.

The degree of entrapment of ^{99m}Tc-I was expressed as the percentage of the total activity eluted from the column found in the liposome peak. The variability of entrapment due to lipid composition was determined using positive or negative liposome formulations and a sonication time of 45 min. The variability due to sonication time was determined using the positive liposome formulation and sonication times of 15, 30, 45, and 60 min.

Size Determinations Using Electron Microscopy—The preparation of liposomes for electron microscopy studies was identical to that discussed previously except for the addition of 1 ml of 3.3 *mM* potassium iodide solution to the pear-shaped flask prior to the entrapment of ^{99m}Tc-I. A negative staining technique was employed by diluting the liposome preparation with an equal volume of a 0.5% solution of phosphotungstic acid and allowing this mixture to stand for 1 hr. A small amount of this preparation was placed on a carbon-coated grid[6] and allowed to air dry. The grid was then examined on an electron microscope[7].

Animal Studies—*In vivo* distribution studies of positively charged liposomes were performed using male Swiss mice, 23–29 g. The positive liposome formulation was used because of the apparent increase entrapment of ^{99m}Tc-I over the negative liposome formulation. After vortex mixing of the injection preparation to obtain a homogeneous suspension, 0.2 ml was administered intravenously in the caudal vein; the mouse was sacrificed by cervical separation after the allotted distribution time. Organs of interest were weighed and counted along with blood samples and 0.2-ml samples of the injection preparation. Corrections were made

[1] Previously referred to as diethylenetriaminepentaacetic acid.
[2] *Sigma Chemical Co., St. Louis, Mo.*
[3] K & K Laboratories, Plainview, N.Y.
[4] Model B-12, Bransonic, Shelton, Conn.
[5] Sephadex G-50 (fine), Pharmacia Fine Chemicals, Piscataway, N.J.
[6] Formvar, Ernest F. Fullam, Inc., Schenectady, N.Y.
[7] Hitachi model HU-11A.

Table II—Localization of a Colloidal Particle-Size Range Sample of Liposomes in Mice at Various Time Intervals

Minutes after Injection	Run	Blood (1 g)	Liver	Kidneys	Spleen	Lungs
5	1	1.4[a] ± 0.7	91.7 ± 0.7	0.9 ± 0.1	5.1 ± 1.2	0.9 ± 0.1
	2	3.1 ± 0.1	87.8 ± 0.8	1.6 ± 0.1	2.6 ± 0.5	1.3 ± 0.2
10	1	1.2 ± 0.1	93.4 ± 3.1	0.9 ± 0.1	3.3 ± 1.1	0.8 ± 0.1
	2	2.3 ± 0.3	90.1 ± 1.8	1.6 ± 0.3	3.3 ± 1.6	1.0 ± 0.2
15	1	1.3 ± 0.7	92.1 ± 2.9	0.8 ± 0.1	4.5 ± 0.9	0.5 ± 0.1
	2	1.8 ± 0.2	91.6 ± 1.2	1.6 ± 0.1	2.6 ± 0.2	1.1 ± 0.2

[a] Percentage of injected dose per organ or per gram ± *SD*. Mean of three mice.

for the physical decay of technetium Tc 99m, and the results were expressed as percentage of injected dose per organ or per gram of tissue.

In one study, organ distribution of a wide particle-size range sample of liposomes was performed by combining the cloudy liposome fractions into one injection preparation. A 45-min sonication time was used to produce the wide particle-size distribution of liposomes. In a second study, liver localization of liposomes was accomplished using the fraction containing colloidal particle-size range liposomes for injection. The volume of the injection preparation was increased by the addition of eluant buffer.

The 45-min sonication time was used in the animal studies because it provided the greatest number of liposomes in the colloidal size range in comparison with 15-, 30-, and 60-min sonication periods. Preliminary studies involving light and electron microscopy techniques showed that colloidal size particles were being prepared with each sonication time period. Sonication for 15 min resulted in a large number of liposomes approaching 10 μm in diameter. Longer periods of sonication produced small diameter structures whose passage through the gel chromatography medium was retarded during separation attempts. The 45-min sonication time produced few liposomes greater than 0.5 μm, and separation of the labeled liposomes from the free ^{99m}Tc-I using gel chromatography provided the colloidal size structures in the first fraction following the void volume.

RESULTS

Preparation of Liposomes of ^{99m}Tc-I—Separation of the liposome preparation from the free ^{99m}Tc-I by column chromatography provided a determination of the degree of entrapment along with a liposome preparation for animal studies. Figure 1 shows the degree of entrapment of ^{99m}Tc-I in two preparations differing in lipid composition. The mean percentage of ^{99m}Tc-I activity in the liposome fractions of negatively charged liposomes, as calculated from a total of six entrapment studies, was 1.86 ± 0.34. The mean percentage of ^{99m}Tc-I activity in the liposome fractions of positively charged liposomes, as calculated from 12 entrapment studies, was 8.14 ± 1.66. A significant increase in the degree of entrapment was seen with positively charged liposomes.

Figure 2 summarizes the results of entrapment of ^{99m}Tc-I into positively charged liposomes produced with various sonication time intervals. The sonication time intervals were chosen because they produced liposomes in the colloidal size range. Application of the Newman–Keuls test to the data led to the conclusion that the 60-min sonication time produced significantly lower (p = 0.05) entrapment yields than did 15-, 30-, and 45-min times, which did not differ from each other significantly. The slight decrease in entrapment with a 60-min sonication time was expected since the larger liposomes produced at the other times can entrap more aqueous solution between their numerous lipid bilayers.

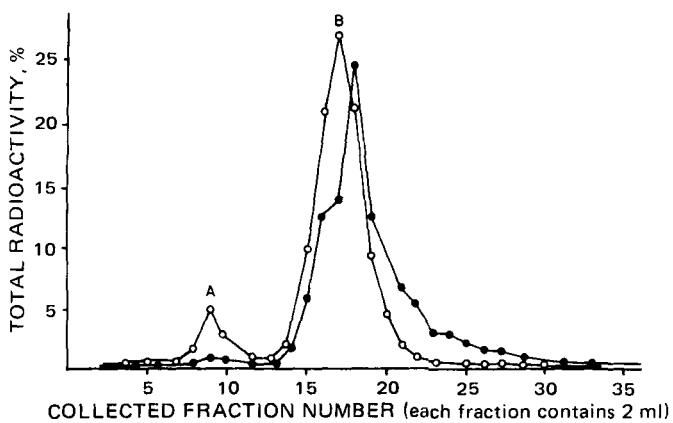

Figure 1—*Elution pattern of positively* (○) *and negatively* (●) *charged liposomes showing the liposome peak* (*A*) *and the ^{99m}Tc-I peak* (*B*).

Size Determination—Figure 3 shows the concentric lamellar structures enclosing aqueous channels of the liposome. The electron micrograph shows two liposomes from the injection preparation made from the first fraction obtained from gel filtration. Subsequent studies of the liposomes contained in the first fraction after the void volume showed the presence of liposomes ranging from 0.1 to 2 μm in diameter. Successive fractions contained small diameter liposomes, which remained in the circulation for extended periods.

Animal Studies—Table I illustrates the organ distribution of liposome from the pooled fractions of a chromatographed liposome preparation. Four animals were used for each time interval. A rapid initial decrease in circulating blood activity was seen within 3 min following the partial clearance of liposomes from the blood. The blood activity did remain somewhat elevated for at least 15 min, apparently because of the presence of small diameter liposomes that were not removed by the liver.

After 30 min, the liver activity decreased with an increase in the urinary bladder (urine) activity. Biodegradation of the liposomes allowed the release of free ^{99m}Tc-I into the circulation, which eventually was excreted in the urine. Hepatic activity decreased slowly, probably because of the biodegradation of liposomes competing with a continuous uptake of liposomes from the circulation. Distribution intervals of 5, 10, and 15 min provided optimum liver uptake of the formed liposomes and were used for successive animal studies.

The use of liposomes from the first fraction after the void volume as the injection suspension resulted in maximum uptake by the liver (Table II). The study was initially completed using three animals per time interval and then repeated in identical fashion. The ability of gel filtration to fractionate the prepared liposomes according to their size is evident when comparing these results with those of Table I. By using an injection suspension composed of the larger liposome structures, the blood activity was significantly reduced. In addition, preferential uptake by the liver

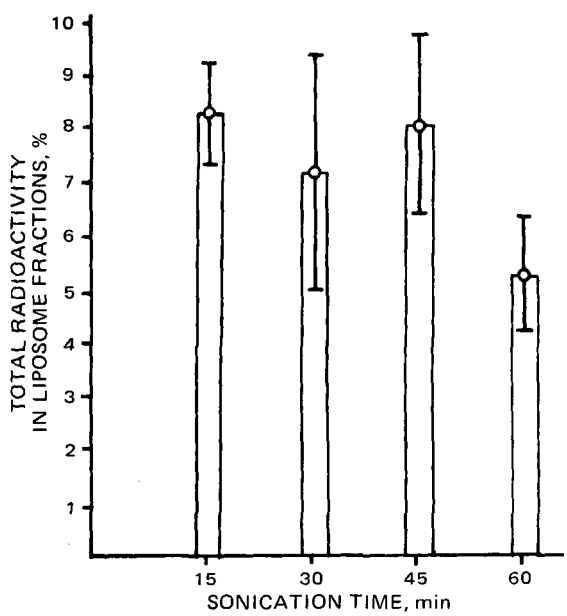

Figure 2—*Effect of sonication time on the entrapment of ^{99m}Tc-I into positively charged liposomes* (*mean* ± SD).

Figure 3—*Electron micrograph of liposomes (80,000×).*

occurred, providing 93.4% of the total dose in the liver at the maximum uptake level. In this particular situation, this localization of the liposomes yielded an organ to background ratio of uptake suitable for imaging.

DISCUSSION

The preparation of a liposome suspension that provided maximum localization in the organ of interest depended primarily upon the use of liposome structures of suitable particle size. Liposomes were distributed to other tissue sites when the particle size of the injection material was not restricted. An injection preparation of the desired particle-size range was prepared by the use of controlled sonication times and gel filtration column chromatography to separate the different particle-size ranges. Column chromatography also provided the means for determining the degree of entrapment of the labeled chelate in the liposomes. Fractionation of the resultant liposome particles provided an injection preparation that yielded a maximum uptake in the liver of over 93% of the total dose.

The ability of this radiopharmaceutical to provide hepatic blood flow data, γ-camera images of the liver, and liver function data appears promising. The fast blood clearance along with target organ accumulation of the activity would decrease the background activity around the liver, producing quality images.

An important advantage of this radiopharmaceutical over those currently used for liver imaging is its biodegradation property. The breakdown *in vivo* of the liposome preparation is quite rapid, with over 50% of the total dose appearing in the urine after 60 min. The first important result of this breakdown is a shorter biological half-life than that of technetium Tc 99m–sulfur colloid, which has an effective half-life dependent on the physical half-life of technetium Tc 99m. In addition to the reduced radiation dose to the patient, additional diagnostic procedures, if necessary, may be performed sooner when liposomes are used for liver studies.

Furthermore, the ability of liposomes to provide information on liver function may lead to additional use of this preparation. After localization in the liver, it appears the liver enzymes disrupt the liposomal membranes, allowing the entrapped aqueous material to be released. By monitoring the amount of radioactivity remaining in the liver with time, an indication of the functioning capacity of the liver may be obtained.

Investigations concerning improvement of the entrapment yield along with the production of a liposome product with an increased radioactive concentration are being planned. In addition, decreasing the labeling procedure time, which could lead to the development of a kit preparation suitable for human use, is being studied.

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